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The efficacy trial of some antibiotic combinations against multi-drug resistant *Escherichia coli*

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Abstract: A study was designed to evaluate some antibiotic dual combinations against *Escherichia coli in situ*. The 11 subtypes of *E. coli* with multiple drug resistance were isolated from sewage water. The i*n situ* trial with some dual antibiotic combination against isolated *E. coli* was conducted, their minimum inhibitory concentration (MIC) determined and time kill studies were performed. A synergistic effect was obtained by chloramphenicol and gentamycin combination. Ampicillin combination with ciprofloxacin (FIC index 0.625) or tetracycline (FIC index 0.5) or gentamycin (FIC index 0.75) was synergistic and antagonistic with chloramphenicol (FIC index 1.5). Similar results in the form of cfu drop were obtained in time kill study.

Key words: Antibiotic combinations, *Escherichia coli*, Multi-drug resistance

Introduction

Escherichia coli is a common intestinal microflora of humans and animals. Most strains of *E. coli* serve as an avirulent commensal in the large bowel of mammals. It is also an important pathogen which can cause a variety of diseases including diarrhea, urinary tract infections, bacteraemia, pneumonia and meningitis (Levine, 1984). The diseases caused by *E. coli* are severe and require antibiotic therapy for treatment. The increased and indiscriminate use of antibiotics, especially third and fourth generation cephalosporins and poor patient compliance has led to the development of bacterial resistant strains. From the past three decades the bacterial resistance to β-lactam based antibiotics are on the steep hike due to production of plasmid mediated extended spectrum beta lactamase (ESBL). Antibiotic resistance is frequently observed in Enterobacteriaceae and has worldwide occurrence (Forssten *et al*., 2010). Some bacterial strains have become resistant to all the know drugs (Lister, 2006). The 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. It is in this backdrop a study was designed with an objective to test the efficacy of some antibiotic dual combinations against *E. coli*.

Materials and Methods

Collection of bacterial isolates : A study was carried out at Department of Microbiology, S.B.S. (PG) Institute, Dehradun, India. The sample for the study was collected from sewage water from a nearby locality. The bacterial types were isolated and characterized morphologically, physiologically, biochemically and tested for antibiotic sensitivity against nine antibiotics.

Identification of E. coli isolates: Sewage water samples were analyzed as per the standard method for total and fecal coliform counts on eosin methylene blue (EMB) agar plates and incubated at 37 °C for 24 h. The colonies with blue-green color and metallic-sheen on EMB agar plates were selected and purified by re-streaking on EMB plates. Plates were incubated at 37 °C for 24 h and stored for further use. Isolates were Gram stained according to standard methods and Gram-negative isolates were subjected to biochemical identification. *Escherichia coli* MTCC 443 was used as a positive control in the experiment.

Antibiotics: Nine antibiotics, namely, ampicillin, amikacin, cephaloridine, doxycycline, colistin, carbenicillin, enrofloxacin, streptomycin and tetracycline were used in sensitivity testing against *E. coli* biovars. Whereas dual combinations of eight antibiotics namely, ampicillin, chloramphenicol, ciprofloxacin, gentamycin, norfloxacin, tetracyclin, and trimethoprim were tried in the MIC (mg L^{-1}) study.

Antibiotic Sensitivity testing : Antibiotic sensitivity profiles of *E. coli* isolates were studied against eight different antibiotics by Kirby-Bauer disc diffusion test. Overnight grown *E. coli* culture was used in the study. The antibiotic susceptibility of bacteria was tested in Mueller-Hinton Agar (MHA). The bacteria were seeded on MHA plates and eight different antibiotic discs of tetracycline (30 µg $disc^{-1}$), cephaloridine (30 µg $disc^{-1}$), amikacin (10µg $disc^{-1}$), streptomycin (25µg $disc^{-1}$), carbenicillin (100 µg $disc^{-1}$), doxycycline (30 µg $disc^{-1}$), ampicillin (25 µg $disc^{-1}$), enrofloxacin (10 µg $disc^{-1}$) and colistin (25 µg $disc^{-1}$) were placed on the plates and incubated at 37° C for 24 h.

The zones of inhibition were recorded and results in terms of sensitivity or resistance were interpreted. *Escherichia coli* MTCC 443 was used as control in all assays. The biovars having resistance to more than three different antibiotics were designated multiple drug resistant.

Determination of minimum inhibitory concentration (MIC): The MIC of eight antibiotics, alone or in combination, was determined against *E. coli* isolates by standard broth micro-dilution method (Andrews, 2001). Serial dilution of antibiotic drug or antibiotic drug combinations was prepared. A series of broth tubes containing antibiotic concentrations in the range of 0.25 mg to 4 mg L^{-1} (0.25, 0.5, 1, 2 and 4 mg L^{-1}) were taken. The standard inocula were prepared from incubated MHB (HiMedia) by diluting it with sterile distilled water until the turbidity matched a 0.5 McFarland standard. The wells were inoculated with the diluted *E. coli* suspension of ~10^5 cfu mL^{-1} strength. The lowest concentration of drug on plate, which restricted the bacterial growth of around the plate wells incubated at 24 h at 37°C, designated its MIC. A checkerboard method was used to find the synergy between two drugs (Fig. 1). A total of 50 µL MHB was transferred to each well of the micro-dilution plates. The first drug of the combination was serially diluted along the ordinate, while the second antibiotic was diluted along the abscissa. This checkerboard contains each combination of five dilutions of two antibiotics with combinations that contained highest concentration of each antibiotic at extreme opposite corners.

Time kill study: Time kill study was performed to test the efficacy of eight antibiotics, namely amoxicillin, ampicillin, chloramphenicol, ciprofloxacin, gentamycin, norfloxacin and tetracyclin on bacterial survival (Pankuch *et al.*, 1994). A log phase culture was adjusted to approximately 6 $\log_{10}$ cfu mL^{-1} in Mueller-Hinton broth (HiMedia), which contained either one antibiotic or combination of two antibiotics with no antibiotic as control. Five mL cultures in glass tubes were incubated in a shaking water bath at 37°C for up to 24 h and viable bacteria were counted after 24 h. After vortexing a 0.5 mL sample was removed from the cultures and serial ten-fold dilutions were made in cold phosphate-buffered saline. Bacterial viability was measured by pour plating 0.5 mL of the appropriate dilution in nutrient agar in triplicate and incubating plates for 24 h at 37°C. Bacterial counts were expressed as the mean of three plates. The difference between plate counts was always less than 10%.

Results and Discussion

Identification of E. coli isolates: Of the 121 samples isolated from sewage water of various sites in Dehradun (Table 1), eleven biovars of *E. coli* were identified using biochemical tests. The isolates were named BL3, BL5, BL6, BL11, BL15, BL16, BL21, BL25, BL32, BL35 and BL39, respectively, as per the location name "Balawala" of the isolates.

Antimicrobial drug sensitivity testing: All the eleven isolates were resistant to at least two antibiotics (Table 2). Biovar *E. coli* BL5 was resistant to maximum (six) antibiotics whereas BL16 to only two. Five *E. coli* isolates (BL3, BL15, BL21, BL32 and BL39) were resistant to five antibiotics. A study conducted on urinary tract infection (UTI) patients revealed that there is a high ampicillin resistance rate (55.4%) followed by gentamicin (45.1%), amikacin (41.4%) and co-trimoxazole (30.5%) (Anandkumar *et al.*, 2003). In our study *E. coli* isolates had highest resistance to ampicillin (81.8%) followed by tetracyclin (81.8%). Aamikacin found moderate resistance (54.5%) whereas colistin had no resistance against *E. coli*. The evidence shows that the proportion of Gram-negative organisms resistant to commonly used antibiotics is increasing at an alarming rate. Our study demonstrates the similar feature of *E. coli*, which could be supported by the fact that plasmid bearing extended-spectrum β-lactamase (ESBL) and fosfomycin resistance have been discovered, which can rapidly spread in Enterobacteriaceae (Zhao *et al.*, 2015).

Determination of MIC : With single antibiotic test maximum antibiotic activity was shown by ciprofloxacin, norfloxacin and trimethoprim (MIC 0.25 mg L^{-1}, Table 3). In combination, minimum doses of ciprofloxacin with trimethoprim, ciprofloxacin with norfloxacin and trimethoprim with norfloxacin (MIC 0.125 + 0.125 mg L^{-1}) showed antibiotic effect whereas combination of amoxicillin with chloramphenicol (MIC 2 + 1 mg L^{-1}) and tetracycline with amoxicillin (MIC 1 + 2 mg L^{-1}) required highest dosage. The

Table-1: Morphological and biochemical characteristics of the *E. coli* biovars

Isolate number	BL3	BL5	BL6	BL11	BL15	BL16	BL21	BL25	BL32	BL35	BL39
Morphology	rods	rods	rods	rods	rods	rods	rods	rods	rods	rods	rods
Gram's reaction	-	-	-	-	-	-	-	-	-	-	-
Motility	+	+	+	+	+	-	-	+	+	+	+
Acid production	+	+	+	+	+	+	+	+	+	+	+
Catalase test	+	+	+	+	+	+	+	+	+	+	+
H_2S production	-	-	-	-	-	-	-	-	-	-	-
Indole test	+	+	+	+	+	+	+	+	+	+	+
Methyl red test	+	+	+	+	+	+	+	+	+	+	+
Oxidase	-	-	-	-	-	-	-	-	-	-	-
Urea hydrolysis	-	-	-	-	-	-	-	-	-	-	-
VP test	-	-	-	-	-	-	-	-	-	-	-
Carbon source											
Arabinose	+	+	+	+	+	+	+	+	+	+	+
Citrate	-	-	-	-	-	-	-	-	-	-	-
Glucose	+	+	+	+	+	+	+	+	+	+	+
Glycerol	+	+	+	+	+	+	+	+	+	+	+
Inositol	-	-	-	-	-	-	-	-	-	-	-
Mannitol	+	+	+	+	+	+	+	+	+	+	+
Melibiose	+	+	+	+	+	+	+	+	+	+	+
Raffinose	+	+	-	+	+	+	-	-	-	-	-
Rhamnose	+	+	+	+	+	+	+	+	+	+	+
Sorbitol	+	+	+	+	+	+	+	+	-	+	+
Sucrose	+	+	-	+	-	+	+	-	-	-	-
Xylose	+	+	+	+	+	+	+	+	-	+	+

Table-2: Sensitivity/resistance of *E. coli* biovars

Antibiotic	BL3	BL5	BL6	BL11	BL15	BL16	BL21	BL25	BL32	BL35	BL39
Ampicillin (A) (10 µg disc^{-1})	s	r	s	r	r	r	r	r	r	r	r
Amikacin (AK) (10 µg disc^{-1})	r	r	s	r	r	s	s	r	r	s	r
Cephaloridine (30 µg disc^{-1})	s	r	s	r	s	s	r	s	s	s	s
Doxycycline (30 µg disc^{-1})	r	r	r	s	r	s	s	r	r	r	r
Colistin (10 µg disc^{-1})	s	s	s	s	s	s	s	s	s	s	s
Carbenicillin (10 µg disc^{-1})	r	s	r	s	r	s	r	s	s	r	s
Enrofloxacin (EX) (10 µg disc^{-1})	s	r	s	s	s	s	r	s	s	s	s
Streptomycin (S) (10 µg disc^{-1})	r	r	r	s	s	r	s	r	r	s	r
Tetracycline (T) (10µg disc^{-1})	r	s	r	r	r	s	r	r	r	r	r

s = sensitive, r = resistant

Table-3: The *in situ* activity of antibiotics against *E. coli* BL5

Antibiotic Treatment	MIC (mg L^{-1})	Change in log cfu mL^{-1}*
Control	-	3.00
Amoxicillin	4.00	-1.86
Ampicillin	1.00	-1.75
Chloramphenicol	2.00	-1.61
Ciprofloxacin	0.25	-2.52
Gentamycin	0.50	-1.71
Norfloxacin	0.25	-2.55
Tetracyclin	2.00	-1.85
Trimethoprim	0.25	-1.88

* Observation of 24 h (inoculum < 6 log cfu mL^{-1})

Table-4: The *in situ* activity of antibiotic combinations against *E. coli* BL5

Antibiotic combinations	MIC (mg L^{-1})	FIC index	FIC A:B	Inference	Change in log cfu mL^{-1}*
Chl.+ Amo.	1.000 + 2.000	1.0	1.0	Indifference	-1.78
Chl.+ Cip.	1.000 + 0.125	1.0	1.0	Indifference	-2.08
Chl.+ Trimetho.	1.000 + 0.125	1.0	1.0	Indifference	-1.80
Chl.+ Nor.	1.000 + 0.125	1.0	1.0	Indifference	-2.09
Chl.+ Ampi.	1.000 + 1.000	1.5	0.5	Antagonist	-1.72
Chl.+ Genta.	0.500 + 0.250	0.75	0.5	Synergistic	-1.68
Cip.+ Amo.	0.125 + 2.000	1.0	1.0	Indifference	-2.24
Cip.+ Trimetho.	0.125 + 0.125	1.0	1.0	Indifference	-2.26
Cip.+ Nor.	0.125 + 0.125	1.0	1.0	Indifference	-2.54
Cip.+ Ampi.	0.125 + 0.125	0.625	4.0	Synergistic	-2.18
Cip.+ Tet.	0.125 + 1.000	1.0	1.0	Indifference	-2.24
Cip.+ Genta.	0.125 + 0.250	1.0	1.0	Indifference	-2.15
Trimetho.+ Amo.	0.125 + 2.000	1.0	1.0	Indifference	-1.87
Trimetho.+ Nor.	0.125 + 0.125	1.0	1.0	Indifference	-2.27
Trimetho.+ Ampi.	0.125 + 0.500	1.0	1.0	Indifference	-1.84
Trimetho.+ Tet.	0.125 + 1.000	1.0	1.0	Indifference	-1.87
Trimetho.+ Genta.	0.125 + 0.250	1.0	1.0	Indifference	-1.83
Nor.+ Amo.	0.125 + 2.000	1.0	1.0	Indifference	-2.26
Nor.+ Ampi.	0.125 + 0.500	1.0	1.0	Indifference	-2.19
Nor.+ Tet.	0.125 + 1.000	1.0	1.0	Indifference	-2.25
Nor.+ Genta.	0.125 + 0.250	1.0	1.0	Indifference	-2.16
Ampi.+ Amo.	0.500 + 2.000	1.0	1.0	Indifference	-1.83
Ampi.+ Tet.	0.250 + 0.500	0.5	1.0	Synergistic	-1.82
Ampi.+ Genta.	0.250 + 0.250	0.75	0.5	Synergistic	-1.74
Tet.+ Amo.	1.000 + 2.000	1.0	1.0	Indifference	-1.86
Tet.+ Chl.	1.000 + 1.000	1.0	1.0	Indifference	-1.78
Tet.+ Genta.	1.000 + 0.250	1.0	1.0	Indifference	-1.81
Genta.+ Amo.	0.250 + 2.000	1.0	1.0	Indifference	-1.82

Amo. – amoxicillin, Ampi. – ampicillin, Chl. – chloramphenicol, Cip.- ciprofloxacin, Genta. –gentamycin, Nor. – norfloxacin, Tetra. – tetracyclin, Trimetho. – trimethoprim
* Observation of 24 h (inoculum < 6 log cfu mL^{-1})

combination of chloramphenicol with gentamicin (MIC 0.5 + 0.25 mg L^{-1}), ampicillin with ciprofloxacin (MIC 0.125 + 0.125 mg L^{-1}), ampicillin with tetracycline (MIC 0.25 + 0.5 mg L^{-1}) and ampicillin with gentamycin (MIC 0.25 + 0.25 mg L^{-1}) was synergistic against *E. coli* BL5 (Table 4).

As per the common belief the synergism results due to bactericidal drug combination and antagonism due to bacteriostatic drugs (Abu-Basha *et al.*, 2012). Synergism can also occur due to bacteriostatic-bacteriostatic or bactericidal-bactericidal drug combinations; synergistic interactions between the bactericidal penicillin/cephalothin and bacteriostatic tetracycline have previously been reported (Abu-Basha *et al.*, 2012). The interaction of β-lactams and aminoglycosides has synergistic effect *in situ* (Cercenado *et al.*, 1995; Nichols and Maki, 1985). The combination of luoroquinolone with β-lactam/amikacin provides synergistic antimicrobial activity and could therefore reduce occurrence of ESBL-producing *E. coli* strains (Drago *et al.*, 2001). Aminoglycoside based drug, gentamicin, causes bacterial mRNA misreading by binding with 30S ribosomal subunit (Knowles *et al.*, 2002). Similar effect was observed in current study where the effect of ampicillin combination with gentamicin was synergistic against *E. coli*. Similarly, combination of penicillin with chloramphenicol has been reported to show antagonistic effect in clinical cases (Mellado *et al.*, 1991). Chloramphenicol binds with 50S ribosomal subunit to cause bacterial mRNA misreading (Wilson, 2014). In our observation similar results were found where combined MIC of ampicillin and chloramphenicol showed antagonism against *E. coli* BL5. Ampicillin and ciprofloxacin have been reported to show synergistic antibacterial effect against strains of *E. coli* (Nworu and Esimone, 2007). Similar observation has been reported in the current study. Synergism has also been reported with the combinations of clindamycin/erythromycin with gentamicin/colimycin against Gram negative bacteria and hence their combinations can be used in lower doses (Leng *et al.*, 1975). One report shows antagonism due to combination of gentamicin with chloramphenicol *in situ* (Klastersky and Husson, 1977). A contrasting report shows synergism due to gentamicin with chloramphenicol against

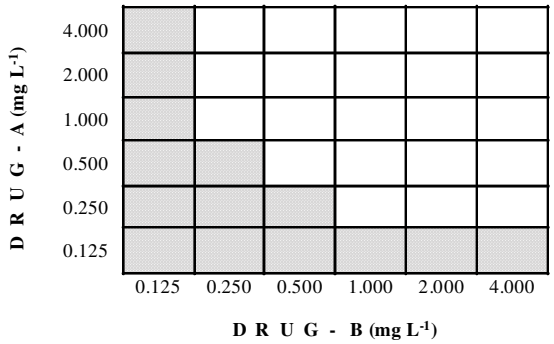

Fig. 1: The checkerboard method showing synergy of two drugs

Haemophilus influenzae (Dabernat *et al.*, 1979). In our study, combination of gentamicin with chloramphenicol yielded synergistic effect. Antimicrobial combinations can provide broad spectrum empirical coverage in the treatment of *E. coli* borne infections. Neomycin can be combined with ampicillin, penicillin, gramicidin, bacitracin, polymixin-B and erythromycin for effective expulsion of coliform infections (Grzybowska *et al.*, 2003).

Time kill studies: Synergistic, antagonistic and indifference activities were demonstrated with 28 combinations of eight antibiotics. Bactericidal activity was achieved against *E. coli* by all the tested antibiotic combinations (Table 4). Table summarises the results of time kill studies of different antibiotic combinations for *E. coli* BL5. Both ciprofloxacin and independently alone showed good bactericidal activity (> 2 $\log_{10}$ decrease in cfu mL^{-1} at 24 h). The combination of chloramphenicol with ampicillin was antagonistic to *E. coli* BL5 with >1$\log_{10}$ cfu mL^{-1} difference, whereas combination of chloramphenicol with gentamicin, ampicillin with tetracycline and ampicillin with gentamycin was found synergistic with > 1.5 $\log_{10}$ mL^{-1} cfu difference. All the combinations of ciprofloxacin or norfloxacin had >2 $\log_{10}$ cfu difference. Ciprofloxacin in combination with norfloxacin had second largest cfu drop of 2.55 $\log_{10}$ (largest drop was with norfloxacin treatment, Table 3) but synergy can not be demonstrated.

The *in situ* results showed that ampicillin provide synergistic effect with ciprofloxacin, tetracycline and gentamycin combinations. The combination of two antibiotics provide extended spectrum coverage against infections by producing a greater antibacterial effect. Sometimes combinations of two low potent antibiotics could be equally effective as one higher potent antibiotic. Although the risk associated with multiple drug usage in patients could not be denied, but the lower doses of toxic drugs can reduce concentration related risk (Leng *et al.*, 1975). The antibiotic combination therapy can help patients with more than one infection types. The authenticity of results need to be checked *ex-situ*.

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