

Research Article

***In Vitro* and *In Vivo* Evaluation of Antibiotic Combinations against Multidrug Resistant *Proteus Mirabilis* Isolated from Admitted Patients of Dhaka Medical College Hospital, Dhaka**

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Abstract

Emergence of MDR *P. mirabilis* is a threat for high morbidity and mortality with catheter-associated urinary tract infections leading to urolithiasis and permanent renal damage, bacteremia and sepsis. Global shortage of new effective antibiotic with reduced susceptibility of *P. mirabilis* to imipenem, tigecycline and resistance to colistin needs antimicrobial combinations. So, different antimicrobial combinations were used to see their efficacy both *in vitro* and *in vivo* with their resistance in Bangladesh.

MIC of antibiotics alone and in combination and efficacies of different antibiotic combinations *in vivo* (mice model) were evaluated. PCR and sequencing of resistance genes were done.

Among the 500 samples, 70% were culture positive and out of these, 10.57% were *P. mirabilis*. Among isolated *P. mirabilis*, 24.32%, 21.62%, 27.03% and 78.38% were resistant to fosfomycin, imipenem, tigecycline and amikacin respectively and 75.68%, 13.51% and 10.81% were MDR, XDR and PDR respectively. Among fosfomycin resistant *P. mirabilis*, 77.78%, 44.44% and 22.22% were positive for *fosA*, *fosA₃* and *fosA₄* respectively. Among imipenem

resistant *P. mirabilis*, 62.50% were positive for NDM-1 and OXA-10, 50% for NDM-2, OXA-23 and OXA-48, 25% for OXA-58.

In vitro combination of imipenem-amikacin and imipenem-fosfomycin showed 100%, and 75% synergism respectively. The best *in vivo* results appeared in the group treated with imipenem-amikacin and imipenem-fosfomycin combinations where 100% bacterial clearance was observed.

As *P. mirabilis* showed high level resistance towards commonly used antimicrobial agents, it is very difficult to treat by a single agent. So, emphasis should be given on using antibiotic combinations.

Keywords: Antibiotic combination, FIC, MDR, MIC, PDR, XDR.

Highlights: Drug combinations are more efficacious than single drug therapy

1. Introduction

The emergence and spread of carbapenem resistant *Enterobacteriaceae* have become an increasing concern for healthcare services worldwide. Infections caused by these bacteria including *P. mirabilis* cause significant morbidity and mortality [3]. Broadly carbapenemases are categorized into three groups: Class A (penicillinases), Class B (metallo- β -lactamases [MBL]), and Class D (Oxacillinases) [6]. The New Delhi metallo- β -lactamase (NDM) is one of the class B metallo- β -lactamases. They are present largely in *Enterobacteriaceae* [30]. Decreased susceptibility to imipenem, intrinsic resistance to polymyxins and tetracyclines along with the acquisition of β -lactam resistance traits by *P. mirabilis* seriously limits treatment options [34].

Recently, the use of fosfomycin has attracted renewed interest for the treatment of serious systemic infections caused by multidrug-resistant *Enterobacteriaceae* [38]. The WHO has classified fosfomycin in the category of a ‘critically important’ and has been identified as an antimicrobial which holds great promise worldwide for MDR gram negative infection, due to its affordable cost and efficacy as carbapenem sparing regimen [45]. It is among the limited treatment options for carbapenem resistant *Enterobacteriaceae* (CRE) infections [2].

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 [46, 29].

So, until better antibiotics are being developed, novel antibiotic combinations yield some *in vitro* and *in vivo* synergistic activity are perhaps the best options we have in our hands to manage multidrug resistance. Combination of two antibiotics may provide broader spectrum coverage, decrease the emergence of resistance & also dose related toxicity [24]. In addition, animal models provide not only an important resource for the development and testing of new therapies, but also for studies into the initiation and maintenance of bacterial infections [22].

To the best of knowledge, no study has been carried out in Bangladesh regarding use of different antibiotic combinations on multidrug resistant *Proteus mirabilis* both *in vitro* and *in vivo*. This study seeks to determine multidrug resistant *Proteus mirabilis* among patients of DMCH and to evaluate the effectiveness of different antibiotic combinations both *in vitro* and *in vivo* (in mouse model) along with their PCR assays.

2. Methods

2.1 Clinical isolates and identification

A cross sectional study was conducted from January 2019 to December 2019 among 500 samples of urine, wound swab, pus and blood of adult patients having clinically suspected infections admitting in Dhaka Medical College Hospital or were received in the microbiology department for culture and sensitivity after taking informed written consent irrespective of sex and antibiotic intake. Patients who did not give consent were excluded from this study. Out of these 500 samples, 350 (70%) samples were culture positive, of which 37 (10.57%) were identified as *Proteus mirabilis* by cultural, biochemical and phenotypic tests.

2.2 Antimicrobial susceptibility test

Susceptibility to antimicrobial agents of all isolated organisms were determined by Kirby-Bauer modified disc diffusion technique using Mueller-Hinton plates and zones of inhibition were interpreted according to CLSI guidelines [9]. The criteria for the United States Food and Drug Administration was used for the interpretation of zone of inhibition of tigecycline. Antibiotic discs were obtained from commercial sources (Oxoid Ltd, UK). Following antimicrobial discs were used: amikacin (30µg), piperacillin-tazobactam (100/10µg), imipenem (10µg), tigecycline (15µg), ciprofloxacin (30µg), cefepime (30µg), ceftazidime (30µg), ceftriaxone (30µg), ceftiofur (30µg), cefuroxime sodium (30µg), amoxiclav (amoxicillin 20µg & clavulanic acid 10µg) and aztreonam (10µg). Fosfomycin susceptibility was tested by agar dilution method of MIC.

2.3 Agar dilution method of MIC

MIC of imipenem, piperacillin-tazobactam (Reneta Pharma Limited, Dhaka), tigecycline (Incepta Pharma Limited, Dhaka), amikacin (ACI Pharma Limited, Dhaka), fosfomycin (Beximco Pharma Limited) was determined by agar dilution method [1].

2.3.1. Inoculums preparation & Inference of MIC

As 0.5 McFarland turbidity standard contains 1×10^8 cfu/ml [7]. 10 times dilution of test inoculums was done to achieve 1×10^7 cfu/ml. All the inoculated plates were incubated aerobically at 37°C overnight. The lowest concentration of antibiotic impregnated Mueller-Hinton agar showing no visible growth on agar media was considered as MIC of the drug of that strain of bacteria. *Escherichia coli* ATCC 25922 were used as control organisms.

2.3.2. Effects of antibiotic combinations *in vitro*

Combination of antibiotics was done to see synergistic, additive, indifference or antagonistic effects by agar dilution

method. By the agar dilution method synergy was present 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 were considered additive. Indifference was found when neither drug exhibited a decrease in MIC and an increase in the MIC was considered antagonism [18].

Synergy between different antibiotic combinations also was assessed by fractional inhibitory concentration (FIC) by using the formula of FICI (Fractional inhibitory concentration index) by agar dilution method. So, FICI =

$$\frac{\text{MIC of antibiotic in combination}}{\text{MIC of antibiotic alone}} + \frac{\text{MIC of other antibiotic in combination}}{\text{MIC of antibiotic alone}}$$

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 ≤ 4 ; antagonism, FICI of >4 [39].

2.3.3. Preparation of Mueller Hinton agar plate containing combination of two antibiotics

For each sample, four plates were prepared with 50ml Mueller Hinton agar media in each plate. The first plate contained the MIC value of two antibiotics, for that sample. The second plate contained two folds lower dilution than the MIC of two antibiotics, for that sample. The third plate contained four folds lower dilution than the MIC of both antibiotics and the fourth plate contained eight folds lower dilution than the MIC of both antibiotics for that sample. The Mueller-Hinton agar plate was impregnated with antibiotic stock solution according to the above description.

2.4. Molecular method [13, 21]

Polymerase chain reaction (PCR) was done for the detection of multidrug resistance genes in *Proteus mirabilis*.

2.4.1. Procedure of Bacterial pellet formation

A loop full of bacterial colonies from MHA media was inoculated into a microcentrifuge tube having sterile TSB and incubated overnight at 37°C. Incubated tube was centrifuged at 4000g for 10 minutes. Supernatant was discarded and tubes containing bacterial pellets were kept at -20°C for DNA extraction.

2.4.2. DNA extraction

Three hundred microlitre of sterile distilled water was added to microcentrifuge tubes having pellets and vortexed until mixed well. Then the mixture was heated at 100° C for 10 minutes in a heat block. After heating, tubes were immediately placed on ice for 5 minutes and centrifuged at 14000 g for 6 minutes at 4°C. Finally, the supernatant was taken into another microcentrifuge tube. This extracted DNA was preserved at 4°C for 7-10 days and -20°C for a long time.

2.4.3. Mixing of mastermix with primer and DNA template

PCR was performed in a final reaction volume 25 µl in a PCR tube, containing 12.5 µl of master mix (mixture of dNTP, taq polymerase, MgCl₂ and PCR buffer), 2 µl forward primer, 2 µl reverse primer (Promega Corporation, USA), 2 µl of extracted DNA and 6.5 µl of nuclease free water. After a brief vortex, the tubes were centrifuged.

2.4.4. Amplification in thermal cycler (Gene Atlas, Master cycler gradient, Japan, Model482)

PCR assays were performed in a DNA thermal cycler. After amplification products were processed for gel documentation or kept at -20°C till tested.

2.4.5. Agarose gel electrophoresis and visualization

PCR products were detected by electrophoresis on 1.5% agarose gel. Gel was prepared with 1 X TBE buffer (Tris EDTA). For 1.5% agarose gel preparation, 0.18 gram agarose powder (LE, analytic grade, Promega, Madison, USA) was mixed with a 1.25 ml TBE buffer. A comb was placed in a gel tray, the gel was poured. After solidification, 1 µl of loading dye and 5 µl of amplicon was mixed on parafilm and was loaded in agarose well. Similarly, 2 µl of 100bp DNA ladder was mixed with 1µl loading dye and was loaded. Gel electrophoresis was done in 230 voltages for 30 minutes. After electrophoresis, the gel was stained with ethidium bromide (20µl ethidium bromide in 200 ml distilled water). The gel was observed under UV transilluminator (Gel Doc, Major Science, Taiwan) for DNA bands. The DNA bands were identified according to their molecular size by comparing with the molecular weight marker (100bp DNA ladder) loaded in a separated lane.

2.4.6. Procedure of DNA Sequencing

For sequencing of bacterial DNA, purification of amplified PCR products was done by using DNA purification kits (FAVOGEN, Biotech Corp). Purified PCR products of *Proteus mirabilis* were sent to 1st Base Laboratories, Malaysia for sequencing by capillary method (ABI PRISM 3500). BLAST analysis was performed to search for homologous sequences into the GenBank at www.ncbi.nlm.nih.gov.

2.4.7. Primers used in this study [20, 5, 4, 28]

Primer	Forward	Reverse	Size (bp)
<i>fosA</i>	ATC TGT GGG TCT GCC TGT CGT	ATGCCCGCATAGGGCTTCT	271
<i>fosA3</i>	CTTAGGCTTACCA GCAGT	CGGTTATCTTTCCATACCTCAG	221
<i>fosA4</i>	CTGGCGTTTTATCAGCGGT	CTTCGCTGCGGTTGTCTTT	230
<i>fosA5</i>	TATTAGCGAAGCCGATTTTG CT	CCCCTTATACGGCTGCTCG	177
<i>fosB</i>	CAGAGATATTTTAGGGGCTG ACA	CTCAATCTATCTTCTAAACTTCCT G	312
<i>fosB2</i>	CCTGGCCGAGAAAGAGATGA G	AACCGGTTTTGCAAAAGTGCC	392
<i>fosC</i>	CCTTGCTC ACT GGA CTG	TACAAGACCCGACGCACTTC	354

<i>fosC2</i>	TGGAGGCTACTTGGATTG	AGGCTACCGCTATGGATT	209
<i>fosX</i>	TGTCCCTCACCTTCGACTCT	TGTGTGTG ATTTGC	
<i>bla</i> NDM-1	ACC GCC TGG ACC GAT GAC CA	GCCAAAGTTGGGCGCGGTTG	264
<i>bla</i> NDM-2 like	ATG ACCAGACCGCCAGA	GAG ATT GCC GAG CGA CTTG	380
<i>bla</i> OXA-10	ACG GAA AGC CAA GAG CCAT	CCCACACCAGAAAAACCAGT	354
<i>bla</i> OXA-23	GATCGGATTGGAGAACCAGA	ATT TCT GAC CGC AT TTC CAT	501
<i>bla</i> OXA-48	ATGCGTGTATTAGCCTTA TCG	AAC TAC AAG CGC ATC GAG CA	888
<i>bla</i> OXA-58	GCCATTCCCCAGCCACTTT A	CACGCATTTAGACCGAGCA	599

2.5. In vivo studies

The experiment was performed in immunocompetent male and female Swiss albino mice weighing 12-15 grams. Mice were infected by intraperitoneal injection of 12.5 units of approximately 10^4 cfu/ml bacterial inoculums using a 100 IU insulin syringe in the lower right abdomen [41]. Bacterial inoculum was obtained through a 24 hours subculture of multidrug resistant *Proteus mirabilis* in MHA media at 37°C. The animals were observed for 72 hours and the survival rates were recorded every 12 hours. Blood samples were taken as detailed below. All the samples were processed for microbiological studies.

2.5.1. In vivo different antibiotic regimens studies

To evaluate the effectiveness of the different antibiotic regimens, 50 mice were divided into 10 groups with 5 mice in each group. In group A imipenem (dose- 60mg/kg/day, in group B tigecycline (dose-20mg/kg/day), in group C amikacin (dose-15mg/kg/day), in group D imipenem and amikacin, in group E imipenem and tigecycline, in group F tigecycline and amikacin, in group G fosfomycin (dose-400mg/kg/day), in group H fosfomycin and imipenem were injected. Group I was positive control and group J was negative control. Group A, B, C, D, E, F, G, H and I were inoculated with bacterial inoculums. But Group J was not inoculated with bacterial inoculums. Group A, B, C, D, E, F, G, H and J received antimicrobial treatment. Group I was inoculated with bacterial inoculums but did not receive antimicrobial treatment. Fosfomycin was given orally and all other drugs were given intraperitoneally 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 toxic to the animal, another group of five uninfected mice (Group J) were given each antibiotic for 72 hours (uninfected treatment group/ negative control). The infected animals were observed for 72 hours of treatment and the cumulative survival rates were recorded every 12 hours. Blood samples were taken as described below.

After 72 hours of antibiotic treatment, blood samples were collected from mice by cardiac puncture aseptically. At first, the upper part of the chest was shaved by razor, washed with hexisol. After palpating the cardiac 61 pulsation with the finger pulp, the area was washed with povidone iodine, 100IU insulin syringe needle was introduced

through the skin in the heart of the mouse blindly. For blood culture 1.5 ml of each mouse's blood was collected and then inoculated in sterile conical flask with 5 ml of TSB and incubated for 24 hours at 37°C. Subculture was done in blood agar and MacConkey agar media and incubated for 24 hours at 37°C. Then the incubated plates were observed for positive or negative growth. Subculture was done after 48 hours and 72 hours if culture yielded no growth after 24 hours.

3. Results

Among 350 culture positive samples, 244 were wound swab and pus, 84 were urine and 22 were blood samples. Among 244 wound swab and pus samples, 26 (10.65%) were *P. mirabilis*, among 84 urine samples, 8 (9.52%) were *P. mirabilis* and among 22 blood samples, 3 (13.64%) were *P. mirabilis* (Table-I).

Table I: Distribution of *P. mirabilis* isolated from different culture positive samples (N=350).

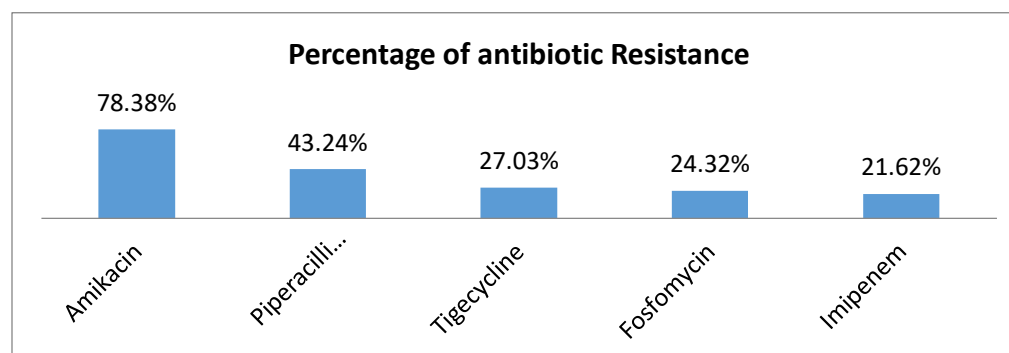
Samples	<i>P. mirabilis</i> n(%)
Wound swab and pus (N=244)	26 (10.65)
Urine (N=84)	8 (9.52)
Blood(N=22)	3 (13.64)
Total (N=350)	37 (10.57)

N= Number of culture positive samples.

n = Number of isolated *P. mirabilis* from different culture positive samples.

Among 37 isolated *Proteus mirabilis*, 78.38%, were resistant to amikacin, 43.24% were resistant to piperacillin-tazobactam, 27.03% were resistant to tigecycline, 24.32% were resistant to fosfomycin and 21.62% were resistant to imipenem (Table-II).

Table II: Antibiotic resistance pattern of *P. mirabilis*.



Of the isolated 37 *Proteus mirabilis*, 28 (75.68%) were MDR of which 18 (64.29%) were detected from wound

swab and pus, 8 (28.57%) from urine and 2 (7.14%) from blood samples; 5 (13.51%) were XDR, of which 2 (40%) were detected from wound swab and pus, 2 (40%) from urine and 1 (20%) from blood samples and 4 (10.81%) were PDR, among them 3 (75%) were detected from wound swab and pus and 1 (25%) from urine samples (Table-III).

Table III: Distribution of multidrug resistant (MDR) extensively drug resistant (XDR) and pan drug resistant (PDR) *P. mirabilis* isolated from different samples.

Sample	Total isolates	MDR isolates n(%)	XDR isolates n(%)	PDR isolates n(%)
Wound swab and pus	26	18 (64.29)	2 (40)	3(75)
Urine	8	8 (28.57)	2 (40)	1(25)
Blood	3	2 (7.14)	1 (20)	0(0.00)
Total	37	28 (75.68)	5 (13.51)	4(10.81)

N= Total number of bacteria.

n= Number of MDR/XDR/PDR *P. mirabilis* isolated from different samples.

Among 9 fosfomycin resistant *P. mirabilis*, 6 (66.67%) were from wound swab and pus and 3 (33.33%) were from urine. Among 8 imipenem resistant *Proteus mirabilis*, 4 (50%) were from wound swab and pus and 4 (50%) were from urine. Among 10 tigecycline resistant *Proteus mirabilis*, 4 (40%) were from wound swab and pus and 6 (60%) was from urine. Among 28 amikacin resistant *P. mirabilis*, 17 (60.71%) were from wound swab and pus, 9 (32.14%) were from urine and 2 (7.14%) were from blood (Table-IV).

Table IV: Distribution of fosfomycin resistant, imipenem resistant, tigecycline resistant and amikacin resistant *P. mirabilis* in different samples.

Samples	Fosfomycin resistant N=9 n (%)	Imipenem resistant N=8 n (%)	Tigecycline resistant N=10 n (%)	Amikacin resistant N=28 n (%)
Wound swab and pus	6 (66.67)	4 (50)	4 (40)	17 (60.71)
Urine	3 (33.33)	4 (50)	6 (60)	9 (32.14)
Blood	0 (0.00)	0 (0.00)	0 (0.00)	2 (7.14)
Total	9 (100.00)	8 (100.00)	10 (100.00)	28 (100)

N=Total number of resistant *P. mirabilis*.

n= Number of antibiotic resistant *P. mirabilis* from different samples.

Out of 4 fosfomycin and imipenem resistant *P. mirabilis*, 2(50%) had 8 fold reduction of MIC, one (25%) had 4 fold

reduction of MIC and one (25%) had 2 fold reduction of MIC. Among 4 imipenem and amikacin resistant *P. mirabilis*, 3(75%) had 8 fold reduction of MIC and one (25%) had 4 fold reduction of MIC; Out of 4 amikacin and tigecycline resistant *P. mirabilis*, one (25%) had 4 fold reduction of MIC, 2(50%) had 2 fold reduction of MIC and 1(25%) had no difference on MIC and Of the 4 imipenem and tigecycline resistant *P. mirabilis*, one (25%) had 4 fold reduction of MIC, one (25%) had 2 fold reduction of MIC and 2(50%) had no difference on MIC (Table-V).

Table V: Efficacy of antibiotic combinations against MDR *P. mirabilis* identified by agar dilution method (N=4).

Antibiotics in combinations	8 fold reduction Number(%)	4 fold reduction Number(%)	2 fold reduction Number(%)	At the MIC Number(%)
Fosfomycin and imipenem	2(50.00)	1(25.00)	1(25.00)	0(0.00)
Imipenem and amikacin	3(75.00)	1(25.00)	0(0.00)	0(0.00)
Amikacin and tigecycline	0(0.00)	1(25.00)	2(50.00)	1(25.00)
Imipenem and tigecycline	0(0.00)	1(25.00)	1(25.00)	2(50.00)

Out of 4 fosfomycin and imipenem resistant strains, 3 had synergism in combination as their FICI value were ≤ 0.5 and one had addition in combination as its FICI value was 1. Of the, 4 imipenem and amikacin resistant strains, all showed synergism in combinations as their FICI value were ≤ 0.50 . Out of 4 amikacin and tigecycline resistant strains, one had FICI value 0.05(synergistic), 2 had FICI value 1 (additive) and one had FICI value 2 (indifference). Out of 4 imipenem and tigecycline resistant strains, one had FICI value 0.05 (synergistic), one had FICI value 1 (additive) and 2 had FICI value 2 (indifference) (Table-VI).

Table VI: Comparison of efficacy of different antibiotic combinations by FICI formula in MDR *P. mirabilis*.

Antimicrobial Combination	MIC value by agar dilution method (µg/ml)				FIC _a + FIC _b	FICI	Effects	Mean FICI
Fosfomycin + Imipenem	Fosfomycin Alone		Imipenem Alone					0.5
	Combination		Combination					
	4096	512	64	8	0.125+0.125	0.25	Synergistic	

	1024	128	64	8	0.125+0.125	0.25	Synergistic	
	1024	256	32	8	0.25+0.25	0.50	Synergistic	
	128	64	16	8	0.5+0.5	1	Additive	
Imipenem + Amikacin	Imipenem Amikacin							0.31
	Alone Combination Alone Combination							
	64	8	32768	4096	0.125+0.125 5	0.25	Synergistic	
	64	8	2048	256	0.125+0.125 5	0.25	Synergistic	
	32	4	1024	128	0.125+0.125 5	0.25	Synergistic	
	16	4	256	64	0.25+0.25	0.50	Synergistic	
Amikacin + Tigecycline	Amikacin Tigecycline							1.125
	Alone Combination Alone Combination							
	32768	8192	128	32	0.25+0.25	0.50	Synergistic	
	2048	1024	64	32	0.5+ 0.5	1	Additive	
	1024	512	32	16	0.5+ 0.5	1	Additive	
	256	256	16	16	1+1	2	Indifference	
Imipenem + Tigecycline	Imipenem Tigecycline							1.375
	Alone Combination Alone Combination							
	64	16	128	32	0.25+0.25	0.50	Synergistic	
	64	32	64	32	0.5+ 0.5	1	Additive	
	32	32	32	32	1+1	2	Indifference	
	16	16	16	16	1+1	2	Indifference	

Note: FIC_a is fractional inhibitory concentration of one antibiotic in combination.

FIC_b is a fractional inhibitory concentration of other antibiotics in combination.

After antibiotic therapy, survival of mice by periodic observation after 12, 24, 36, 48, 60 and 72 hours of infection

were recorded. After 48 hours 2 mice from the positive control group and after 72 hours another one died. After 60 hours 4 mice from group B treated with tigecycline alone died. However, the mouse group treated with amikacin alone became profoundly sick with advancement of time in comparison to other groups (Table-VII).

Table VII: Survival rate of mouse after antibiotic therapy found by periodic observation.

Time after infection (hour)	12	24	36	48	60	72
Positive Control n(%)	5(100.00)	5(100.00)	5(100.00)	3(60.00)	3(60.00)	2(40.00)
Negative Control n(%)	5(100.00)	5(100.00)	5(100.00)	5(100.00)	5(100.00)	5(100.00)
Imipenem n(%)	5(100.00)	5(100.00)	5(100.00)	5(100.00)	5(100.00)	5(100.00)
Tigecycline n(%)	5(100.00)	5(100.00)	5(100.00)	5(100.00)	1(20.00)	1(20.00)
Amikacin n(%)	5(100.00)	5(100.00)	5(100.00)	5(100.00)	5(100.00)	5(100.00)
Imipenem+ Amikacin n(%)	5(100.00)	5(100.00)	5(100.00)	5(100.00)	5(100.00)	5(100.00)
Imipenem+Tigecycline n(%)	5(100.00)	5(100.00)	5(100.00)	5(100.00)	5(100.00)	5(100.00)
Tigecycline+ Amikacin n(%)	5(100.00)	5(100.00)	5(100.00)	5(100.00)	5(100.00)	5(100.00)
Fosfomycin n(%)	5(100.00)	5(100.00)	5(100.00)	5(100.00)	5(100.00)	5(100.00)
Fosfomycin+imipenem n(%)	5(100.00)	5(100.00)	5(100.00)	5(100.00)	5(100.00)	5(100.00)

Among the mice treated with different antibiotics, 100% of the imipenem treated group, 80% of the fosfomycin treated group and 60% of the tigecycline treated group and amikacin treated group were culture negative. Among the mice treated with different antibiotic combinations, 100% of the imipenem+amikacin treated group and fosfomycin+imipenem treated group, 80% of the imipenem+tigecycline treated group and 60% of the tigecycline+amikacin treated group were culture negative (Figure-1).

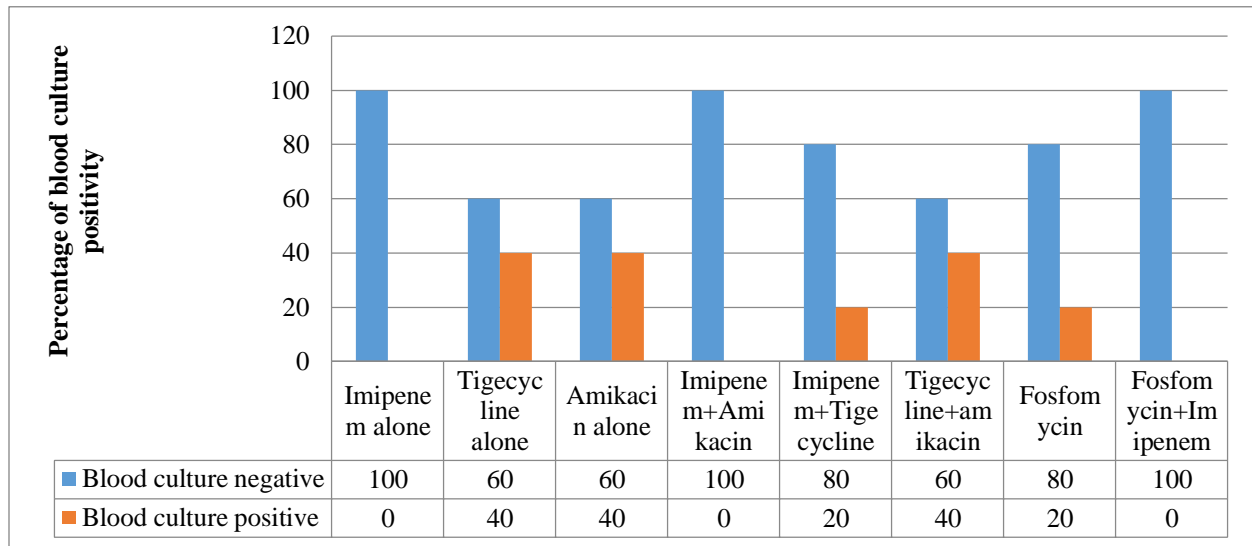


Figure 1: Result of antibiotic therapy on the clearance of MDR *P. mirabilis* from the blood of mice.

The comparison between synergism of different antibiotic combinations in MDR *P. mirabilis* *in vitro* and *in vivo* was documented (Table-VIII).

Table VIII: Comparison of synergism among different antibiotic combinations between MDR *P. mirabilis* *in vitro* and *in vivo*.

Group of combination	Synergy positive <i>in vitro</i> n (%)	Synergy positive <i>in vivo</i> n (%)
Imipenem+ Amikacin	100	100
Imipenem+ Tigecycline	25	80
Tigecycline+ Amikacin	25	60
Fosfomycin+ Imipenem	75	100

Among 8 imipenem resistant *P. mirabilis*, 5 (62.50%) were positive for *bla*NDM-1 and *bla*OXA-10, 4 (50%) were positive for *bla*NDM-2, *bla*OXA-23 and *bla*OXA-48 and 2 (25%) were positive for *bla*OXA-58 (Table-IX). Among 9 fosfomycin resistant *P. mirabilis*, 7 (77.78%) were positive for *fosA*, 4 (44.44%) were positive for *fosA*₃ and 2 (22.22%) for *fosA*₄. No *fosA*₅, *fosB*, *fosB*₂, *fosC*, *fosC*₂ and *fosX* were detected (Table-X).

Table IX: Detection of *bla*NDM-1, *bla*NDM-2 like, *bla*OXA-10, *bla*OXA-23, *bla*OXA-48, *bla*OXA-58 genes among imipenem resistant *P. mirabilis* by PCR (N=8).

Genes	Wound swab and pus n(%)	Urine n(%)	Total n(%)
<i>bla</i> NDM-1	2 (25.00)	3 (37.50)	5 (62.50)
<i>bla</i> NDM-2 like	2 (25.00)	2 (25.00)	4 (50.00)
<i>bla</i> OXA-10	3 (37.50)	2 (25.00)	5 (62.50)
<i>bla</i> OXA-23	2 (25.00)	2 (25.00)	4 (50.00)
<i>bla</i> OXA-48	1 (12.50)	3 (37.50)	4 (50.00)
<i>bla</i> OXA-58	0 (0.00)	2 (25.00)	2 (25.00)

N= Total number of imipenem resistant *P. mirabilis*.

n= Total number of imipenem resistance gene in different samples.

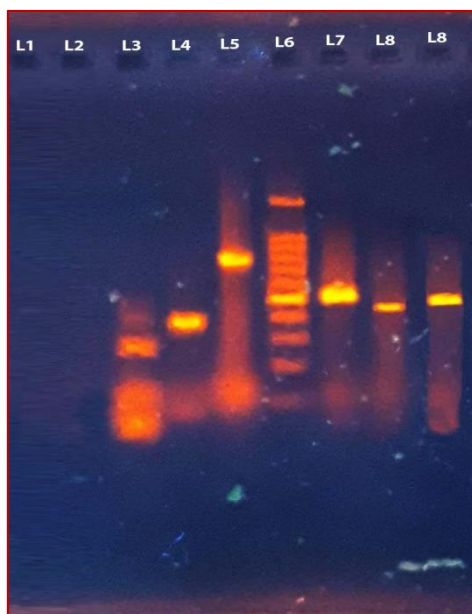
**Figure 2:** Photograph of gel electrophoresis of amplified DNA of 264bp for NDM-1 gene (lane3), amplified DNA of 354bp for OXA-10 gene (lane 4), amplified DNA of 888bp for OXA-48 gene (lane 5), 1500bp DNA ladder (lane 6), amplified DNA of 599bp for OXA-58 gene (lane 7), amplified DNA of 380bp for NDM-2 like gene (lane 8), amplified DNA of 501bp for OXA-23 gene (lane 9), negative control without DNA (TEbuffer) (lane 1), negative control *Escherichia coli* ATCC 25922 (lane 2).

Table X: Detection of *fosA*, *fosA₃*, *fosA₄*, *fosA₅*, *fosB*, *fosB₂*, *fosC*, *fosC₂* and *fosX* genes among fosfomycin resistant *P. mirabilis* by PCR (N=9).

Genes	Wound swab and pus n(%)	Urine n(%)	Total n(%)
<i>fosA</i>	5 (55.56)	2 (22.22)	7 (77.78)
<i>fosA₃</i>	2 (22.22)	2 (22.22)	4 (44.44)
<i>fosA₄</i>	1 (11.11)	1 (11.11)	2 (22.22)
<i>fosA₅</i>	0 (0.00)	0 (0.00)	0 (0.00)
<i>fosB</i>	0 (0.00)	0 (0.00)	0 (0.00)
<i>fosB₂</i>	0 (0.00)	0 (0.00)	0 (0.00)
<i>fosC</i>	0 (0.00)	0 (0.00)	0 (0.00)
<i>fosC₂</i>	0 (0.00)	0 (0.00)	0 (0.00)
<i>fosX</i>	0 (0.00)	0 (0.00)	0 (0.00)

Sequencing of *fosA*, *fosA₃*, *fosA₄*, *NDM-1*, *NDM-2 like*, *OXA-10*, *OXA-23* and *OXA-58* were done. Of this, *NDM-2 like* gene got a GenBank accession number and that was [MW672265](#).

4. Discussion

Multidrug resistance is the most important problem in antibiotic resistance due to difficulty in treating multidrug resistant microorganisms and exponential increase in MDR over the last decade [36]. *Proteus mirabilis* is responsible for a wide range of infections that affect the urinary tract, the respiratory tract, burns, wounds and the feet of individuals with diabetes. They are highly resistant to antimicrobial agents, and new therapeutic options are therefore needed to combat this pathogen [17].

There is limited data regarding *Proteus mirabilis* as few studies in Bangladesh have been conducted previously. Therefore the present study was designed to find out multidrug resistance among the isolated *Proteus mirabilis* phenotypically and genotypically with their antibiotic resistance pattern and evaluation of the efficacy of antibiotic combination against MDR *Proteus mirabilis* both *in vitro* and *in vivo*.

24.32% MDR *Proteus mirabilis* were resistant to fosfomycin. Clinical use of fosfomycin in Bangladesh is rare and there is very little data regarding fosfomycin resistance. The reason behind such finding in present study might be due to horizontal transfer of resistance genes between different species. Recent studies indicate that the

recombination of plasmid- encoding carbapenemase and fosfomycinase occurs via mobile elements, presenting new treatment challenges [43].

Resistance to imipenem which is used as a last resort of drugs in the health care settings was found 21.62% in *P. mirabilis*. *P. mirabilis* isolates present an elevation in the resistance level to imipenem due to many reasons: the loss of outer membrane porins, decreased expression of PBP1a or reduced binding of imipenem by PBP2 [16]. ImpR (outer membrane protein) over expression resulted in increased carbapenem MICs in the laboratory strain and clinical isolates. Development of resistance against imipenem in *P. mirabilis* is also due to the absence of 24 kDa OMP [42].

Though colistin and tigecycline are considered as the most sensitive drugs against CRE but *P. mirabilis* is intrinsically resistant to tigecycline and colistin [25]. 27.03% *P. mirabilis* were found resistant to tigecycline. For tigecycline, the genome of *P. mirabilis* contains a homolog of the *Esch. coli* AcrAB efflux system and this gene cluster appears to be responsible, at least in part, for the intrinsic reduced susceptibility to tigecycline in *P. mirabilis* [44].

Multidrug resistance to *P. mirabilis* could be a result of continuous use of broad spectrum antimicrobials and non adherence to hospital antibiotic therapy, horizontal transfer of drug resistance genes among different bacteria as most patients already have been harboring the resistant organisms in their hospital staying period [33].

The present study observed 100% synergism with the combination of imipenem plus amikacin, 75% with fosfomycin plus imipenem and 25% with amikacin plus tigecycline and imipenem plus tigecycline. Fosfomycin in combination with either amino glycosides and carbapenems or piperacillin-tazobactam is the effective combination against MDR *Enterobacteriaceae* [15]. As with infections with gram-negative bacteria, antimicrobial synergy has traditionally been seen with β -lactam-aminoglycoside combinations. β -lactam mediated disturbance of the cell walls of gram-negative bacilli facilitates passage of amino glycosides into the periplasmic space [27]. Interestingly, time-kill assays disclosed a bactericidal synergism between tigecycline and amikacin against *P. vulgaris*. Such combinations could be of clinical relevance as the organisms they target belong to potentially problematic multi resistant species and to bacteria inadequately inhibited by tigecycline [12].

In the present study, mice were observed periodically for survival for 72 hours after intervention. Mice of the positive control group became profoundly sick and after 48 hours 2 mice had died and one more died after 72 hours. 4 mice of the tigecycline alone group died after 60 hours. In animal studies, a common outcome of therapy with tigecycline in combination was prolonged survival and/or reductions in tissue colony counts [12].

In vivo combination of imipenem plus amikacin and imipenem plus fosfomycin showed 100% synergism, imipenem plus tigecycline showed 80% synergism and amikacin and tigecycline showed 60% synergism. Fosfomycin is

reported to mitigate *in vivo* and *in vitro* synergy with carbapenem against KPC producing multidrug resistant *Enterobacteriaceae* [40, 32]. The best *in vivo* result appeared in the group treated with imipenem combinations. Among imipenem resistant *P. mirabilis*, 62.50% and 50% were positive for *bla*NDM-1 and *bla*NDM-2 like genes detected by PCR. In Bangladesh, *bla*NDM-1 was first identified in *K. pneumoniae* in 2008 thereafter some reports described detection of *bla*NDM-1 in some GNR species, [13, 4]. Since the first identification of *bla*NDM-1 in 2008, worldwide attention was attracted to this carbapenemase because of its rapid dissemination among *Enterobacteriaceae* and *Acinetobacter* spp. clinical isolates, as well as those colonizing humans and contaminating environments [10].

Among imipenem resistant *P. mirabilis* 62.50% were positive for *bla*OXA-10, 50% were positive for *bla*OXA-23 and *bla*OXA-48 and 25% were positive for *bla*OXA-58. It was found, *bla*OXA-10 was horizontally transferable in *Enterobacteriaceae* family which was supported by transformation and conjugation [26]. Considering the transferable elements surrounding *bla*OXA-23 as well as the phenotype of relative susceptibility of this isolate, *bla*OXA genes may be spreading silently “under the radar” [23]. The *bla*OXA-48 gene is relatively common in Europe, especially in Mediterranean countries [35]. In a study from Turkey, 92% of carbapenem resistant *Enterobacteriaceae* were OXA-48 like producers [3]. In addition to Europe, OXA-48-like enzymes have been found worldwide in *Enterobacteriaceae* [31]. The *bla*OXA-58 represents one of the class D carbapenemase types that are major carbapenem resistance determinants in *Acinetobacter* spp. being extremely rare in *Enterobacteriaceae*. The first report of its presence in diverse enterobacteria was from Sierra Leone, followed by recent papers on *Proteus mirabilis* from Belgium and Germany. Only 6 such isolates, exclusively *P. mirabilis*, have been reported in Europe to date, of which German and Polish isolates shared unique plasmids [8].

Among the fosfomycin resistant *P. mirabilis*, 77.78% were positive for *fosA*, 44.44% for *fosA*₃ and 22.22% were positive for *fosA*₄ genes. The fosfomycin resistance protein FosA, are metalloenzymes that catalyse the nucleophilic addition of the tripeptide 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 FosA₃, FosA₄, FosA₅ and FosC₂ [14].

DNA sequence of amplified PCR product and translated nucleotide base sequence of *fosA*, *fosA*₃ and *fosA*₄ showed point mutations including base substitution and frameshift mutation at multiple positions. Large and small deletions are the main source of gene-inactivating mutations followed by insertions/duplications [19]. In addition a considerable number of point mutations had been detected, including truncation by nonsense and missense mutations [37].

5. Conclusion

From *in vivo* and *in vitro* experiments, it may be clear that imipenem and amikacin is the best combination for MDR *P. mirabilis*. The second best *in vivo* and *in vitro* effective combination is imipenem and fosfomycin.

Repurposing of older antimicrobials like fosfomycin and imipenem combination therapy may be good options for the treatment of infection caused by them.

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