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Research Article

Comparative Evaluation of Three *In Vitro* Techniques in the Interaction of Ampicillin and Ciprofloxacin against S*taphylococcus aureus* and E*scherichia coli*.

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Abstract

Purpose*: The study was designed to evaluate the consistency of interpretation of results of interaction between ampicillin and ciprofloxacin against S. aureus and E. coli using three in vitro techniques.*
Methods*: The interaction between ampicillin and ciprofloxacin was studied using three in vitro methods-Checkerboard technique, Overlay Inoculum Susceptibility Disc technique (OLISD) and the Decimal Assay for Additivity technique (DAA).*
Results*: In the Checkerboard technique, fractional inhibitory concentration (FIC) indices show that the ampicillin/ciprofloxacin combination is synergistic against the test organisms. In the DAA approach, a target IZD of 15 mm yielded Biological Equivalent Factors (BEF) of 1.35 µg (amp/Staph), 6.74 µg (cipro/Staph), 9.62 µg (ampicillin/E. coli), and 5.45 µg (cipro/E. coli). Statistical analyses show that all decimal combinations of ampicillin and ciprofloxacin were additive ($p < 0.05$). The overlay inoculum susceptibility disc method shows inhibition zone diameter increments ranging between 36 ± 8.00 % to 69.2 ± 23.08 % for S. aureus and 28.12 ± 3.13 % to 50 ± 12.50 % for E. coli. These increments are consistent with reported criteria for synergism in the OLISD method.*
Conclusion*: The study suggests a possible clinical use for the combination of ampicillin and ciprofloxacin against infections caused by these organisms. Equally, the apparent disagreement between DAA and the other two methods raises questions as to the consistency of inferences drawn on interaction studies when different techniques are used.*

Key words: *Ampicillin, Ciprofloxacin, Interaction, Decimal Assay, Checkerboard, OLISD method.*

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INTRODUCTION

The simultaneous use of two or more antimicrobial agents has certain rationale and is recommended in specifically defined situations[1,2] Several reasons have been advanced to justify the use of combination of two or more antibiotic treatment[2,3] For many years now, combination of two or more antibiotics has been recognized as an important method for, at least, delaying the emergence of bacterial resistance[4.] Besides, antibiotic combinations may also produce desirable synergistic effects in the treatment of bacterial infections[5].

However, the selection of an appropriate combination requires an understanding of the potential for interaction between the antimicrobial agents. Accordingly, methods have been developed to quantify the effect of antimicrobial combinations on bacterial growth *in vitro*. Two very distinct traditional methods of testing *in vitro* antibiotic interaction are the checkerboard technique and the time killing curve method[6]. Operational limitations inherent in the usual methods of evaluating bacterial susceptibility to antibiotic combinations make the need for an improved method imperative. Chinwuba *et al*[7] developed with a technique-Overlay Inoculum Susceptibility Disc (OLISD) method. This is essentially a modification of the disc agar diffusion method. Sanders *et al*[8] observed that there is no quantitative dose-effect relationship and no precisely defined end-points of additivity in the traditional methods. They went ahead to describe a new *in vitro* test for antimicrobial agents used in combination[8]. This new method, the Decimal Assay for Additivity (DAA) was based on disc diffusion assay and was designed to have a precisely defined end point for additivity so that interactions greater or less than additivity could be respectively defined as synergism or antagonism.

Ampicillin is a commonly used broad-spectrum aminopenicillin with known activity against *E. coli* and *Staph. aureus*. Its clinical usefulness is limited by its susceptibility to β-lactamase hydrolysis produced by these organisms. Ciprofloxacin is a broad–spectrum fluoroquinolone and possess good activity against *E. coli* and *Staph. aureus*. Recently, there are reports of resistance to this, hitherto, effective group of antibiotics. Chromosomal mutation in subunit A of DNA-gyrase has been identified as a possible cause of bacterial resistance[9]. Resistance to quinolones by efflux mechanisms has equally been described in *Staph aureus*[10].

In this study, the interaction between ampicillin and ciprofloxacin is investigated using three *in vitro* methods-the Checkerboard titration technique, the Decimal Assay for Additivity, and the Overlay Inoculum Susceptibility Disc (OLISD) method. The result of the study could provide rational basis for clinical use of these two antibiotics against infections caused by these organisms. The study will also provide an insight into the degree of consistency of inferences drawn from results of *in vitro* antibiotic interactions using different methods of evaluation.

MATERIALS AND METHODS

Culture media

The media employed for the study are McConkey agar (Oxoid), agar-agar (Oxoid) and nutrient broth (Merck)

Test microorganisms

The organisms used were type strains of *Staphylococcus aureus* (ATCC 12600) and *E. coli* (ATCC 11775) obtained from the Bioresources Development and Conservation Programme (BDCP) Centre, Nsukka, Enugu State, Nigeria.

Drugs and Disc

Pure samples of Ampicillin powder was kindly supplied by Doyin Pharmaceutical Company, Limited, Lagos, Nigeria. Ciprofloxacin hydrochloride was extracted from the tablet dosage form (Ciproflox)®, Orange drugs limited, Nigeria. These drugs were employed to prepare

the antibiotic disc using whatmann No 1 filter paper in accordance with the NCCL standards[11].

Maintenance and standardization of test organisms

The organisms were maintained by weekly sub-culturing on McConkey agar-slants (*E. coli*) and nutrient agar slant (*S. aureus)* stored at 4 ˚C after previous 24 h incubation at 37 ˚C. Before each experiment, the microorganisms were activated by successive sub-culturing and incubation. Twenty-four hour old culture of test organism was always used. Standardization of test micro organism was according to previously reported method[7,12]

Sensitivity of test micro-organisms

The sensitivity of test microorganisms to ampicillin and ciprofloxacin hydrochloride was evaluated by determining the minimum inhibitory concentration (MIC) of the antibiotics using the two-fold broth dilution technique previously described[11,12].

Evaluation of Combined Activity of ciprofloxacin and ampicillin using the Checkerboard-technique

Stock solutions of ampicillin (400 µg/ml) and ciprofloxacin (5 µg/ml) prepared in double-strength nutrient broth and autoclaved at 121˚C for 15 minutes were employed. Thereafter, varying proportions of ampicillin (A) and ciprofloxacin (C) were prepared according to the continuous variation checkerboard method previously described [11].

Each proportion of antibiotic combination was serially diluted (2 –fold), inoculated with 0.1ml of 10^6 cfu/ml culture of the test microorganism and then incubated for 24 h at 37˚C. Interaction was assessed algebraically by determining the fractional inhibitory concentration (FIC) indices according to the relationship:

$$FICindex = FICamp + FICcipro \qquad Eqn1$$

$$FICamp = Fractional inhibitory concentration\ of\ ampicillin$$

$$= \frac{MIC of\ ampicillin\ in\ combination\ with\ ciprofloxacin}{MIC\ of\ ampicillin\ alone} \qquad Eqn2$$

$$FICcipro = Fractional\ inhibitory\ concentration\ of\ ciprofloxacin$$

$$= \frac{MIC of\ ciprofloxacin\ in\ combination\ with\ ampicillin}{MIC of\ ciprofloxacin\ alone} \qquad Eqn3$$

Evaluation of combined activity of ciprofloxacin and ampicillin using Decimal Assay for Additivity (DAA)

This method was first described by Sanders *et al*[8]. Antibiotic discs of graded drug concentrations were prepared in compliance with the NCCL standards[11].

Standard dose effect-curves. A standard dose-effect relationship was obtained for each antibiotic against each test organism using discs of graded drug concentrations in a range capable of yielding linear relationship for log dose versus IZD plot.

Biological Equivalence Factor (BEF). For each micro-organism, a common target zone of inhibition was selected at the mid-range of standard dose-effect curve of each antibiotic. A suitable IZD of 15mm was chosen as target. Using the linear regression equations of the standard plot, the quantities of the various drug required to produce the target inhibition zone were calculated. This quantity for each antibiotic represents what has been described as biological equivalence factor (BEF)[8].

Interaction by DAA technique. Once the BEFs of the antibiotics against each organism have been determined, series of eleven decimal mixtures of the 10 parts BEF of (A) +10 parts BEF of (C) were prepared. These solutions were used to prepare the disc on sterilized Whatman No. 1 fitter paper discs of diameter 6mm. Thereafter, the discs were aseptically placed on nutrient agar plates previously seeded with a standardized inoculums of the test organism and the plates incubated at 37˚C for 24 h. The procedures were replicated and mean values of IZDs recorded after incubation. The nature of interaction was judged statistically according to

the recommendation of Sanders *et al*[8]. The level of significance was decided based on a 95% confidence interval set up for a target zone of 15mm.

Table 1. combined activity of ampicillin and ciprofloxacin against *S. aureus* and *E. coli* by the checkerboard technique

S. aureus				*E. coli*				
Drug ratio (A:C)	**MIC (µg/ml) (A:C)**	**FIC (A:C)**	**FIC Index**	**Inference**	**MIC (µg/ml) (A:C)**	**FIC (A:C)**	**FIC Index**	**Inference**
10:0	125: ----	1.0: ---	1.00	----	125:---	1.0: ---	1.00	----
9:1	225:0.0313	1.80:0.1	1.90	Indifference	11.25:0.0313	0.45:0.05	0.5	Indifference
8:2	200:0.00625	1.60:0.02	1.80	Indifference	50.00:0.0313	0.20:0.05	0.25	Synergism
7:3	87.5:0.0469	0.70:0.15	0.85	Synergism	8.75:0.0938	0.34:0.15	0.50	Synergism
6:4	37.5:0.0313	0.30:0.10	0.40	Synergism	3.75:0.0625	0.15:0.10	0.25	Synergism
5:5	62.5:0.0781	0.50:0.25	0.75	Synergism	3.215:0.0781	0.125:0.125	0.25	Synergism
4:6	25.0:0.0469	0.20:0.15	0.35	Synergism	2.50:0.0938	0.10:0.15	0.25	Synergism
3:7	18.75:0.0547	0.15:0.175	0.33	Synergism	1.8:0.1094	0.075:0.175	0.25	Synergism
2:8	25.0:0.125	0.20:0.399	0.60	Synergism	5.0:0.5	0.20:0.80	1.00	Additivity
1:9	12.5:0.406	0.10:0.449	0.55	Synergism	1.5:0.563	0.10:0.90	1.00	Additivity
0:10	---: 0.313	---: 1.00	1.00	Additivity	---: 0.625	---: 1.00	1.00	----

A = Ampicillin
C = Ciprofloxacin
FIC = Fractional Inhibitory Concentration

Evaluation of combined activity of ciprofloxacin and ampicillin by OLISD technique

The method was first reported by Chinwuba *et al*[7]. The procedure is basically a modification of the disc agar diffusion method. A solution of ampicillin was prepared in molten nutrient agar to yield about 50% of the MIC of the ampicillin against each test microorganism. Then 20 ml of the antibiotic seeded agar was poured in the Petri-dish to form the base agar-layer and 5 ml of molten antibiotic free agar containing 10^6 cfu/ml was applied as a thin overlay inoculum-agar layer and allowed to solidify. The ciprofloxacin antibiotic discs of varying drug concentrations were placed on the solidified surface and the plates incubated at 37^0C for 24 h.. The petri dishes so treated were taken as the test plates. The control plates were similarly prepared without any antibiotic on the base agar-layer. The mean percentage increases in IZD's of the test over the control groups were determined. The interaction results were then determined according to recommended criteria [7].

RESULTS AND DISCUSSION

E. coli was more sensitive than *Staph. aureus* to ampicillin (MICs of 25 ± 1.20 µg/ml and 125 ± 2.50 µg/ml, respectively) while *Staph. aureus* was more sensitive than *E. coli* to ciprofloxacin (MICs of 0.3 ± 0.15 µg/ml and 0.625 ± 0.25 µg/ml, respectively) as indicated by their respective MIC values.

In the Checkerboard technique, the interaction between pair combinations of ampicillin and ciprofloxacin against *Staph aureus* and *E. coli* were predominantly synergistic, although there were few variations (Tables 1). FIC_{index} values < 1were considered as synergy and the degree of synergy increases as the value tends towards zero. FIC-index value of one show additivity, values greater than one, but less than two represent indifference while values greater than two show antagonism[7,12]. Seven out of nine pair

Table 2: Interactions between ampicillin and ciprofloxacin against *Staph. aureus by DAA* technique

Drug	Diameter of target zone inhibition (mm)	BEF	Practical mean IZD (mm)	95% CI[a]	Type of inference
10(A)+0(C)	15	1.353µg	15.0	13.04-16.96	---
0(A)+10(C)	15	6.74µg	15.25	14.14-16.01	---
9(A)+1(C)-		-	17.25	16.75-17.74	Additive (P>0.05)[b]
8(A)+2(C)-		-	15.5	14.52-16.48	Additive (P>0.05)
7(A)+3(C)-		-	16.5	15.52-17.48	Additive (P>0.05)
6(A)+4(C)-		-	16.5	15.25-17.48	Additive (P>0.05)
5(A)+5(C)-		-	16.25	15.25-17.48	Additive (P>0.05)
4(A)+6(C)-		-	15.5	15.76-16.74	Additive (P>0.05)
3(A)+7(C)-		-	14.5	14.52-16.48	Additive (P>0.05)
2(A)+8(C)-		-	14.5	13.52-15.48	Additive (P>0.05)
1(A)+9(C)-		-	14.5	13.52-15.48	Additive (P>0.05)

[a]CI, Confidence interval, [b]probability value for analysis of variance at ά = 0.05

Table 3: Interactions between ampicillin and ciprofloxacin against E. coli by DAA technique

Drug	Diameter of target zone inhibition (mm)	BEF	Practical mean IZD (mm)	95% CI[a]	Type of inference
10(A)+0(C)	15	9.62	16.0	14.04-15.48	---
0(A)+10(C)	15	5.45	14.5	13.52-15.48	---
9(A)+1(C)-		-	17.5	14.56-20.44	Additive (P>0.05)[b]
8(A)+2(C)-		-	16.5	13.56-19.44	Additive (P>0.05)
7(A)+3(C)-		-	18.5	17.52-19.48	Additive (P>0.05)
6(A)+4(C)-		-	19.0	17.04-20.96	Additive (P>0.05)
5(A)+5(C)-		-	17.5	14.56-20.44	Additive (P>0.05)
4(A)+6(C)-		-	16.0	14.04-17.96	Additive (P>0.05)
3(A)+7(C)-		-	13.5	10.56-16.44	Additive (P>0.05)
2(A)+8(C)-		-	12.5	11.52-13.48	Additive (P>0.05)
1(A)+9(C)-		-	18.0	14.08-21.92	Additive (P>0.05)

[a]CI, Confidence interval 95%
[b]probability value for analysis of variance at ά = 0.05

Table 4. Interaction between ampicillin and ciprofloxacin against *S. aureus* and *E. coli* by OLISD-technique.

Microorganism	Ciprofloxacin (Disc drug)	Mean inhibition zone diameter (IZD± SEM)		Increase in IZD ±SEM (%)	Inference
		Control (mm)	Test (mm)		
S. aureus	5.0	14.25± 0.25	21.0±1.00	47.37± 8.77	Synergism
	1.25	11.0± 0.50	36.36± 0.50	36.36± 0.50	Synergism
	0.31	6.5±0.50	69.23±15.38	69.23±15.38	Synergism
	0.078	6.25± 0.25	36.0±8.00	36.0±8.00	synergism
E. coli	10.0	16.0± 0.00	20.5±0.50	28.12±3.13	Synergism
	5.0	14.5± 0.50	19.5± 0.50	34.486±6.90	Synergism
	2.5	11.5±0.50	15.5±0.00	34.78±8.70	Synergism
	1.25	0.0± 0.50	12.0±0.50	50.00±12.50	Synergism

combinations of ampicillin and ciprofloxacin

produced synergistic effect against *Staph aureus*. In E. coli, the antibiotic combination resulted in the MIC of ampicillin decreasing from 125 µg/ml to a very low value of 1.5 µg/ml in 1 (A) + 9(c) mixture) and that of ciprofloxacin decreasing from 0.625 µg/ml to 0.0313 µg/ml. Only two decimal mixtures [9 (A) + 1 (C) and 1 (A) + 9 (C)] deviated from synergism and were, respectively, indifference or additive.

The Biologic Equivalent Factors [calculated on a target IZD of 15 mm from regression equations of plots of IZD versus log (disc drug concentrations)] are 1.353 µg (ampicillin/*Staph. aureus*), 11.41 µg (ampicillin/*E. coli*), 8.994 µg (ciprofloxacin/*S. aureus),* 5.45 µg (ciprofloxacin/ *E. coli*). In the interaction study by DAA, all the decimal combinations produced an additive interaction against both organisms (Table 2 and 3). The inhibition zone diameters produced by the pair combinations were similar to that produced by each of the antibiotics acting alone. At 95 % confidence interval, there is no statistically significant difference between the IZD's of pair combinations and that of ampicillin and ciprofloxacin alone[5]. Although, additivity is a positive interaction, the inference from this method is not in agreement with that of the Checkerboard technique.

In the OLISD technique (Table 4), all the IZD increments were above 19 % and is interpreted as synergism[7]. This agrees with the inference obtained in the Checkerboard method and differs with that of the DAA approach.

Ampicillin and ciprofloxacin are both bactericidal, but they act through different mechanisms and at different sites on bacteria cells. Ampicillin inhibit the formation and integrity of the bacteria cell wall while ciprofloxacin inhibit DNA – gyrase[4,9]. Jawtez *et al*[13] observed that concomitant use of two bactericidal antibiotics is likely to produce synergistic effect. The results of this study have demonstrated either synergism or additive interaction against the test organisms. Although, these inferences are all potentiation of activity, the apparent disagreement between the DAA approach and the other two methods call to question the issue of uniformity and standardization of techniques to avoid conflicting results in interaction studies. It is hoped that these approaches, if well standardized and adopted, will not only provide useful alternatives to pre-existing time-kill and checkerboard titration method, but will also circumvent problems and methodological limitations inherent in their use.[8]

The possibility of a combination of these two antibiotics limiting the development and spread of resistance strains is yet to be investigated in greater detail.

CONCLUSION

Based on results obtained, ampicillin interacts with ciprofloxacin to produce a synergistic antibacterial activity against strains of *Staph. aureus* and *Escherichia coli* in the majority of the cases. Actual clinical experiences are needed to conclude on possible clinical benefits of using these two antibiotics in combination. The apparent disagreement between DAA approach and the other two methods needs further investigation with a view to standardizing techniques to avoid variation of inferences and interpretation of results between different methods.

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