Original Article

Evaluation of Effectiveness of Antibiotic Combination Therapy in Multi Drug Resistant *Escherichia Coli in Vitro* and *in Vivo*.

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Abstract

The antimicrobial resistance to commonly used antibiotics including broad spectrum antibiotics like carbapenem and colistin and aminoglycosides are increasing among *Escherichia coli* which has resulted in emergence of MDR strains as well as limiting therapeutic options to treat infection by them. The use of combination therapy has been found to be an effective strategy to overcome this situation. The aim of the present study was to see the efficacy of combination of imipenem-colistin, imipenem-amikacin and colistin-amikacin both *in vitro* and *in vivo* MDR *Escherichia coli* resistant to all of them.

Total 65 *Escherichia coli* were included in this study isolated from urine, wound swab and blood samples of infected patients. *Escherichia coli* were identified by biochemical test followed by PCR. Antibiotic susceptibility pattern was performed by disc-diffusion method. MIC of imipenem, colistin and amikacin were done by agar dilution method. Antibiotic combinations (imipenem+colistin, imipenem+amikacin and colistin+amikacin) effectiveness in treating resistant *Escherichia coli* were evaluted *in vitro* by agar dilution method and *in vivo* by using a mouse model.

Among 65 *Escherichia coli* 11 were MDR among which 2 were resistant to imipenem& colistin; 4 were resistant to imipenem & amikacin; 4 were resistant to amikacin & colistin; one was resistant to all the three drugs. Combination of two antibiotics of these three *in vitro* against resistant *Escherichia coli* showed synergistic effect when 1/4 or 1/8

MIC for each antimicrobial were used. The best *in vivo* efficacy appeared with imipenem-colistin and imipenem-amikacin combination which showed 100% blood culture negative results after 72 hours of antibiotic combination therapy.

Keywords: Antibiotic combination, MDR, FIC, *E.coli*.

1. Introduction

Escherichia coli is one of the most common human pathogen. They are the leading cause of urinary tract infections, wound infections, diarrhoea and other illnesses [15]. The diseases caused by *Escherichia coli* are severe and require antibiotic therapy for treatment [22]. Bacterial resistance to commonly prescribed antimicrobials are raising both in developing as well as in developed countries [20]. *Escherichia coli* has been found highly resistant to commonly used antibiotics like penicillin, cephalosporins, fluroquinolones, Trimethoprim Sulfamethoxazole and other ß-lactam drugs [8]. The prevalence of carbapnenem-resistant *Escherichia coli* in human patients has increased in Bangladesh [3]. Carbapenemase-producing bacteria are usually multidrug resistant [16]. Polymyxin drugs, such as polymyxin B and colistin, remain active against carbapenemase producing pathogens and are considered to be last-resort antimicrobials in treating carbapenem resistant infections [24]. The recent increase in the use of colistin in clinical practice accompanied by its unbridled use in agriculture have contributed to the rapid dissemination of resistance [13]. This problem of wide scale resistance of bacteria against antibiotics is severe and need to be checked by employing effective measures. As a preventive measure the combined use of two or more antibiotics could be employed for killing multiple drug resistant bacteria. Treatment with more than one drug simultaneously can lower the survival rate of bacteria against resistance stemming from the occurrence of fortuitous mutations [22].

2 Materials and Methods

2.1 Isolation and identification of organisms:

Total 350 urine, blood and wound swab samples were included from the patients admitted in Dhaka Medical College Hospital after taking informed written consent. *Escherichia coli* were isolated and identified by observing pink colonies in Mac Conkey's agar media, motility, indole positive, urease and citrate negative reactions [5] and confirmed by detecting uidA gene by PCR [4].

2.2 Antimicrobial susceptibility test

Susceptibility of isolates to 10 antimicrobials (ciprofloxacin, ceftriaxone, cefepime, amoxicillin/clavullenic acid, piperacillin/tazobactam, aztreonam, cefoxitin, amikacin, colistin, imipenem) were done by modified Kirby–Bauer disc diffusion method [2] and zones of inhibition were interpreted according to CLSI guidelines [6]. *Escherichia coli* ATCC 29212 was used as control strain to assess the performance of the method.

2.3 Determination of MIC

Minimum inhibitory concentration (MIC) of amikacin, imipenem and colistin were determined using agar dilution method [1][9]. Eighty mg base of commercially available colistin injection vial was added to 10 ml distilled water to

make a concentration of 8 mg/ml to prepare colistin stock solution. For each plate 50 ml Muller-Hinton agar media was prepared. 50 ml sterile Muller-Hinton agar was impregnated with 12.5 µl, 25 µl, 50 µl, 100 µl, 200 µl, 400 µl, 800 µl, 1600 µl of colistin stock solution to achieve concentration of 2µg/ml, 4µg/ml, 8µg/ml, 16µg/ml, 32µg/ml, 64µg/ml, 128µg/ml, 256µg/ml per plate respectively. Imipenem stock solution was prepared by adding 100 ml distilled water in 500mg base of commercially available imipenem injection vial to get 5mg/ml. 50 ml sterile Muller-Hinton agar was impregnated with 20 µl, 40 µl, 80 µl, 160 µl, 320 µl, 640 µl, 1280 µl, and 2560 µl of imepenem stock solution to achieve concentration of 2µg/ml, 4µg/ml, 8µg/ml, 16µg/ml, 32µg/ml, 64µg/ml, 128µg/ml, 256µg/ml per plate respectively. Commercially available amikacin injection vial was used as amikacin stock solution and the concentration was 250 mg/ml. 50 ml sterile Muller-Hinton agar was impregnated with 3.2 µl, 6.4 µl, 12.8 µl, 25.6 µl, 51.2 µl, 102.4 µl, 204.8 µl, 409.6 µl, 819.2 µl, 1638.4 µl, 3276.8 µl, 6553.6 µl, 13107.2 µl and 26214.4 µl of amikacin stock solution to achieve concentration of 16µg/ml, 32µg/ml, 64µg/ml, 128µg/ml, 256µg/ml, 512 µg/ml, 1024 µg/ml and 2048 µg/ml, 4096 µg/ml, 8192 µg/ml, 16384 µg/ml, 32768 µg/ml, 65536 µg/ml and 131072 µg/ml per plate respectively. 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 10^4 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.

2.4 Antibiotic combinations *in vitro*

Combinations of imipenem-colistin, imipenem-amikacin and amikacin-colistin 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 dilution than the MIC of both antibiotics for that sample. The 3rd plate contained four fold lower dilution than the MIC of both antibiotics for that sample. The 4th plate contained eight fold lower dilution 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 [9]. 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 [14].

$$\text{FICI} = \frac{\text{MIC of drug A in combination}}{\text{MIC of drug A alone}} + \frac{\text{MIC of drug B in combination}}{\text{MIC of drug B alone}}$$

All the tests were performed in triplicate.

2.5 *In vivo* study

Forty mice (swis albino) were used for this purpose. The experiments were performed in immunocompetent female mouse weighting 15-20 grams. The mouse were purchased from icddr,b 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 8 groups (A, B, C, D, E, F,G, H) 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 [21]. 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 (colistin, imipenem and amikacin resistant) *Ecsherichia coli* in MacConkey agar media at 37ºC. Group A, B, C, D, E, F received antimicrobial treatment intraperitoneally after 4 hours of infection at 12 hours interval for 3 days. Group A, B and C were treated individually only with amikacin (15mg/kg), imipenem (15mg/kg) and colistin (3.4mg/kg) respectively. Group D received amikacin plus imipenem (15mg/kg + 15mg/kg), Group E received amikacin plus colistin (3.4mg/kg + 15mg/kg) and Group F received imepenem plus colistin (3.4mg/kg + 15mg/kg) combination. Group G did not receive antimicrobial treatment. Group G 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 H) were given each antibiotic for 72 hours (uninfected treat group/negative control). The animals were observed for 72 hours and the survival mouse 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.

3. 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, 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 [10].

4. Results

Out of 350 samples 65 were *Escherichia coli* among which 11 (N1 to N11) were multidrug resistant. Among the

MDR *E. coli* 90.91%, 81.82% and 45.45% were resistant to amikacin, imipenem and colistin respectively. The MIC value for imipenem, amikacin and colistin ranged from 16µg/ml to ≥128 µg/ml, 1,024 µg/ml to ≥ 65,536 µg/ml and 4 µg/ml to ≥ 256 µg/ml respectively (Table-1).

Table: 1 MIC of colistin, imipenem and amikacin in MDR *Escherichia coli* (N=11)

Colistin		Imipenem		Amikacin	
MIC (µg/ml)	Total number	MIC (µg/ml)	Total number	MIC (µg/ml)	Total number
≥ 256	2	≥128	1	≥ 65,536	1
128	0	64	0	32,768	1
64	1	32	2	16,384	0
32	0	16	6	8,192	0
16	1	8	0	4,096	0
8	0	4	0	2,048	6
4	3	2	0	1,024	1
≤ 2	4	≤1	2	512	0
-	-	-	-	256	1
-	-	-	-	128	0
-	-	-	-	64	0
-	-	-	-	32	0
-	-	-	-	≤ 16	1

Combination of imipenem and amikacin against four MDR isolates which were resistant to both drugs showed reduction of their MIC value by four fold and eight fold after combination which were synergistic effect and this finding was strengthened by FICI≤0.5 (Table-3). Two MDR species found resistant to imipenem and colistin and MIC value of these drugs reduced by four fold after combination against these two species and FICI were 0.5, which proved synergistic effect (Table-2).

Table-2: Results of combination of imipenem with amikacin in MDR *E. coli* resistant to both drugs.

E. coli isolates	MIC of imipenem before combination (µg/ml)	MIC of imipenem after combination (µg/ml)	MIC of amikacin before combination (µg/ml)	MIC of amikacin after combination (µg/ml)	FICI	Inference

N1	16	2	2048	256	0.25	synergism
N5	16	2	65536	8192	0.25	synergism
N6	32	8	2048	512	0.5	synergism
N10	16	4	2048	512	0.5	synergism

Table-3: Results of combination of imipenem with colistin in MDR *E. coli* resistant to both drugs.

E. coli isolates	MIC of imipenem before combination (µg/ml)	MIC of imipenem after combination (µg/ml)	MIC of colistin before combination (µg/ml)	MIC of colistin after combination (µg/ml)	FICI	Results
N5	16	4	4	1	0.5	synergism
N8	16	4	8	2	0.5	synergism

Four MDR isolates were resistant to amikacin and colistin and combination of these two showed reduction of MIC by four fold in 3 isolates with FICI value 0.5 which was synergistic effect and by two fold in one isolates with FICI value 1 which was additive effect (Table-4).

Table-4: Result of combination of amikacin with colistin in MDR *E. coli* resistant to both drugs.

E. coli isolates	MIC of amikacin before combination (µg/ml)	MIC of amikacin after combination (µg/ml)	MIC of colistin before combination (µg/ml)	MIC of colistin after combination (µg/ml)	FICI	Results
N3	1024	256	256	64	0.5	synergism
N5	2048	512	256	64	0.5	synergism
N7	2048	1024	4	2	1	synergism
N11	65536	16384	4	2	0.5	synergism

Combination of imipenem with amikacin and imipenem with colistin found more effective than colistin with amikacin combination as well as single durg therapy (table-5).

Table 5: Results of antibiotic therapy on the clearance of *Escherichia coli* from the blood of mice.

Group	Blood culture positive n(%)	Blood culture negative n(%)	Total n(%)
Positive control	5 (100.00)	0 (0.00)	5 (100.00)
Negative control	0 (0.00)	5 (100.00)	5 (100.00)
CL	3 (60.00)	2 (40.00)	5 (100.00)

IMP	4 (80.00)	1 (20.00)	5 (100.00)
AK	5 (100.00)	0 (0.00)	5 (100.00)
CL+IMP	0 (0.00)	5 (100.00)	5 (100.00)
CL+AK	3(60.00)	2 (40.00)	5 (100.00)
IMP+AK	0 (0.00)	5 (100.00)	5 (100.00)

CL= Colistin IMP= Imipenem AK= Amikacin.

5. Discussion

Multidrug-resistant gram-negative bacteria are a major public health threat. However, intense efforts to limit their spread, the number of multidrug resistant gram-negative bacteria continues to increase globally [16]. Combined use of antimicrobial could be a better choice [18] in such cases. In the present study, combination of imipenem with colistin against MDR *E. coli* revealed synergistic effect *in vitro* as well as hundread percent blood culture negative result appeared *in vivo* using this combination. This finding is consistent with the previously reported combination therapy against MDR gram negative bacteria [23]. Colistin is a cationic peptide which disrupts bacterial cell membrane which may potentiate action of other antimicrobials in combination. Combination of imipenem with amikacin also showed synergism in all tested MDR isolates *in vitro* and hundread percent blood culture negative result appeared *in vivo*. Carbapenems were previously reported effective in combination with aminoglycosides against MDR gram negative bacteria [11][12]. Imipenem is cell wall inhibitor and amikacin is protein synthesis blocker. So, this combination work well when used in combination. In this study Colistin and amikacin combination showed synergistic effect in 3 of the 4 test isolates and one showed additive effect. Synergism was also reported regarding this combination by other studies [25]. But *in vivo* experiment in this study showed 40% blood culture negative result. But this combination was reported as promising therapeutic option to treat infection by carbapenem resistant *E. coli* and *Pseudomonas aeuroginosa* [25][19].

In the present study, mice were observed periodically for survival for 72 hours after intervention. In the present study the best *in vivo* result appeared in the group treated with imipenem-colistin and imipenem-amikacin combination. Hundread percent blood culture negative results were seen for these two combination *in vivo*. Previous *in vivo* studies reported these combination to be effective in infection by gram negative bacteria [23][7].

5. Conclusion

The present study observed that combination therapy could be a good option for MDR *Escherichia coli*. Combination of imipenem with colistin, imipenem with amikacin and colistin with amikacin showed synergistic effect *in vitro*. Combination of colistin with amikacin found less effective (40% bacterial clearance) than other two combination (100% bacterial clearance) *in vivo.*

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