Original Research Article

Evaluation of efficacy of fosfomycin combined with other antimicrobials against MDR proteus mirabilis in vitro and in vivo

ABSTRACT

Aims: The aim of this study is to evaluate antibiotic combinations Against MDR Proteus Mirabilis.

Study design: This was a cross sectional study.

Place and Duration of Study: Department of Microbiology Dhaka Medical College Dhaka Bangladesh from June 2019 to July 2020.

Methodology: Total 570 urine, blood or wound swab and pus samples were collected from the patients admitted at different words in Dhaka Medical College Hospital after taking informed written consent. Proteus mirabilis were isolated and identified by observing pale of colorless colonies in Mac Conkey's agar media and biochemical tests. Antimicrobial susceptibility test of various drugs were done by modified Kirby–Bauer disc diffusion method (11) and zones of inhibition were interpreted according to CLSI guidelines (12). Escherichia coli ATCC 29212 was used as control strain to assess the performance of the method. Minimum inhibitory concentrations (MIC) of various drugs were determined using agar dilution method (13), (14). To prepare bacterial inoculum, the turbidity of bacterial suspension in normal saline was compared with 0.5 McFarland turbidity standards. Antibiotic Combinations of various drugs were used to see synergistic, additive, indifferent or antagonistic effects by agar dilution method both in vivo and in vitro. After 72 hours of antibiotic treatment, blood samples were collected from mouse by cardiac puncture aseptically and subculture was done in Blood agar and MacConkey agar media which was incubated for 24 hours at 37ºC .Then the incubated plates were observed for positive or negative growth (17).

Results: Out of 44 proteus mirabilis 29 were multidrug resistant (MDR). Among the MDR proteus mirabilis 7(24.14%), 20(68.97%), 11(37.93%) and 13(44.83%) were resistant to fosfomycin, amikacin, piperacilin- tazobactam and tigecycline, respectively. The MIC value for fosfomycin, amikacin, piperacilin- tazobactam and tigecycline ranged from 64 µg/ml to 4096µg/ml, from 256 µg/ml to 16,384µg/ml, from 128/4 µg/ml to 1024/4µg/ml and from 8 µg/ml to 64µg/ml, respectively. Out of 44 isolated *P. mirabilis* 13 (29.55%) were resistant to fosfomycin. Out of 30 amikacin resistant *P. mirabilis*, 3 (10.00%) had MIC of 16,384µg/ml, 4 (13.33%) had MIC of 8,192µg/ml, 11 (36.67%) had MIC of 4,096µg/ml, 3 (10.00%) had MIC of 2,048µg/ml, 4 (13.33%) had MIC of 1,024 µg/ml, and 5 (16.67%) had MIC of 256 µg/ml (Table-2). Out of 18 piperacillin-tazobactam resistant *P. mirabilis*, 4 (22.22%) had MIC of 1024/4µg/ml, 4 (22.22%) had MIC of 512/4µg/ml, 5 (27.78%) had MIC of 256/4µg/ml and 5 (27.78%) had MIC of 128/4 µg/ml (Table-3). Nineteen (43.18%) were tigecycline resistant and 6 (13.64%) sensitive *P. mirabilis* were detected by this method. Out of 4 fosfomycin and amikacin resistant *P. mirabilis,* one (25%) had 8 fold reduction of MIC, 3 (75%) had 4 fold reduction of MIC (Table-5). Out of 4 fosfomycin and amikacin resistant strains, all showed synergism in combination as their FICI value were ≤0.50. Out of 4 fosfomycin and tigecycline resistant strains, one had FICI value 0.50 (synergistic), two had FICI value 1 (additive) and one had FICI value 0.25 (synergistic). Out of 4 fosfomycin and piperacillin-tazobactam resistant strains, 2 had FICI value 0.50 (synergistic) and one had FICI value 0.25 (synergistic), one had FICI value 1 (additive). All the mice in the positive control group were bacteramic. All the mice in the negative control were blood culture negative. In the group treated with fosfomycin, 20% were culture negative. In the group treated with tigecycline,

piperacillin- tazobactam none was culture negative. In the group treated with amikacin, 20% were culture negative. In the group treated with fosfomycin and amikacin, 100% were culture negative. In the group treated with fosfomycin and tigecycline, 80% were culture negative. In the group treated with fosfomycin and Piperacilin- tazobactam 80% were culture negative. Comparison between synergism of different antibiotic combinations in MDR *P. mirabilis in vitro* and *in vivo* was showed in (Table-10). While combining fosfomycin with amikacin, they showed 100% synergistic effect both *in vitro* and *in vivo*, while combining tigecycline with fosfomycin, they showed 50% synergistic effect *in vitro* and 80% synergistic effect in vivo and while combining fosfomycin with piperacillin-tazobactam, they showed 75% synergistic effect *in vitro* and 80% synergistic effect *in vivo*.
Conclusion: Combination therapy is good treatment option for MDR *P. mirabilis* both *in vitro* and *in vivo*. Fosfomycin and amikacin was the most effective combination in both *in vitro* and *in vivo* which showed 100% synergism. From the present study it appeared that combination of fosfomycin and amikacin may be a good option for treating infection by MDR *P. mirabilis*.

Keywords: Antibiotic combination; MDR; FICI; proteus mirabilis, fosfomycin.

1. INTRODUCTION

Proteus species are third most common causes of hospital-acquired infections (1) and the primary infection in patients with indwelling urinary catheters (2). It is also known as opportunistic pathogens that involve in various infections (3). Among *Proteus* species 90% of proteus infection is caused by *P. mirabilis which* shows swarming motility and urease activity (4). It is an important cause of community-acquired and health care-associated infections, including those involving the urinary tract (46%), surgical wound (24%), lower respiratory tract (30%) and the bloodstream (17%) itself (5). It is also a common cause of complicated urinary tract infections (UTIs) in patients with anatomical or functional problems (6). The use of fosfomycin has attracted renewed interest for the treatment of serious systemic infections caused by multidrug-resistant *Enterobacteriaceae* (7). The WHO has classified fosfomycin in the category of a 'critically important' antimicrobial for investigation in light of its efficacy MDR gram negative organism (8) . Recently fosfomycin resistance has been reported in MDR *P.mirabilis* in DMCH (9). Combination of two antibiotics may provide broader spectrum coverage, decreases the emergence of resistance & dose related toxicity (10). The increasing antimicrobial resistance of *P. mirabilis* causing nosocomial infection is a great threat to us. It has already showed high level of resistance (more than 60%) to some reserve group drugs like polymyxin B, tigecycline, and nitrofurantoin. Biofilm formation further complicates the treatment options by resisting antimicrobial penetration and protecting bacteria.

2. MATERIAL AND METHODS

2.1 Isolation and identification of organisms: Total 570 urine, blood or wound swab and pus samples were collected from the patients admitted at different words in Dhaka Medical College Hospital after taking informed written consent. Proteus mirabilis were isolated and identified by observing pale of colorless colonies in Mac Conkey's agar media and biochemical tests.

2.2 Antimicrobial susceptibility test: Susceptibility of isolates to 10 antimicrobials (amikacin (30µg), piperacillin-tazobactam (100/10µg), imipenem (10µg), ciprofloxacin (30µg), cefepime (30µg), ceftazidime (30µg), ceftriaxone (30µg), cefoxitin (30µg), amoxiclav (amoxicillin 20µg & clavulanic acid 10µg), Sulphamethoxazole/ Trimethoprim were done by modified Kirby–Bauer disc diffusion method (11) and zones of inhibition were interpreted

according to CLSI guidelines (12). Escherichia coli ATCC 29212 was used as control strain to assess the performance of the method. Fosfomycin and tigecycline susceptibility were tested by agar dilution method of minimum inhibitory concentration (MIC).

2.3 Determination of MIC: Minimum inhibitory concentration (MIC) of amikacin, piperacilin- tazobactam, tigecycline, and fosfomycin were determined using agar dilution method (13), (14). Commercially available 4g/0.5g piperacillin-tazobactam injection vial (Renata limited, Gazipur, Bangladesh) was added to 20 ml normal saline used as piperacillin-tazobactam stock solution and the concentration was 4500mg/20 ml (225/1 ml). For each plate 50 ml Mueller-Hinton media was prepared. 50 ml sterile Mueller-Hinton agar was mixed with 4 µl, 8 µl, 16 µl, 32 µl, 64 µl, 128 µl, 256 µl and 512 µl of piperacillin-tazobactam stock solution to achieve concentration of 16 µg/ml, 32 µg/ml, 64 µg/ml, 128 µg/ml, 256 µg/ml, 512 µg/ml, 1024µg/ml and 2048 µg/ml per plate respectively. 50 mg base of commercially available tigecycline injection vial (Incepta Pharma Ltd, Dhaka) was added to 50 ml normal saline to make a concentration of 1mg/ml.For each plate 50 ml Mueller-Hinton medium was prepared. 50 ml sterile Mueller-Hinton agar was mixed with 100 µl, 200 µl, 400 µl, 800 µl, 1600 µl, 3200 µl, 6400 µl, 12800 µl of tigecycline stock solution to achieve concentration of 2 µl/ml, 4 µg/ml, 8 µg/ml, 16 µg/ml, 32 µg/ml, 64 µg/ml, 128 µg/ml and 256 µg/ml per plate, respectively. For Preparation of fosfomycin stock solution three thousand mg base of commercially available fosfomycin (Beximco Pharma Limited) was added 150 ml of distilled water to make a concentration of 20mg/ml. For preparation of Mueller-Hinton agar plate containing different concentration of fosfomycin for each plate 50 ml Mueller-Hinton media containing 1.25mg glucose-6-phosphate was prepared. 50 ml sterile Mueller-Hinton agar was mixed with 80µl, 160µl, 320µl, 640µl, 1280µl, 2560µl, 5120µl, 10240µl of fosfomycin stock solution to achieve the concentration 32µg/ml, 64µg/ml, 128µg/ml, 256µg/ml, 512µg/ml, 1024 µg/ml, 2048µg/ml and 4096µg/ml, respectively. For preparation of amikacin stock solution commercially available amikacin injection ampoule (ACI Pharma Limited, Dhaka) was used as stock solution and the concentration was 250mg/ml. For preparation of Mueller-Hinton agar plate containing different concentration of amikacin and each plate 50 ml Mueller-Hinton media was prepared. 50 ml sterile Mueller Hinton agar was mixed with 3.2µl, 6.4µl, 12.8µl, 25.6µl, 51.2µl, 102.4µl, 204.8µl and 409.6µl of amikacin stock solution to achieve the concentration 16µg/ml, 32µg/ml, 64µg/ml, 128µg/ml, 256µg/ml, 512µg/ml, 1024 µg/ml, 2048 µg/ml, respectively.

2.4 Inoculum preparation: To prepare bacterial inoculum, the turbidity of bacterial suspension in normal saline was compared with 0.5 McFarland turbidity standard and as 0.5 McFarland turbidity standard contain 1×10^8 cfu/ml, 10 times dilution (one ml test inoculums compared to turbidity standard added when with 9 ml of normal saline) of test inoculums was done to achieve 1×10^7 cfu/ml. To obtain 104 cfu/spot on agar surface one µl of 10 times diluted inoculums were placed on Muller-Hinton agar plate. All the inoculated plates were incubated aerobically at 37ºC overnight. The lowest concentration of antibiotic impregnated Muller-Hinton agar media showing no visible growth was considered as MIC of that drug for that strain. Escherichia coli ATCC strain 25922 was used as control organism.

2.5 Antibiotic combinations:

2.5.1 in vitro: Combinations of fosfomycin- amikacin, fosfomycin-piperacilin- tazobactam and fosfomycin-tigecycline against MDR species including resistance to the drugs used in combination were undertaken to see synergistic, additive, indifferent or antagonistic effects by agar dilution method. For each sample 4 plates were prepared with 50ml Muller-Hinton agar media in each plate. The first plate of combination contained the MIC of each antibiotic for that sample. The 2nd plate contained two fold lower dilutions than the MIC of both antibiotics for that sample. The 3rd plate contained four fold lower dilutions than the MIC of

both antibiotics for that sample. The 4th plate contained eight fold lower dilutions than the MIC of both antibiotics for that sample. The Muller-Hinton agar plate was impregnated with respective amount of antibiotic stock solution according to above description. Then inoculum was prepared as mentioned above and all the plates were inoculated with 1 µl of inoculum followed by incubation at 37º overnight. In antibiotic combination Synergy was considered by agar dilution method when there was a fourfold or greater reduction in the MICs of both antibiotics. A reduction of less than four fold in the MICs of both antibiotics was considered additive. Indifference was found when neither drug exhibited a decreasing MIC, and an increase in the MIC was considered antagonism (14). The fractional inhibitory concentration index (FICI) was also determined to evaluate the effects of antimicrobial combination as follow: synergistic (FICI≤0.5), partial synergistic (0.5< FICI <1), additive (FICI=1), indifferent (1< FICI ≤ 4), antagonistic (FICI >4), and calculated using the following equation (15). FICI = MIC of drug A in combination MIC of drug A alone. All the tests were performed in triplicate.

2.5.2 In vivo study: Forty five mice (swis albino) were used for this purpose. The experiments were performed in immune competent female mouse weighting 15-20 grams. The mice were purchased from ICDDRB breeding house Dhaka, Bangladesh. Animals were maintained under adequate temperature (22-24º C) and humidity. The mice received a standard diet obtained from ICDDRB and sterile water. Mice were divided into 9 groups (A, B, C, D, E, F,G, H, I) with 5 mouse in each group. Group A, B, C, D, E, F, G were infected by intra-peritoneal injection of 250 µl of approximately 10^4cfu/ml bacterial inoculums using a 100 unit insulin syringe in the lower right abdomen (16). Group H was not inoculated with bacterial inoculums. Group H was regarded as negative control group. Bacterial inoculums were obtained through a 24 hours subculture of a MDR (fosfomycin, amikacin and piperacilin- tazobactam resistant) p. mirabilis in MacConkey agar media at 37ºC. Group A, B, C, D, E, F,G received antimicrobial treatment intra-peritonealy after 4 hours of infection at 12 hours interval for 3 days. Group A, B, C, and D were treated individually only with fosfomycin (400mg/kg), tigecycline (20mg/kg) and amikacin (15mg/kg) and piperacilin tazobactam (90mg/kg), respectively. Group E received fosfomycin plus tigecycline (400mg/kg + 20mg/kg), Group F received fosfomycin plus amikacin (400mg/kg + 15mg/kg) and Group G received fosfomycin plus piperacilin-tazobactam (400mg/kg + 90mg/kg) combination. Group H did not receive antimicrobial treatment. Group H was regarded as positive control. In order to confirm that these drugs were not toxic to the animal, another group of five uninfected mouse (Group I) were given each antibiotic for 72 hours (uninfected treat group/negative control). The animals were observed for 72 hours and the survival mice were recorded every 12 hours. Blood samples were taken as detailed below. All the blood samples were processed for microbiological studies. The infected animals were observed for 72 hours of treatment and the cumulative survival rates were recorded every 12 hours.

2.6 Microbiological study: After 72 hours of antibiotic treatment, blood samples were collected from mouse by cardiac puncture aseptically. At first, upper part of the chest was shaved by razor, and then washed with alcohol pad followed by povidon iodine. After palpating the cardiac pulsation with the finger pulp, the area was washed with povidon iodine, then 100 unit insulin syringe needle was introduced through the skin in the heart of the mouse blindly. For blood culture 1.5ml of each mouse's blood was collected and then incubated in sterile conical flask with 5 ml of TSB and incubated for 24 hours at 37ºC. Subculture was done in Blood agar and MacConkey agar media and incubated for 24 hours at 37ºC .Then the incubated plates were observed for positive or negative growth (17).

3. RESULTS

Out of 570 samples 44 were proteus mirabilis among which 29 were multidrug resistant. Among the MDR proteus mirabilis 7(24.14%), 20(68.97%), 11(37.93%) and 13(44.83%) were resistant to fosfomycin, amikacin, piperacilin- tazobactam and tigecycline, respectively. The MIC value for fosfomycin, amikacin, piperacilin- tazobactam and tigecycline ranged from 64 µg/ml to 4096µg/ml, from 256 µg/ml to 16,384µg/ml, from 128/4 µg/ml to 1024/4µg/ml and from 8 µg/ml to 64µg/ml, respectively.

MIC of fosfomycin among isolated *Proteus mirabilis* was detected by agar dilution method. Out of 44 isolated *P. mirabilis* 13 (29.55%) fosfomycin resistant *P. mirabilis* were detected. Among the fosfomycin resistant *P. mirabilis,* 2 (4.55%) had MIC **≥** 4096µg/ml, one (2.27%) had 2048 µg/ml, one (2.27%) had MIC 1024 µg/ml, 2 (4.55%) had MIC 512 µg/ml**,** one (2.27%) had MIC 256 µg/ml, one (2.27%) had MIC 128 µg/ml and 5 (38.46%) had MIC 64 µg/ml. 31 (70.45%) had MIC of 32µg/ml. (Table-1)

Table-1: MIC of fosfomycin among isolated *P. mirabilis* detected by agar dilution method (N=44).

MIC of fosfomycin (µg/ml)	Fosfomycin susceptibility of *P.mirabilisn* (%)
≥ 4096	2 (4.55)
2048	1 (2.27)
1024	1 (2.27)
512	2 (4.55)
256	1 (2.27)
128	1 (2.27)
64	5 (11.36)
32	31 (70.45)
Total	**44 (100.00)**

EUCAST (2020) breakpoint of MIC of fosfomycin for *Enterobacteriaceae*.
Sensitive: ≤ 32 µg/ml.
Resistant: >32 µg/ml.

MIC of amikacin by agar dilution method among amikacin resistant *P. mirabilis* was detected by disc diffusion method. Out of 30 amikacin resistant *P. mirabilis*, 3 (10.00%) had MIC of 16,384µg/ml, 4 (13.33%) had MIC of 8,192µg/ml, 11 (36.67%) had MIC of 4,096µg/ml, 3 (10.00%) had MIC of 2,048µg/ml, 4 (13.33%) had MIC of 1,024 µg/ml, and 5 (16.67%) had MIC of 256 µg/ml.(Table-2)

Table-2: MIC of amikacin among amikacin resistant *P. mirabilis* detected by disc diffusion method (N=30).

MIC of amikacin (µg/ml)	Amikacin resistant *P. mirabilis* n (%)

≥ 65,536	0 (0.00)
32,768	0 (0.00)
16,384	3 (10.00)
8,192	4 (13.33)
4,096	11 (36.67)
2,048	3 (10.00)
1,024	4 (13.33)
512	0 (0.00)
256	5 (16.67)
128	0 (0.00)
64	0 (0.00)
32	0 (0.00)
≤ 16	0 (0.00)
Total	**30 (100)**

CLSI (2020) breakpoint of MIC of amikacin for *Enterobacteriaceae.*
Sensitive: ≤ 16 µg/ml.
Intermediate: 32 µg/ml.
Resistant: ≥ 64 µg/ml.

MIC of piperacillin-tazobactam by agar dilution method among piperacillin-tazobactam resistant *P. mirabilis,* was detected by disc diffusion test. Out of 18 piperacillin-tazobactam resistant *P. mirabilis*, 4 (22.22%) had MIC of 1024/4µg/ml, 4 (22.22%) had MIC of 512/4µg/ml, 5 (27.78%) had MIC of 256/4µg/ml and 5 (27.78%) had MIC of 128/4 µg/ml.(Table-3)

Table-3: MIC of Piperacillin-tazobactam among piperacillin-tazobactam resistant *P. mirabilis* (N=18)

MIC of piperacillin-tazobactam(µg/ml)	**Piperacillin-tazobactam resistant *P.mirabilis*n(%)**
2048/4	0 (0.00)
1024/4	4 (22.22)
512/4	4 (22.22)
256/4	5 (27.78)
128/4	5 (27.78)
64/4	0 (0.00)
32/4	0 (0.00)

16/4	0 (0.00)
Total	**18 (100.00)**

CLSI (2020) breakpoint of MIC of piperacillin-tazobactam for *Enterobacteriaceae*
Sensitive ≤16µg/ml
Intermediate 32µg/ml
Resistant ≥ 64µg/ml

MIC of tigecycline was detected by agar dilution method among the isolated *P. mirabilis*. Nineteen (43.18%) were tigecycline resistant and 6 (13.64%) sensitive *P. mirabilis* were detected by this method. Out of 44 isolated *P. mirabilis*, 3 (6.82%) had MIC of 64µg/ml, 8 (18.18%) had MIC of 32 µg/ml, 4 (9.09%) had MIC of 16 µg/ml, 4 (9.09%) had MIC of 8 µg/ml, 19 (43.18%) had MIC of 4 µg/ml and 6 (13.64%) had MIC of 4 µg/ml. (Table-4)

Table-4: MIC of tigecycline among isolated *P. mirabilis* detected by agar dilution method (N=44)

MIC of Tigecycline (µg/ml)	**Tigecycline susceptibility of *P.mirabilis* n (%)**
≥256	0 (0.00)
128	0 (0.00)
64	3 (6.82)
32	8 (18.18)
16	4 (9.09)
8	4 (9.09)
4	19 (43.18)
2	6 (13.64)
Total	**44 (100.00)**

FDA breakpoint of MIC of tigecycline for *Enterobacteriaceae*
Sensitive ≤ 2 µg/ml
Intermediate 4 µg/ml
Resistant ≥ 8 µg/ml

Efficacy of fosfomycin and amikacin combination against multidrug resistant *P. mirabilis was* identified by agar dilution method. Out of 4 fosfomycin and amikacin resistant *P. mirabilis,* one (25%) had 8 fold reduction of MIC, 3 (75%) had 4 fold reduction of MIC. (Table-5)

Table-5: Efficacy of fosfomycin and amikacin combination against MDR *P. mirabilis* identified by agar dilution method (N=4).

Reduction of MIC	Number (%)
8 fold reduction	1 (25.00)
4 fold reduction	3 (75.00)
2 fold reduction	0 (0.00)
At the MIC	0 (0.00)

Efficacy of fosfomycin and tigecycline combination against multidrug resistant *P. mirabilis* was identified by agar dilution method. Out of 4 fosfomycin and tigecycline resistant *P. mirabilis* one (25%) had 8 fold reduction of MIC, one (25%) had 4 fold reduction of MIC, 2 (50%) had 2 fold reduction of MIC. (Table-6)

Table-6: Efficacy of fosfomycin and tigecycline combination against MDR *P. mirabilis* identified by agar dilution method (N=4).

Reduction of MIC	Number (%)
8 fold reduction	1 (25.00)
4 fold reduction	1 (25.00)
2 fold reduction	2 (50.00)
At the MIC	0 (0.00)

Efficacy of fosfomycin and piperacillin-tazobactam combination against multidrug resistant *P. mirabilis* was identified by agar dilution method. Out of 4 fosfomycin and piperacillin-tazobactam resistant *P. mirabilis* one (25%) had 8 fold reduction of MIC, 2 (25%) had 4 fold reduction of MIC, one (25%) had 2 fold reduction of MIC. (Table-7)

Table-7: Efficacy of fosfomycin and piperacillin-tazobactam combination against MDR *P. mirabilis* identified by agar dilution method (N=4).

Reduction of MIC	Number (%)
8 fold reduction	1 (25.00)
4 fold reduction	2 (50.00)
2 fold reduction	1 (25.00)
At the MIC	0 (0.00)

Comparison of efficacy of different antibiotic combinations by using fractional inhibitory concentration index (FICI) formula in 4 MDR *P. mirabilis* was showed in Table-8. Out of 4 fosfomycin and amikacin resistant strains, all showed synergism in combination as their FICI value were ≤0.50. Out of 4 fosfomycin and tigecycline resistant strains, one had FICI value 0.50 (synergistic), two had FICI value 1 (additive) and one had FICI value 0.25 (synergistic). Out of 4 fosfomycin and piperacillin-tazobactam resistant strains, 2 had FICI value 0.50 (synergistic) and one had FICI value 0.25 (synergistic), one had FICI value 1 (additive).

Table-8: 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 + Amikacin	Fosfomycin		Amikacin					0.44
	Alone	Combination	Alone	Combination				
	512	128	16384	4096	0.25+0.25	0.50	Synergistic	
	4096	512	8192	1024	0.125+0.125	0.25	Synergistic	
	4096	1024	2048	512	0.25+0.25	0.50	Synergistic	
	256	64	1024	256	0.25+0.25	0.50	Synergistic	
Fosfomycin + Tigecycline	Fosfomycin		Tigecycline					0.69
	Alone	Combination	Alone	Combination				
	1024	2048	16	32	0.50+0.50	1	Additive	
	1024	4096	32	128	0.25+0.25	0.50	Synergistic	
	512	1024	16	32	0.50+0.50	1	Additive	
	512	4096	8	64	0.125+0.125	0.25	Synergistic	
Fosfomycin +	Fosfomycin		piperacillin-tazobactam					

Piperacillin-tazobactam	Alone	Combination	Alone	Combination			0.56
	512	64	512/4	64/4	0.125+0.125	0.25	Synergistic
	2048	512	1024/4	256/4	0.25+0.25	0.50	Synergistic
	4096	1024	512/4	128/4	0.25+0.25	0.50	Synergistic
	2048	1024	1024/4	512/4	0.50+0.50	1	Additive

Effects of antibiotic therapy on survival of mice were found by periodic observation after 12, 24, 36, 48, 60 and 72 hours of infection. However, after 48 hours 2 mice of positive control group and after 60 hours another one died. After 48 hours mice treated with only tigecycline 2 mice died and one mice of each group treated with only fosfomycin and piperacilin-tazobactam died and after 72 hours another one treated by only fosfomycin and piperacilin-tazobactam died. After 60 hours one mouse treated with only amikacin group died. (Table-9)

Table-9: Survival rate of mouse after antibiotic therapy found by periodic observation.

Antibiotics		**Time in hour after infection**				
	12 n (%)	**24 n (%)**	**36 n (%)**	**48 n (%)**	**60 n (%)**	**72 n (%)**
Fosfomycin	5 (100.00)	5 (100.00)	5 (100.00)	4 (80.00)	4 (80.00)	3 (60.00)
Tigecycline	5 (100.00)	5 (100.00)	3 (60.00)	3 (60.00)	3 (60.00)	3 (60.00)
Amikacin	5 (100.00)	5 (100.00)	5 (100.00)	5 (100.00)	4 (80.00)	4 (80.00)
Piperacillin-tazobactam	5 (100.00)	5 (100.00)	5 (100.00)	4 (80.00)	4 (80.00)	3 (60.00)
Fosfomycin + Tigecycline	5 (100.00)	5 (100.00)	5 (100.00)	5 (100.00)	5 (100.00)	5 (100.00)
Fosfomycin + Amikacin	5 (100.00)	5 (100.00)	5 (100.00)	5 (100.00)	5 (100.00)	5 (100.00)
Fosfomycin+ piperacillin-tazobactam	5 (100.00)	5 (100.00)	5 (100.00)	5 (100.00)	5 (100.00)	5 (100.00)
Positive control	5 (100.00)	5 (100.00)	3 (60.00)	3 (60.00)	2 (40.00)	2 (40.00)
Negative control	5 (100.00)	5 (100.00)	5 (100.00)	5 (100.00)	5 (100.00)	5 (100.00)

n= Number of mice that survived.

Effects of antibiotic therapy on the clearance of MDR *P. mirabilis* from the blood among different groups of mouse were showed in fig-1. All the mice in the positive control group were bacteramic. All the mice in the negative control were blood culture negative. In the group treated with fosfomycin, 20% were culture negative. In the group treated with tigecycline, piperacillin- tazobactam none was culture negative. In the group treated with amikacin, 20% were culture negative. In the group treated with fosfomycin and amikacin, 100% were culture negative. In the group treated with fosfomycin and tigecycline, 80% were culture negative. In the group treated with fosfomycin and Piperacilin- tazobactam 80% were culture negative.

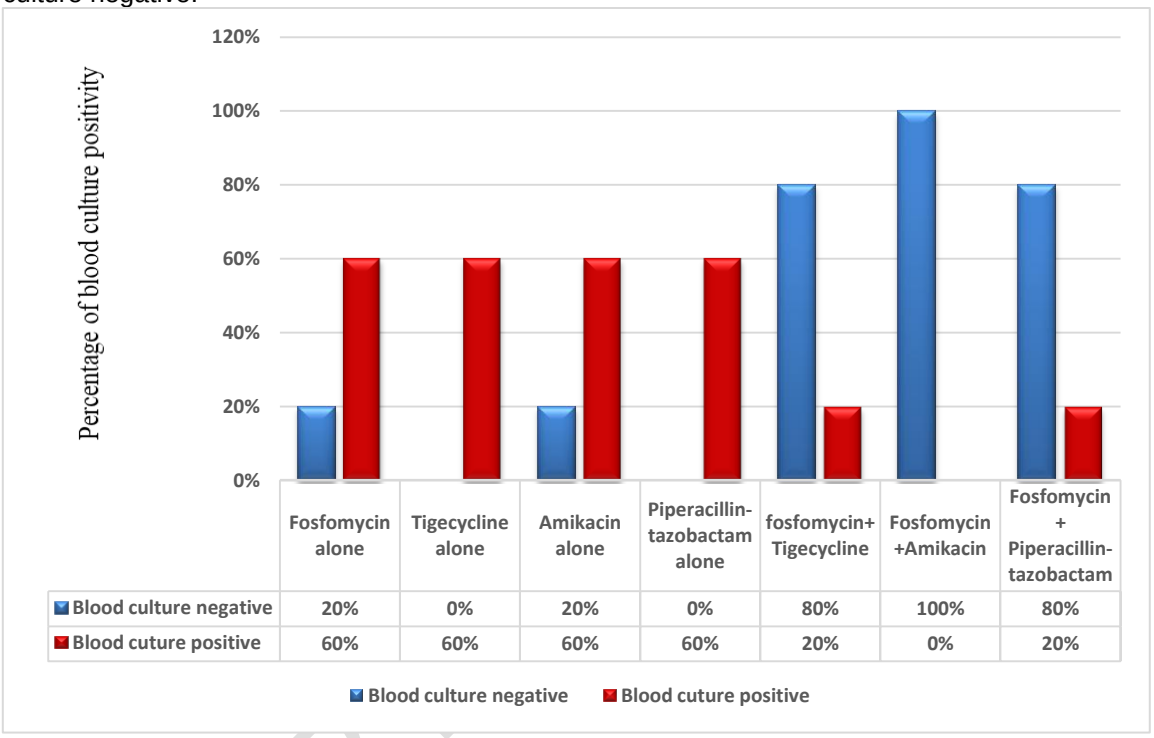

Figure-1: shows result of antibiotic therapy on the clearance of MDR *P. mirabilis* from the blood of mouse.

Comparison between synergism of different antibiotic combinations in MDR *P. mirabilis in vitro* and *in vivo* was showed in (Table-10). While combining fosfomycin with amikacin, they showed 100% synergistic effect both *in vitro* and *in vivo*, while combining tigecycline with fosfomycin, they showed 50% synergistic effect *in vitro* and 80% synergistic effect in vivo and while combining fosfomycin with piperacillin-tazobactam, they showed 75% synergistic effect *in vitro* and 80% synergistic effect *in vivo*.

Table-10: Comparison of synergism among different antibiotic combinations between MDR *P. mirabilis in vitro* and *in vivo*.

Group of combination	**Synergy positive *in vitro*** **n(%)**	**Synergy positive *in vivo*** **n(%)**
Fosfomycin + Tigecycline	50	80

Fosfomycin + Amikacin	100	100
Fosfomycin + Piperacillin-tazobactam	75	80

4. DISCUSSION

In this present study synergy were observed in 4 MDR *P. mirabilis* for fosfomycin and amikacin combination (mean FICI value 0.44), fosfomycin and tigecycline combination (mean FICI value 0.69) and fosfomycin and piperacillin-tazobactam (mean FICI value 0.56). No available data was found to compare such study against MDR *P. mirabilis*.

The present study observed 100% synergism with the combination of fosfomycin plus amikacin, 50% synergism and 50% additivity with the combination of fosfomycin plus tigecycline and 75% synergism and 25% additivity with the combination of fosfomycin plus piperacillin-tazobactam were found. Fosfomycin in combination with either aminoglycosides, or carbapenem or piperacillin-tazobactam is the effective combination against MDR *Enterobacteriaceae* (18).

In the present study, survival of mouse was observed periodically for 72 hours after intervention. Mouse of positive control group became profoundly sick and after 72 hours only 2 mice were alive. In contrast, studies carried out by (19) and (20) in DMCH on *in vivo* study of *Acinetobacter baumannii* reported that, 100% rats were alive after 72 hours of antibiotic treatment. This might be due to the fact, that no immunosuppressive agent like cyclophosphamide was used in that study to make the rat neutropenic.

The best *in vivo* result appeared in the group treated with fosfomycin and amikacin combinations. *In vivo* combination of fosfomycin plus amikacin showed 100% synergism and 80% synergism was shown by fosfomycin plus tigecycline and fosfomycin plus piperacillin-tazobactam, respectively. Fosfomycin was reported to mitigate *in vivo* and *in vitro* synergy with carbapenem against KPC producing multidrug resistant *Enterobacteriaceae* (21); (22). No *in vivo* experimental study was available to compare such antibiotic therapies against MDR *P. mirabilis.*

Multidrug resistance is emerging among *P. mirabilis* leaving limited therapeutic options for the management of serious infections. Repurposing of older antimicrobial like fosfomycin and amikacin and combination therapy may be good options for the treatment of infection caused by *P. mirabilis*.

5. CONCLUSION

Combination therapy is good treatment option for MDR *P. mirabilis* both *in vitro* and *in vivo*. Fosfomycin and amikacin was the most effective combination in both *in vitro* and *in vivo* which showed 100% synergism. From the present study it appeared that combination of fosfomycin and amikacin may be a good option for treating infection by MDR *P. mirabilis*.

9. CONSENT

We declare that 'written informed consent was obtained from the patient for publication of obtained information from them.

10. ETHICAL APPROVAL

We declare that all experiments have been examined and approved by the appropriate ethics committee and have therefore been performed in accordance with the ethical standards laid down in the 1964 Declaration of Helsinki."

REFERENCES

1. *The prevalence of antimicrobial resistance among uropathogens causing acute uncomplicated cystitis in young women. .* **Gupta, K., Hooton, T. M., Wobbe, C. L., & Stamm, W. E.** 1999, International journal of antimicrobial agents,, pp. 11(3-4), 305-308.

2. *"Identification of a Proteus penneri isolate as the causal agent of red body disease of the cultured white shrimp Penaeus vannamei and its control with Bdellovibrio bacteriovorus." .* **Cao, Haipeng, et al.** 2014, Antonie Van Leeuwenhoek , pp. 423-430.

3. *Significance and roles of Proteus spp. bacteria in natural environments. .* **Drzewiecka, D.** 2016, Microbial ecology, , pp. 741-758.

4. *Prevalence of multi-drug resistant Proteus species from isolates of urine and pus with their antibiogram. .* **Nachammai, S. M., Sneka, P., & Aswinsayiram, S. J.** 2015, International Journal of Scientific Research,, pp. 223-225.

5. *Genomic fingerprinting using random amplified polymorphic DNA for discrimination between Proteus mirabilis strains. .* **Mansy, M. S. M.** 2001, Egypt. Egypt J Biotech, , pp. 67-79.

6. *PREVALENCE AND RELATION OF URINARY TRACT INFECTION BACTERIAL PATHOGENS TO SEX AND AGES AMONG PATIENTS IN THREE ARAB COUNTRIES. .* **Azab, K.** 2021, Al-Azhar Journal of Pharmaceutical Sciences,, pp. 194-206.

7. *Exploring the hidden potential of fosfomycin for the fight against severe Gram-negative infections. .* **Saiprasad, P. V., & Krishnaprasad, K.** 2016, Indian journal of medical microbiology,, pp. 416-420.

8. **Organization, World Health.** *Report of the 1st meeting of the WHO advisory group on integrated surveillance of antimicrobial resistance,.* Copenhagen, : organization, World Health, 2011.

9. *Predominance of Nosocomial Pathogens among Patients with Post-Operative Wound Infections and Evaluation of the Antibiotic Susceptibility Patterns in rural Hospitals of*

Bangladesh. **Abedin, M. Z., Aktar, M. B., Zaman, M. S. U., Jarin, L., Miah, M. A. S., Ahmed, A. A., ... & Shilpi, R. Y.** 2020, Recent adv biol med, p. 17990.

10. *Novel plasmid-mediated 16S rRNA methylase, RmtC, found in a Proteus mirabilis isolate demonstrating extraordinary high-level resistance against various amynoglycosides.* **Wachino, J. I., Yamane, K., Shibayama, K., Kurokawa, H., Shibata, N., Suzuki, S., ... & Arakawa, Y.** 2006, Antimicrobial agents and chemotherapy, pp. 178-184.

11. *Antibiotic susceptibility testing by a standerized single disc method. .* **Bauer AW, Kirby WMM, Sheris JC, Truck M.** 1996, Am J Clin Pathol , pp. 225-230.

12. *Performance standards for antimicrobial susceptibility testing.* **Wayne, PA.** 2018, Clinical and Laboratory Standard Institute,, pp. 1-2.

13. *Determination of minimum inhibitory concentration.* **JM., Andrews.** 2001, J Antimicrob Chemotherapy, pp. 5-16.

14. *Synergism of imipenem and amikacin in combination with other antibiotics against Nocardia asteroides. .* **Gombert ME, Aulicino TM.** 1983, Antimicrob Agents Chemother , pp. 810–811.

15. *Comparative In Vitro Antimicrobial Susceptibilities of Nosocomial Isolates of Acinetobacter baumannii and Synergistic Activities of Nine Antimicrobial Combinations.* **Marques MB, Brookings ES, Moser SA, Sonke PB, Waites KB.** 1997, J Antimicrob Chemotherapy, p. 41.

16. *Experimental model for treatment of extended spectrum beta lactamase producing Klebsiella pneumoniae. .* **Toledo PVM, Tuon FF, Bail L, Manente F, Arruda P, Arranha-Junior.** 2014, Arq Bras Cir Dig , pp. 168-171.

17. *Imipenem, doxycycline and amikacin in monotherapy and in combination in Acinetobacter baumannii experimental pneumonia. .* **Hernandez MJR, Pachon J, Pichard C, Cuberos L, Martinez JI, Curiel AG, Caballero FJ, Moreno I, Mejias MEJ.** 2000, J Antimicrob Chemother , pp. 493-501.

18. *Assessment of fosfomycin for complicated or multidrug-resistant urinary tract infections: patient characteristics and outcomes. .* **Giancola, S. E., Mahoney, M. V., Hogan, M. D., Raux, B. R., McCoy, C., & Hirsch, E. B.** 2017, Chemotherapy,, pp. 100-104.

19. *ANTIMICROBIAL RESISTANCE PATTERN AMONG DIABETIC PATIENTS WITH URINARY TRACT INFECTION AT BANGLADESH. .* **Mohammad Saifuddin, F. C. P. S., Selim, S., Uddin, N., & Pathan, F.** 2016, Endocrine Practice, , pp. 22, 61.

20. *Microbiological Study of Diabetic Foot Ulcer. .* **Jahan, T., & Al Amin, A.** 2018, BIRDEM Medical Journal,, pp. 251-256.

21. *Evaluation of CHROMagar™ KPC for the detection of carbapenemase-producing Enterobacteriaceae in rectal surveillance cultures. .* **Panagea, T., Galani, I., Souli, M.,**

Adamou, P., Antoniadou, A., & Giamarellou, H. 2011, International journal of antimicrobial , pp. 124-128.

22. *In vitro and in vivo evaluation of antibiotic combinations against multidrug resistant Proteus mirabilis isolated from admitted patients of Dhaka Medical College Hospital, Dhaka. .* **Zaman, R., & Shamsuzzaman, S. M.** 2021, Archives of Microbiology & Immunolo, pp. 243-262.