WORLD JOURNAL OF PHARMACY AND PHARMACEUTICAL SCIENCES

Volume 2, Issue 3, 1-11. Research Article ISSN 2278 – 4357

SHORT TIME KILLING RATE OF COMBINATIONS OF SELECTED ANTIBIOTICS ON TWO STRAINS OF *E. COLI*

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Article Received on
25 March 2013,

Revised on 21 April 2013,
Accepted on 14 May 2013

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ABSTRACT

The killing kinetics of ciprofloxacin and gentamicin against Extended Spectrum β-lactamase (ESBL) positive and negative strains of *E. coli* were investigated in the Pharmaceutical Microbiology laboratory, Faculty of Pharmaceutical Sciences, Nnamdi Azikiwe University, Agulu Campus between March and May 2012. The minimum inhibitory concentrations (MICs) of the drugs were determined using the broth dilution method. The killing rate constant of each drug and their combinations was determined at double the MICs of the drugs singly and in combination using pour plate method. The result was that both antibiotics had activity on the two strains of the *E. coli* when used alone and better activity when used in combination. Also, gentamicin had equal activity on the two strains with the MIC of 3.125µg/ml, while ciprofloxacin had more effect on ATCC strain with MICs of 3.125µg/ml and 6.25µg/ml for ATCC and ESBLs strain respectively. The conclusion is that combining gentamicin and ciprofloxacin in ESBL cases have an advantage of faster microbial death over using each of the drugs alone especially in acute cases where rapid killing or eradication of the infecting organism is needed. Rapid bacterial eradication of pathogenic bacteria is a favourable characteristic of an antibiotic because it minimizes the spread of the disease with tremendous socio-economic impact.

Keywords: killing rate, Ciprofloxacin, Gentamicin, ESBL, *E. coli.*

1. INTRODUCTION

Escherichia coli (E. coli) belongs to the large bacterial family called ***Enterobacteriaceae*** or the **enteric bacteria**, which are facultative anaerobic Gram-negative rods living in the intestinal tracts of animals both in health and disease. The original strain of *E. coli* is believed to be lost and so a new type strain called a neotype has been chosen as a representative [1]. The neotype strain (a specimen selected as the type for naming a species or subspecies when the original strain is no longer in existence) called ATCC 11775, or NCTC 9001, is pathogenic to chickens and has an O1:K1:H7 serotype [2] It does not produce ESBL. However, in most studies either O157:H7 or K-12 MG1655 or K-12 W3110 is used as a representative *E.coli.* Worldwide, the organism has developed resistance to antimicrobial agents and the phenomenon is increasing both in outpatients and hospitalized patients [3, 4]. Among members of the *Enterobacteriaceae* family, resistance to β-lactams has been reported to be associated with ESBL which hydrolyze oxyimino β-lactams like cefotaxime, ceftriaxone, ceftazidime and monobactams but have no effect on cephamycins, carbapenems and related compounds [5, 6] The prevalence of ESBL producing *E. coli* has been observed by several workers and also reported to be between 28 and 67 % [3, 7, 8, 9, 10, 11]. Production of ESBLs is frequently plasmid encoded and confers clinical significance. Plasmids responsible for ESBL production frequently carry genes encoding resistance to other drug classes also; hence, a more appropriate name would be “multidrug resistant organisms”. Major risk factors for infection with ESBL – producing organisms are long term antibiotic exposure, prolonged hospitalization, nursing home residency and severe illness, residence in an institution with high rates of third generation cephalosporin use and instrumentation or catheterization. Other problems due to ESBL-producing bacteria include difficulty in detecting the presence of ESBL, limited treatment options and deleterious impact on clinical outcomes [12, 13].

The presence of ESBL in some *K. pneumonia and E. coli* strains poses an important challenge in clinical practice since these organisms are common causes of serious infections. The conventional treatment for infections caused by such organisms often fails due to the production of extended β-lactamases (ESBLs) [14, 15]. Recent microbiological surveillance studies reported that *E. coli* and *K. pneumonia* were the first four most frequent causes of gram- negative infections [16, 17].

This study aims to determine the kinetic of killing of selected non β-lactam antibiotics singly and in combination on ESBL and non - ESBL producing strains of *E. coli*.

2. EXPERIMENTAL DETAILS

2.1.MATERIALS

2.1.1. Test microorganisms: Laboratory isolates of the extended-spectrum β-lactamases (ESBLs) - beta-lactamases producing *Escherichia coli* and a strain of *Escherichia coli* (non- ESBLs producing) ATCC 11775 were employed in this study. All isolates were obtained, frozen, from the Department of Pharmaceutical Microbiology and Biotechnology Laboratory, Faculty of Pharmaceutical Sciences Nnamdi Azikiwe University Awka, Agulu campus.

2.1.2. Culture media: The culture media used in the study include: Mueller-Hinton agar, Mueller-Hinton broth and McConkey agar [Oxoid Limited, Basingstoke, and Hampshire, England].

2.1.3. Antibiotics: ciprofloxacin HCl powder was kindly donated by Juhel Pharmaceutical Ltd. Enugu, Nigeria. Gentamicin 80mg/2ml injection (by Gland Pharma, Batch: CY142X) was purchased from the model pharmacy, Faculty of Pharmaceutical Sciences, Agulu.

2.1.4. Others: Light Microscope used: XSZ-107BN; #: 000678 and chemicals used: Potassium phosphate, barium chloride, concentrated sulphuric acid, DMSO, distilled water.

2.2. METHODS

2.2.1. Sterilization of materials: The Petri dishes, test tubes plugged with cotton and porcelain cylinders, packed into metal canisters and loaded appropriately, were sterilized in the hot air oven [ov-335, hereaus] at 170□C for one hour at each occasion. Autoclaving at 121□C for 15 minutes, in the Gallencamp Autoclave, was constantly done in the sterilization of culture media and buffer solutions.

2.2.2. Preparation of Culture media: All culture media employed in this study were formulated according to the manufacturers' specifications.

2.2.3. Maintenance and activation of the test micro-organisms: The frozen, isolated, purified and characterized test isolates were sub-cultured in nutrient agar slants. The agar slants were stored in the refrigerator at 4□C after 24 hours of incubation at 37□C. Before carrying out each experiment, the organism involved was activated by successive sub-culturing from the deep agar culture for three consecutive days.

2.2.4. Standardization of test microorganisms: The 0.5 McFarland standard was used in this experiment and corresponds to bacteria density of $1.5x10^8$cfu/ml. A similar diameter (16 mm) tube was used to prepare a bacterial suspension comparable to a McFarland standard using normal saline. Inoculum was added to the suspension diluted to match the standard. The *E. coli* strains used for the susceptibility and killing rate studies were all in their exponential growth phase.

2.2.5. Preparation of stock solutions: A 12.5mg/ml ciprofloxacin concentration was prepared by dissolving an accurately weighed 12.5mg of the drug in 1ml volume of distilled water adjusted to pH 7.4 and sonicated at 37□C for 30 minutes to facilitate dissolution. Stock solution of gentamicin was prepared from ampoules of gentamicin injection of concentration 40mg/ml. A stock solution containing 3.125mg/L was prepared by withdrawing 78µl of the injection with a micropipette and dissolving in 1L of distilled water.

2.2.6. Determination of minimum inhibitory concentration (MIC) of selected antibiotics: Several tubes containing 4ml of sterile Mueller Hinton broth each were arranged in a rack and labeled properly. A 4ml of the stock solution of each of the drugs was diluted serially to produce double dilutions up to the final concentrations of 0.5mg/ml and 0.05µg/ml of ciprofloxacin and gentamicin respectively. A 0.1ml of 0.5McFarland standard inoculum of non ESBL-producing (ATCC) strain was diluted 100 folds by suspending in a 9.9ml of normal saline and a 0.1ml of the resulting bacteria suspension was added to the tubes containing the decreasing concentrations of each of the antibiotics in sterile broth. The above process was done in triplicate. The tubes were labeled appropriately and incubated at 37□C for 24 hours. The lowest concentration of an antibiotic which prevented visible growth of bacteria was interpreted as minimum inhibitory concentration (MIC) of the drug. The whole processes above were repeated with ESBLs producing strain.

2.2.7. Determination of viable counts after exposure to antibiotic combination drugs: A double the MIC concentration of the drugs was used for the single compound studies and also in the drugs combination studies. A 9.9ml of the drug solutions containing 2xMIC of each of drug on each strain of the organism was prepared and labeled appropriately. Identical cultures of each of the strains (ESBLs producing and non-producing) were prepared based on 0.5 McFarland standard scale. The prepared inoculum (volume = 0.1ml) of each strain was diluted in 9.9ml of each of the drug solutions. A 0.1ml samples were drawn from the above mixtures at interval of 2, 5, 15, 30, 45, 60, 90, 120 minutes during incubation. The antibiotic effects of the drugs was neutralized by diluting the 0.1ml sample each in 9.9ml of preset tubes of sterile normal saline. Finally 0.1ml samples were taken from the quenching medium and viable cell count was estimated after 24hours incubation at 37°C using pour plate method. For the combined effect, the drug solution was prepared by combining two times MIC of two drugs at a time. The above processes were completed and the viable cell count determined by simple count. The values obtained were then used to construct curves to demonstrate changes in colony counts with time as a function of exposure to antimicrobials alone and in combination. Results were plotted as $\log_{10}$ [Number of viable cells] against time.

3. RESULTS AND DISCUSSION

Table 1: Minimum inhibitory concentrations of selected (MIC) of selected antibiotics

Escherichia coli Strains	Minimum inhibitory concentration (MIC) (µg/ml)	
	CIPROFLOXACIN	**GENTAMICIN**
NON - ESBL PRODUCING	3.125	3.125
ESBL PRODUCING	6.25	3.125

The MICs of ciprofloxacin for ATCC and ESBL strains tested is 3.125µg/ml and 6.25µg/ml respectively, while that of gentamicin was 3.125µg/ml for the two strains. Higher MIC of ciprofloxacin is therefore needed for the ESBL-producing strains of *E. coli* than for the non-ESBL producing strain. This is comparable with previous work [20] and shows that the ESBL strain is more resistant than the non-producing strain.

3.2. Effect of ciprofloxacin on the two strain of *E. coli*

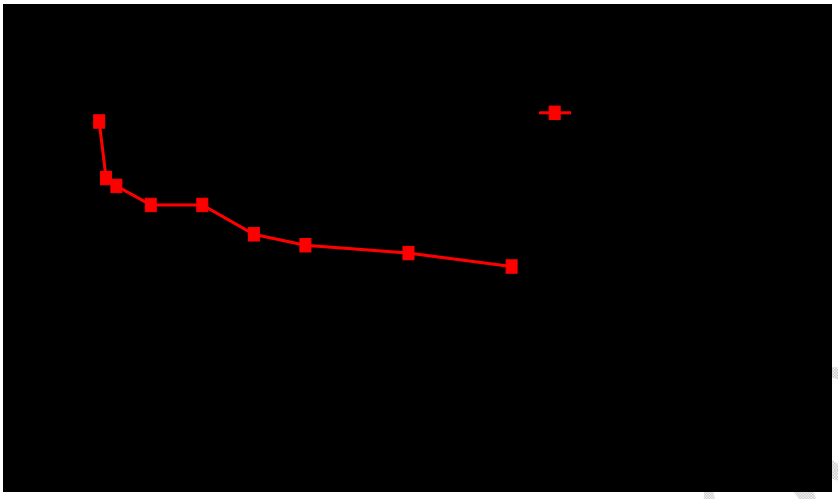

Figure 1: Effect of Ciprofloxacin (2xMIC) on the two strain of *E. coli*

The killing patterns of ciprofloxacin on the ESBLs producing and non-producing strains are shown in figure 1 above. Ciprofloxacin at the concentration of 2xMIC had similar activity on both strains. The kill rate of the drug on both strains are similar but the time taken by the drug to achieve one log reduction in viable cell count is 70 minutes and 90 minutes for ATCC 11775 and ESBLs producing strains respectively. This demonstrates that the ESBLs are less susceptible than the ATCC strain. Production of ESBLs is frequently plasmid encoded and bears clinical significance. Plasmids responsible for ESBLs production frequently carry genes encoding resistance to other drug classes also. Therefore, antibiotic options in the treatment of ESBLs producing organisms are extremely limited [18].

3.3. Effect of Gentamicin on the two strains

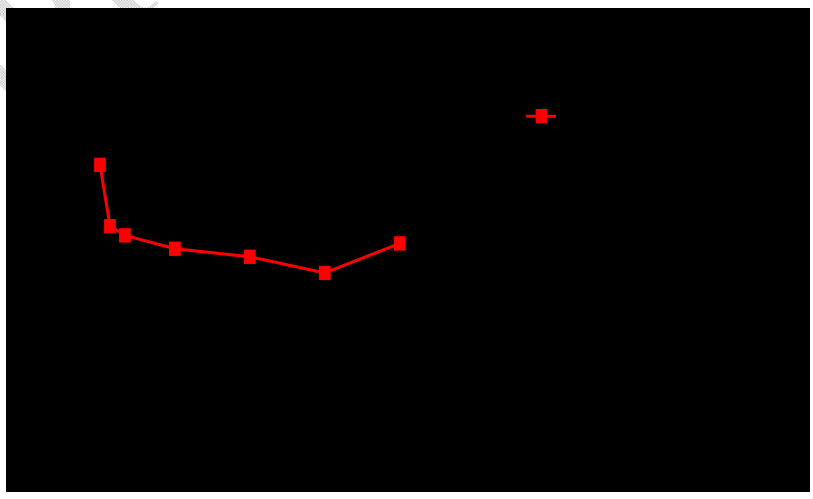

Figure 2: Effect of Gentamicin (2xMIC) on the two strain of *E. coli*

From figure 2 above, gentamicin was able to achieve up to one log reduction in the number of viable cell count at about 45 minutes of exposure with ATCC *E.coli* strain but not with ESBL strain used. ESBLs resistance is plasmid mediated; and aminoglycosides and trimethoprin – sulfamethoxazole resistances are often transferred on same plasmid [14, 18, 19]. This study may suggest that the development of ESBLs only does not affect the organism response to gentamicin or that the ESBLs gene is not on the same plasmid with gene for aminoglycoside resistance. The gene coding for ESBLs and resistance to other class of antibiotics may reside within the same plasmid and therefore be spread together meaning that resistance to two different kinds of drugs may be co selected by the use of either one or all of the antibiotics concerned and there could be a selective pressure for spreading such isolates [3].

3.4. Combined short time kill rate of Gentamicin (2xMIC) and Ciprofloxacin (2xMIC)

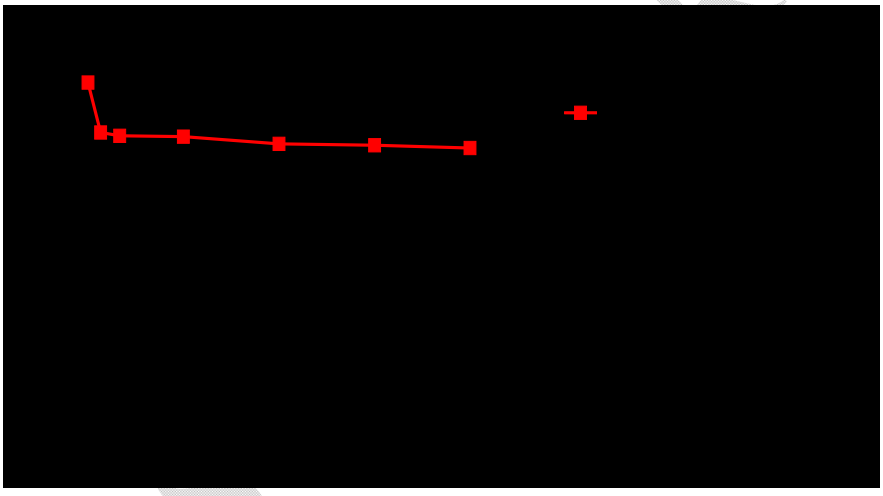

Figure 3: Combined effect of gentamicin 2xMIC) and Ciprofloxacin 2xMIC)

With the non-ESBL producing strain (figure 3 above), there was a very sharp reduction in viable cell count up to about two log reduction in just about 5 minutes after which the number of cells remained constant for the next 50 minutes. This stage was followed by a slope showing further reduction of the cells to zero. This shows that a combination of ciprofloxacin and gentamicin can effectively treat infections due to non-ESBLs producing *E. coli*. With the ESBLs-producing strain, there was also up to one log reduction in the number of viable cell count in less than 5 minutes followed by a plateau showing no change in the number of viable cell count for the remaining periods of the experiment. This study showed that the combination of gentamicin and ciprofloxacin is synergistic as it showed more than two log reduction in viable cell count of non-ESBLs producing strain. When compared with the result

of the single study with each of the drugs, this reduction is greater than what is obtained with either of the drugs when used alone. Also the killing rate constants of the single drugs are lower than that of the combination. All ESBLs producing organism should be considered resistant to all penicillin, cephalosporin, and aztreonam [21]. If the organism is sensitive to β-lactam/β-lactamase inhibitor combination, it is suggested that the organism is reported as being susceptible although use of these combinations to treat patients is not ideal [14, 22]. Aminoglycosides have good activity against clinically important gram negative bacilli [23]. Fluoroquinolones have been recommended as first line therapy in UTI [24] although resistance to fluoroquinolones is increasing throughout the world [24, 25, 26]. The observed resistance in *E. coli* to ciprofloxacin (as shown by the plateau) may be due to misuse of ciprofloxacin in eastern Nigeria.

4. CONCLUSION

Rapid bacterial eradication of pathogenic bacteria is a favourable characteristic of an antibiotic because it minimizes the spread of the disease with tremendous socio-economic impact. Development of ESBLs gene may be associated with fluoroquinolones resistance. Any considerations that favour the clinical use of two or more antimicrobial agents in combination must be based primarily on the presence of synergism between the drugs. Therefore, it is concluded that combining gentamicin and ciprofloxacin in ESBL cases have an advantage of faster microbial death over the use of any of the drugs alone. It is therefore necessary to investigate the prevalence and antimicrobial susceptibility of ESBLs positive strains in hospitals so as to formulate a policy of empirical therapy in high risk units where infections due to resistant organisms are much higher.

ACKNOWLEDGRMENT

The authors wish to acknowledge the financial support of Mr. and Mrs. Linus Icheoku for the work.

COMPETING INTERESTS: None exist.”

Authors’ Contributions

Esimone CO’ Conceptualized and designed the study, ‘Oli AN’ wrote the first draft of the manuscript. ‘Oli AH’ managed the literature searches, ‘Ugwu MC’ managed the analyses of the data, ‘Icheoku UJ’ and ‘Ikegbunam MN’ performed the experiments, did data collection and result interpretation. All authors read and approved the final manuscript.

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