



# Antibiotic combinatorial approach utilized against extended spectrum beta-lactamase (ESBL) bacteria isolates from Enugu, South Eastern Nigeria

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## ABSTRACT

**Introduction:** Antibiotic options in the treatment of extended spectrum beta-lactamase (ESBL) producing bacteria are very limited. The purpose of this study was to analyze several commonly applied antibiotics in quite various novel combinations for use against ESBL-producing bacteria isolates.

**Methods:** Total of 460 samples of urine, throat and anal swab were collected from volunteers and patients from nursery, primary and secondary schools and from other individuals in the community. Hospital and community isolates comprised of 65% and 35% respectively. The identification and characterization of the isolates were done by standard culturing and in vitro antibiotic sensitivity procedures.

**Results:** The antibiotic combination studies showed that the combination of gentamicin with the other antibiotics had predominantly synergistic effects. The percentage synergistic effect for the combinations of gentamicin/pefloxacin was 69%, gentamicin/[Amoxicillin and clavulanic acid] 72%, gentamicin/ceftriaxone 68%, gentamicin/cefuroxime 81.9%, and gentamicin/ciprofloxacin 80.6%, against the community and hospital derived ESBL producing organisms of both *Enterobacteriaceae* and *Pseudomonas species*.

**Conclusion:** Good antimicrobial monitoring exercise and corresponding antimicrobial screening activities should work towards a dynamic approach to generate effective treatment options using combination therapy.

**Keywords:** *Enterobacteriaceae*, *Pseudomonas aeruginosa*, Extended spectrum beta-lactamase (ESBL), Plasmid.

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## INTRODUCTION

Several community-acquired pathogens that commonly cause diarrhoea have been found to be ESBL producers. In the last 3 years there have been reports of true community-acquired infection or colonization with ESBL-producing *Escherichia coli* from

Spain, Israel, the United Kingdom, Canada, and Tanzania (1-4). Typically, patients have developed urinary tract infection with CTX-M-producing *Escherichia coli*. Some urinary tract infections have been associated with bacteraemia. The majority of isolates have been resistant to commonly used first-line agents for urinary tract infection such as trimethoprim-sulfamethoxazole, ciprofloxacin, gentamicin, and ceftriaxone. The cause of this sudden upsurge in community-acquired infections with ESBL-producing organisms is not yet clear, but associations with foodstuffs, animal consumption of antibiotics, and frequent patient contact with health care facilities need to be explored. The ESBL production status of the isolates were previously established through the double disc synergy test (DDST) method involving the use of a combination disc (Amoxicillin 20 µg and Clavulanic acid 10 µg) placed at the centre of the Petri dish and antibiotics (Ciprofloxacin 30 µg and Cefuroxime 30 µg) placed 15 mm apart on both sides of the plates (5). Also, in our report we could establish their community or nosocomial origins (5).

The purpose of this present work was to study some commonly applied antibiotic in quite various novel combinations for use against ESBL-producing bacteria isolates. In this paper, we present the result of our antibiotic susceptibility studies of the ESBL isolates to a cocktail of antibiotics to establish a very realistic antibiogram that may prove useful in clinical practice. The synergies that may exist between the antibiotics involved in each combination were analyzed.

## METHODS

### Microorganisms

From a total of 460 samples collected from volunteers and patients (over a five months period between October 2006 and February 2007) after informed consent and ethical approval, 20 ESBL producers were identified. From these 8 were recruited into the antimicrobial combinatorial therapeutic studies. The samples were processed through the following hospitals: respectively from four hospitals comprising University of Nigeria Teaching Hospital (UNTH); Enugu, National Orthopaedic Hospital, Enugu (NOHE), Ntasiobi Ndinona Afufu

(NONA), and Reego Laboratories, Enugu. Characterization of isolates was according to recommended standard technique by the National Committee for Clinical Laboratory Standard (NCCLS).

### Culture Media and Reagents

Nutrient broth (Oxoid, England), mannitol salt agar (Oxoid, England) nutrient agar (Fluka Spain) and peptone water were used. Sucrose, mannitol, Gram Staining reagents, buffer solution, Tris-ethylene-diamine tetra- acetic acid sodium sulfate (TENS), sodium acetate, peptone, Ethidium bromide and Bromo – phenol blue were all analar grade reagents.

### Antibiotic Discs

Antibiotic discs used were obtained from Oxoid (England) and they include ceftriaxone (30µg), Clindamycin (30µg), Ciprofloxacin (30µg), Cefuroxime (30µg), Augmentin®(Amoxicillin and clavulanic acid) (30µg), Gentamicin (30µg), Pefloxacin (30µg), Imipenem (30µg), Cefotaxime (30µg), Ceftazidime (30µg), Tetracycline (30µg).

### Antibiotic Sensitivity test

Antibiotic sensitivity of the isolates was determined using previously established procedure (6). Briefly, the isolates were cultured in nutrient broth at 37°C for 24 h. Two loopfuls of the suspension of each isolate were inoculated into 20ml of sterile molten agar in 10 cm diameter Petri dishes and mixed. The plates were allowed to set and the antibiotic Sensitivity discs were aseptically placed on their surfaces. The plates were incubated at 37°C for 24 h and the resultant inhibition zone diameters (IZDs) were measured and recorded.

### Combined Activity of Gentamicin with other Antibiotics

A 1000 µg/ml stock solution of gentamicin and 5000 µg/ml each of Augmentin® (Amoxicillin and clavulanic acid), cefoxitin, ceftriaxone, cefotaxime, imipenem and ceftazidime were prepared by dissolving in appropriate quantity of sterile distilled water. Varying proportion of Gentamicin and the other antibiotics ranging from 0:10 to 10:0 were mixed according to the continuous variation checkerboard method (6). Each proportion of the antibiotics in

TABLE 1. Minimum Inhibitory Concentration for Organisms (MIC) ( $\mu\text{g/ml}$ )

S/N	Tag	Isolates/Source	GENT	AMC	CRO	CIP	PEF	CXM
1	Cm1	<i>K. pneumoniae</i>	12.5	3.125	50	100	100	25
2	Cm2	<i>K. pneumoniae</i>	50	25	6.25	100	6.25	1.562
3	Hp3	<i>K. oxytoca</i>	25	3.125	100	50	100	6.25
4	Hp4	<i>K. pneumoniae</i>	3.125	6.250	100	50	25	6.25
5	Cm5	<i>E. coli</i>	1.562	50	1.562	12.5	6.25	12.5
6	Cm6	<i>K. oxytoca</i>	3.125	3.125	100	100	100	6.25
7	Hp7	<i>P. aeruginosa</i>	0.781	0.781	6.25	1.562	1.562	1.562
8	Hp8	<i>K. pneumoniae</i>	1.562	25	6.25	100	1.562	1.562

GEN, Gentamicin; AMC, Amoxicillin/Clavulanic acid; CRO, Ceftriaxone; CIP=Ciprofloxacin; PEF, Pefloxacin; CXM, Cefuroxime; Cm, Community isolates; Hp, Hospital isolates

combination was serially diluted (2 fold) with sterile water. Thereafter, 1 ml of each of the drug combinations was seeded in a Petri dish together with 19 ml of sterile nutrient agar. It was allowed to stand for 1 hour to solidify. An aliquot, 0.1ml equivalent of 0.5 ml MacFarland standard of ESBL producing organisms was streaked on the surface of the Mueller Hinton agar plate. The set up was done in triplicate with a control containing no antibiotics and these were incubated at 37°C for 24 hours and observed for growth. The MICs of the various combinations were determined and interactions between the antimicrobial agents were assessed by determining their Fractional Inhibitory Concentration [FIC] index using the relationship:

$$\text{FIC index} = \text{FIC A} + \text{FIC B}$$

$$\text{FIC A} = \frac{\text{MIC of Drug A in combination with Drug B}}{\text{MIC of Drug A alone}}$$

$$\text{FIC B} = \frac{\text{MIC of Drug B in combination with Drug A}}{\text{MIC of Drug B alone}}$$

The activity index (AI) =log FIC index

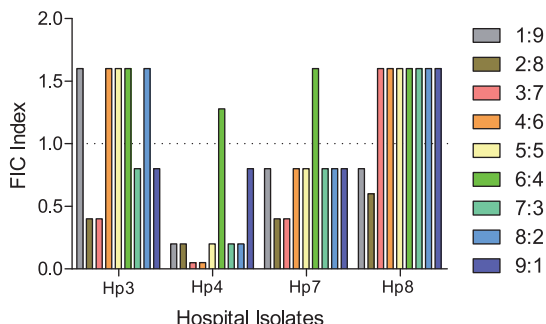
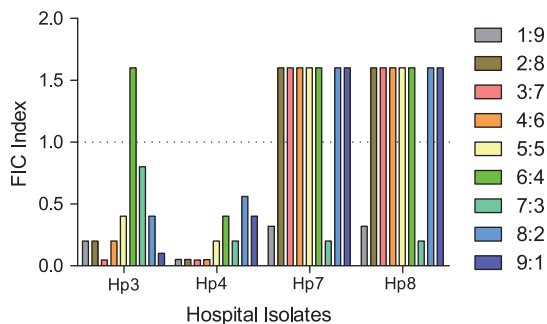
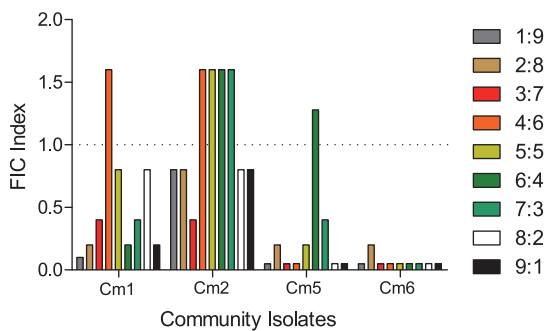
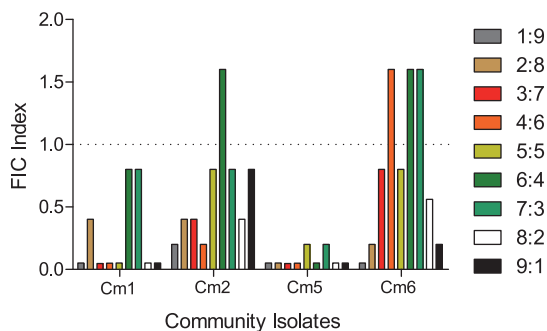
A and B are two antibiotics being combined; MIC, Minimum Inhibitory Concentration; Drug is antimicrobial agent.

## RESULTS

Table 1 shows the result of the antimicrobial susceptibility study and minimum inhibitory concentration (MIC) ( $\mu\text{g/ml}$ ). The MIC values recorded show rather staggered effects however the Klebsiella organisms displayed its values for the conventional cephalosporins and fluoroquinolones antibiotics.

Interestingly, this bacteria genus is associated with tremendous secretion and release of the beta-lactamase enzymes further correlating the thrust of this present research since they were selected (as we earlier reported in Afunwa et al, 2011) for further antibiotics combinatorial studies. We then quickly shifted to develop a simple antibiotics cocktail arrangement to be compatible with global settings including developing countries where cost of antibiotics treatment are crucial considerations towards good and effective clinical outcomes.

Figures 1-5 present the outcome of the antibiotic combinatorial checkerboard assay. Gentamicin-pefloxacin combinations (Figure 1) were largely synergistic against the community isolates in contrast to hospital isolates except Hp 3 and 4. It is a somewhat similar scenario for Gentamicin-Amoxicillin/Clavulanic acid cocktail (Figure 2) where again the community isolates were more susceptible synergistically than the hospital isolates. Thus, the same trend obtains for the Gentamicin-Amoxicillin/Clavulanic acid case. In the case of Gentamicin-Ceftriaxone (Figure 3), their combined effect holds no potential advantage among hospital isolates except in strain Hp4 where synergism is recorded. There is also no remarkable utility against community strains where combined effect simply approached additivity. In a remarkable trend shift, in Figure 4, Gentamicin-Cefuroxime combination was clearly synergistic against community isolates. Nevertheless, hospitals isolates responded differently except isolate Hp4. The most potent synergism was recorded by Gentamicin-Ciprofloxacin admix (Figure 5) against the plethora of



**FIGURE 1.** Susceptibility of ESBL Isolates to Gentamicin-pefloxacin antibiotics cocktail

Varying proportions of Gentamicin and Pefloxacin antibiotics ranging from 1:9 to 9:1 were mixed according to the continuous variation checkerboard method and seeded into Mueller Hinton agar. The ESBL producing organism was streaked on the surface of the agar plate. The set up was done in triplicate with a control containing no antibiotics and these were incubated at 37°C for 24 hours and observed for growth. The MICs of the various combinations were determined and interactions between the antimicrobial agents were assessed by determining their Fractional Inhibitory Concentration [FIC] index ratios where FIC index value below unity signifies synergism,  $\geq 1$ ; indifference, and  $\geq 2$ ; antagonism (Cm1, Cm2, Cm5, and Cm6 represent community isolates, while Hp3, Hp4, Hp7, and Hp8 represent hospital isolates respectively).

**FIGURE 2.** Susceptibility of ESBL Isolates to Gentamicin-Amoxicillin/Clavulanic acid antibiotics cocktail

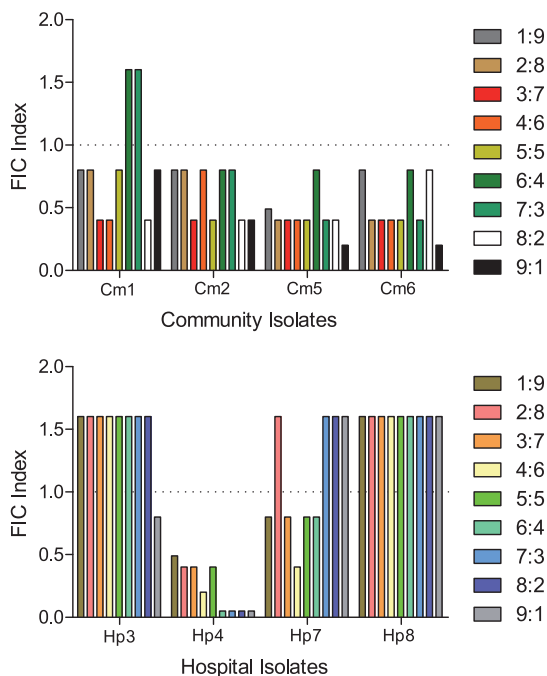
Varying proportions of Gentamicin and Amoxicillin/Clavulanic acid antibiotics ranging from 1:9 to 9:1 were mixed according to the continuous variation checkerboard method and seeded into Mueller Hinton agar. The ESBL producing organism was streaked on the surface of the agar plate. The set up was done in triplicate with a control containing no antibiotics and these were incubated at 37°C for 24 hours and observed for growth. The MICs of the various combinations were determined and interactions between the antimicrobial agents were assessed by determining their Fractional Inhibitory Concentration [FIC] index ratios where FIC index value below unity signifies synergism,  $\geq 1$ ; indifference, and  $\geq 2$ ; antagonism (Cm1, Cm2, Cm5, and Cm6 represent community isolates, while Hp3, Hp4, Hp7, and Hp8 represent hospital isolates respectively).

the community strains. This was nevertheless only reproduced in the strains Hp 3 and 4 of the hospital isolates.

**DISCUSSION**

The wide use of antibiotics in the treatment of bacterial infections has led to the emergence and spread of resistant strains. Combined antibiotic therapy may produce synergistic effects in the treatment of bacterial infection and has been shown to delay the emergence of antimicrobial resistance (7-8). Anti-

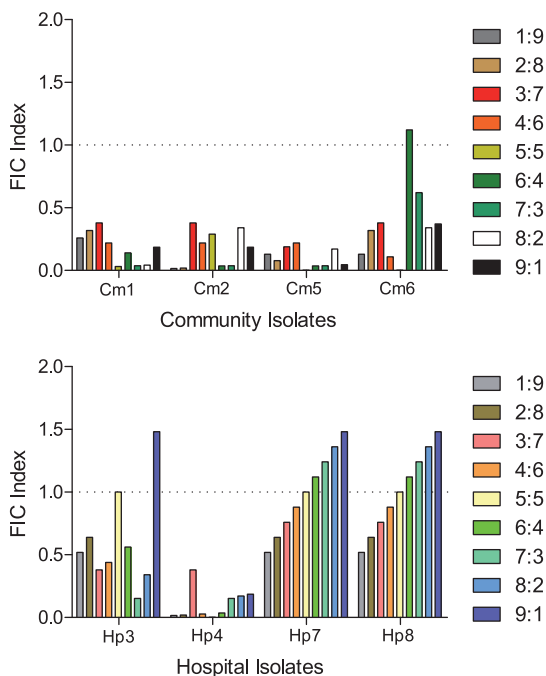
biotic combinations represent a therapeutic option in the treatment of a host of bacterial infections, as a result of the increasing appearance of multi-resistant microorganisms. In treatments involving antibiotics like rifampicin, combination therapy is used to avoid the appearance of antimicrobial resistance in the infectious agent (9-10). In other treatments, combinations are used in order to enhance the effect of individual antimicrobials by means of synergic interactions. The optimal antibiotic combinations are commonly obtained from classical suscepti-



**FIGURE 3.** Susceptibility of ESBL Isolates to Gentamicin-Ceftriaxone antibiotics cocktail

Varying proportions of Gentamicin and Ceftriaxone antibiotics ranging from 1:9 to 9:1 were mixed according to the continuous variation checkerboard method and seeded into Mueller Hinton agar. The ESBL producing organism was streaked on the surface of the agar plate. The set up was done in triplicate with a control containing no antibiotics and these were incubated at 37°C for 24 hours and observed for growth. The MICs of the various combinations were determined and interactions between the antimicrobial agents were assessed by determining their Fractional Inhibitory Concentration [FIC] index ratios where FIC index value below unity signifies synergism,  $\geq 1$ ; indifference, and  $\geq 2$ ; antagonism (Cm1, Cm2, Cm5, and Cm6 represent community isolates, while Hp3, Hp4, Hp7, and Hp8 represent hospital isolates respectively).

bility tests based on diffusion and dilution (broth microdilution test or Checkerboard), as described by the NCCLS. So far, the emergence of ESBL has compromised the utility of some currently preferred antibiotics of choice for some bacteria infectious diseases. Unfortunately, the low turnover rate for newer antibiotics to replace the compromised ones continues to hamper treatment. Therefore, the possibility of re-inventing effective use protocol through fresh, seldom applied antibiotics combinatorial platform may prove a useful alternative approach to overcome

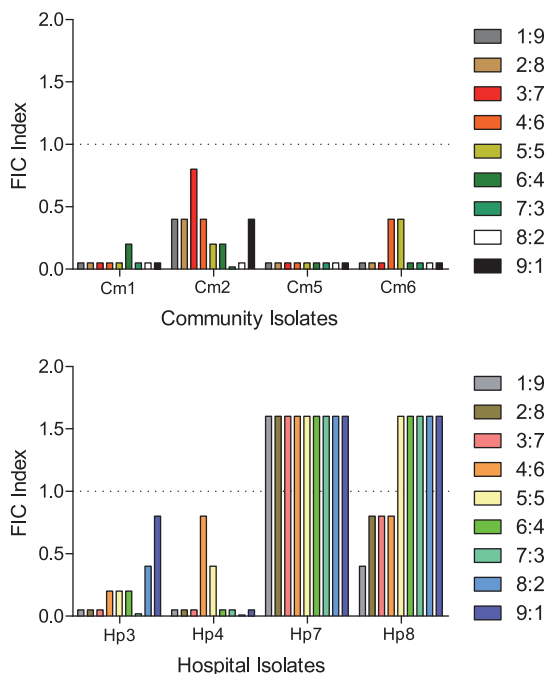


**FIGURE 4.** Susceptibility of ESBL Isolates to Gentamicin-Cefuroxime antibiotics cocktail

Varying proportions of Gentamicin and Cefuroxime antibiotics ranging from 1:9 to 9:1 were mixed according to the continuous variation checkerboard method and seeded into Mueller Hinton agar. The ESBL producing organism was streaked on the surface of the agar plate. The set up was done in triplicate with a control containing no antibiotics and these were incubated at 37°C for 24 hours and observed for growth. The MICs of the various combinations were determined and interactions between the antimicrobial agents were assessed by determining their Fractional Inhibitory Concentration [FIC] index ratios where FIC index value below unity signifies synergism,  $\geq 1$ ; indifference, and  $\geq 2$ ; antagonism (Cm1, Cm2, Cm5, and Cm6 represent community isolates, while Hp3, Hp4, Hp7, and Hp8 represent hospital isolates respectively).

this clinical fisticuffs. There are three potential advantages to using combination therapy: firstly, an increased likelihood that the infecting pathogen will be susceptible to at least one of the components of the regimen; secondly, prevention of emergence of resistance (11-12) and thirdly, reduced mortality, perhaps because of an additive or even synergistic effect of the combination (13-16).

Checkerboard evaluations as a means of monitoring the combined activities of antimicrobial agents is based on the general outcome that FIC index value



**FIGURE 5.** Susceptibility of ESBL Isolates to Gentamicin-Ciprofloxacin antibiotics cocktail

Varying proportions of Gentamicin and Ciprofloxacin antibiotics ranging from 1:9 to 9:1 were mixed according to the continuous variation checkerboard method and seeded into Mueller Hinton agar. The ESBL producing organism was streaked on the surface of the agar plate. The set up was done in triplicate with a control containing no antibiotics and these were incubated at 37°C for 24 hours and observed for growth. The MICs of the various combinations were determined and interactions between the antimicrobial agents were assessed by determining their Fractional Inhibitory Concentration [FIC] index ratios where FIC index value below unity signifies synergism,  $\geq 1$ ; indifference, and  $\geq 2$ ; antagonism (Cm1, Cm2, Cm5, and Cm6 represent community isolates, while Hp3, Hp4, Hp7, and Hp8 represent hospital isolates respectively).

below unity signifies synergism,  $\geq 1$ ; indifference, and  $\geq 2$ ; antagonism. Therefore, using checkerboard evaluation technique our investigation revealed that gentamicin-peffloxacin combinations were largely synergistic against the hospital isolates in contrast to community isolates except Cm 3 and 4. It is however a somewhat opposite scenario for Gentamicin-Amoxicillin/Clavulanic acid cocktail where the community isolates were more susceptible synergistically than the hospital isolates. These observations appear

to have relationship to the use practice of the antibiotics in the settings. Gentamicin and pefloxacin are usually largely abused among the populace within the communities (17) and as such could readily develop resistance against community bacteria isolates while maintaining effectiveness against the hospitals bacteria strains. The reverse trend appears true for the Gentamicin-Amoxicillin/Clavulanic acid case scenario. In the case of Gentamicin-Ceftriaxone, their combined effect holds no potential advantage among hospital isolates except in strain Cm4 where synergism is recorded. There is also no remarkable utility against community strains where combined effect simply approached additivity. In a remarkable trend shift, Gentamicin-Cefuroxime combination was clearly synergistic against community isolates. Nevertheless, hospitals isolates responded differently except isolate Hp4. The most potent synergism was recorded by Gentamicin-Ciprofloxacin admix against the plethora of the community strains. This was notably also reproduced in the strains Hp 3 and 4 of the hospital isolates, thus making this combination a most favourable cocktail for use against these ESBL-producing bacteria.

Developing new therapeutic strategies using existing seldom utilized antimicrobial combinations still present useful option for tackling resistant microbial circumstances including the menace of the ESBL-producing bacteria (18-20). By simply adjusting the combinations and amounts of antibiotics applied could well be the fulcrum to tilt the balance in favour of effective therapy against these categories of infectious agents. Good antimicrobial monitoring exercise and corresponding antimicrobial screening activities should work towards a dynamic approach to generate effective treatment options using combination therapy. This would then fill a gaping void created by the ever-widening scourge of persistent and newer emerging resistant bacteria infections.

## CONCLUSION

This particular study has shown the importance of keeping the wheel of progress in a dynamic state for continued antimicrobial solutions as cheap alternatives expected to find practical usefulness in clinical settings especially in the tropics.



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## COMPETING INTERESTS

The authors declare that they have no competing interests.

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