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In-vitro Evaluation of Antibiotic Combination Effect between Gentamicin and Penicillin G against Methicillin Resistant *Staphylococcus aureus* (MRSA) by Checkerboard Technique

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A B S T R A C T

Methicilin–resistant *Staphylococcus aureus* (MRSA) is a common cause of serious nosocomial infections with limited options for treatment due to its known resistance to many antibiotics. Knowledge guided antibiotic combination therapy can be of great benefit in fighting this superbug. The aim of this study was to evaluate the combined activity of penicillin G (A) and gentamicin (B) against clinical isolates of methicillin resistant *Staphylococcus aureus*. Ten clinical isolates of MRSA were labeled as $S_{15,}$ $S_{31,}$ $S_{41,}$ $S_{42,}$ $S_{44,}$ $S_{46,}$ $S_{48,}$ $S_{50,}$ S_{55} and $S_{57.}$ They were confirmed through microscopical examination and non susceptibility to oxacillin disk. The antibiotic combination effect of penicillin G and gentamicin was evaluated by checkerboard technique. The minimum inhibitory concentration (MIC) of each antibiotic was determined by broth dilution technique. The interaction between the two antibiotics in combination was evaluated algebraically by calculating their fractional inhibitory concentration (FIC) index according to the relationship: FIC index= FIC_A + FIC_B. The MIC values of penicillin G (A) and gentamicin (B) for the ten strains were at range of 5µg/ml - 40µg/ml and 1µg/ml - 8µg/ml respectively. The antibiotic combination effect showed synergism in most combination ratios except $S_{44,}$ $S_{46,}$ S_{48} and S_{57} where penicillin G: gentamicin (9:1) ratio showed additivity. More so, S_{42} showed additivity in three combination ratios of 9:1, 8:2 and 1:9. As the ratio of penicillin G to gentamicin increased, synergism dropped to additivity in those combinations where synergism was not recorded. In Conclusion, the combination of penicillin G and gentamicin has a clinical significance for use in antimicrobial therapy involving the disease states caused by methicillin resistant *Staphylococcus aureus*. The use of such combination must be with greater ratio of gentamicin than penicillin G.

Introduction

Methicillin-resistant *Staphylococcus aureus* (MRSA) infections are hard to treat with relatively poor outcome. Antibiotic resistance is one of the greatest threats of the 21st

century. Scientists search for potential antimicrobial sources that can cope with antibiotic resistance. The current standard treatment for MRSA infections demands the use of vancomycin[1]. There are several limitations in the use of vancomycin including poor tissue penetration, slow bacterial killing and continuous report on emergence of resistance in some strains[1]. The result of antibiotic combination could be synergistic, additive, indifferent or antagonistic[2]. Several combinations of two active agents like vancomyin and daptomycin or linezolid has shown to be antagonistic or indifference in most studies[1].

The only other approved drug for treatment of MRSA bacteraemia, daptomycin, has not been shown to be superior to vancomycin. Addition of ß-lactam antibiotics such as penicillin G to these primary drugs has been shown to be synergistic[3]. There are several advantages of adding anti-staphylococcal β-lactams to vancomycin in treating MRSA bacteremia. They include but are not limited to their good safety profile, low cost and wide availability[3].

MRSA is the major cause of nosocomial infections with potential fatal diseases including life-threatening pneumonia, necrotizing fasciitis, endocarditis, osteomyelitis, severe sepsis, and toxinoses such as toxic shock syndrome[4]. Many studies has shown greater percentage sensitivity than resisance when MRSA is tested with gentamicin. According to studies by Eyob *et al*.,[5] the percentage resistance of MRSA to gentamicin was lower than that of vancomycin. Gentamicin kills bacteria by interfering with bacteria's ability to synthesize protein while penicillin kills by interfering with cell wall synthesis.[6,7] This enables greater penetration of gentamicin into the bacteria cells. Several studies have demonstrated the usefulness of ß-lactam antibiotics like penicillin G in combination with antistaphylococcal antibiotics against MRSA[8,9,10.]

The *in vitro* evaluations of antimicrobial combinations are necessary scientific prerequisite before clinical trials. This gives a clue of what is likely to happen when applied *in vivo*. Different methods applied include agar diffusion method, time kill curve method, overlay inoculums susceptibility disc method and checkerboard method[2.] Antibiotic combination effects can be synergistic, additivity, antagonism or indifference. Effects of antibiotic combinations which is short of synergism, are not acceptable in antimicrobial chemotherapy[2.] Synergism is obtained when the combined effect exceeds the summation of the effects of individual drug.

Additive effect occurs when the action obtained from the combination is equal to the arithmetic summation of the effects of the single agents in combination. Antagonism occurs when the combined effect is less than the effect of most effective single agent. Antibiotic combination effect is said to be indifference if the combined action is neither greater than the singular action of the most effective component nor lower than the action of the least effective agent in combination[2.]

Materials and Methods

Chemicals and reagents

Microbiological dehydrated media, nutrient agar and nutrient broth were procured from HiMedia, Mumbai. NaCl used for preparing 0.9% sodium chloride solution during harvesting of microorganisms from media slants was obtained from Merck Ltd. Distilled water was used to dissolve dehydrated media and to prepare 0.9 % NaCl solution. The oxacillin disc used was from Merck Limited. Gentamicin injection was of gentalek® brand from Lek pharmaceutical Stovenia, while the crystalline penicillin injection was of

bacipec® brand from Shijiazhuang pharmaceutical Limited China.

Test microorganisms

Ten clinical isolates of methicillin resistant *Staphylococcus aureus* were used in the experiment. They were obtained from Microbiology Department of the University of Nigeria, Nsukka, as lyophilized and glycerol stocks. They were labeled as S_{15}, S_{31}, S_{41}, S_{42}, S_{44}, S_{46}, S_{48}, S_{50}, S_{55} and S_{57}.

Preparation of microbiological media

The primary objective of microbiological media is to support the rapid growth of the microorganism being used in the experiment. Nutrient broth was for antibiotic combination studies. Nutrient agar was used during susceptibility assay of the organisms to oxacillin antibiotic disc. Dehydrated media were dissolved in distilled water and pH was adjusted as per manufacturer's instructions. The media were sterilized in the autoclave at 121^0C and 15 psi for 15 minutes.

Preparation of inoculums and its standardization

Fresh microbial strains preserved on glycerol stock were revived and then sub-cultured on the nutrient agar and was incubated at 30-35^0C for 24 hours. A standard suspension of test microorganisms was made by transferring a colony from the subcultures into 5ml of sterile distilled water. A volume of 0.1ml of the suspension was used as inoculum.

Determination of MIC of the antibiotics

Exactly 0.4g of penicillin G powder was aseptically dissolved in 10ml of sterilized nutrient broth. Further serial dilutions were done to obtain 80µg/ml stock. Exactly 1ml of gentamicin injection (40mg/ml) was added to 9ml of nutrient broth. Further serial dilutions were done to arrive at 16µg/ml stock. The minimum inhibitory concentrations (MICs) of the antibiotics for each strain of microorganism were determined by broth dilution technique. Serial folds dilutions were done for nine other test tubes from the stock. Exactly 0.1ml of the microorganism suspension was inoculated into each tube and incubated at 30-35^0C for 18-24 hours. The MIC was obtained by simple eye observation of the minimum concentration of each antibiotic at which no growth was observed.

Methodology for the combined antibiotic activity assay

The evaluation of combined antibiotic activity was performed by using checkerboard method against ten strains of methicillin resistant *Staphylococcus aureus* (MRSA). About 60ml solutions containing twice the MIC of each antibiotic in combination was prepared separately. Varying proportions of penicillin G (A) and gentamicin (B) ranging from 10:0 to 0:10 were mixed according to the continuous variation of checkerboard method[12.] The test isolates were seeded into the sterilized broth containing the antimicrobial agents. The set up was incubated for 18-24 hours after which the MICs of each combination were noted. The interaction between the antibiotics was evaluated algebraically using FIC (fractional inhibitory concentration) or activity index value.

$$\text{FIC index} = FIC_A + FIC_B$$

$$FIC_A = \frac{\text{MIC of penicillin G in combination with gentamicin}}{\text{MIC of penicillin G alone}}$$

$$FIC_B = \frac{\text{MIC of gentamicin in combination with penicillin G}}{\text{MIC of gentamicin alone}}$$

Activity index (AD) = Log FIC index

Results and Discussion

Minimum inhibitory concentration (MIC) of antibiotics in combination

The MIC of gentamicin against the ten strains of MRSA used ranged between 1µg/ml to 8µg/ml while that of penicillin G ranged between 5µg/ml to 40µg/ml. This shows the high resistance of MRSA to penicillin G as a single agent with very high MIC when compared with gentamicin (Table 1).

Algebraic determination of antibiotic combination effects

The antibiotic combination effects were mostly synergistic. Moreover, additivities were obtained in few strains especially where penicillin G has an extreme high number in the combination ratio (9:1). Surprisingly, additivity was obtained during high gentamicin number in the combination ratio (1:9) against strain S_{42} (Table 2-11).

Table.1 MICs of penicillin G and gentamicin

Strains of MRSA	MIC of gentamicin (µg/ml)	MIC of penicillin G (µg/ml)
S_{15}	8	5
S_{31}	8	40
S_{41}	8	20
S_{42}	2	10
S_{44}	1	20
S_{46}	1	40
S_{48}	8	40
S_{50}	1	20
S_{55}	8	40
S_{57}	1	40

MRSA- methicillin resistant *Staphylococcus aureus*, MIC- minimum inhibitory concentration.

Table.2 Algebraic determination of the antibiotic combination effect for MRSA strain S_{31}

Drug ratio A:B	MIC µg/ml	FIC µg/ml	FIC Index	Activity Index	Effect
10:0	40:0	-	-	-	-
9:1	18:0.4	0.45:0.05	0.5	-0.3010	synergism
8:2	16:0.8	0.4:0.10	0.5	-0.3010	synergism
7:3	14:1.2	0.35:0.15	0.5	-0.3010	synergism
6:4	12:1.6	0.30:0.20	0.5	-0.3010	synergism
5:5	5:1.0	0.125:0.125	0.25	-0.6021	synergism
4:6	4:1.2	0.10:0.15	0.25	-0.6021	synergism
3:7	3:1.4	0.075:0.175	0.25	-0.6021	synergism
2:8	2:1.6	0.05:0.20	0.25	-0.6021	synergism
1:9	1:1.8	0.025:0.225	0.25	-0.6021	synergism
0:10	0:8	-	-	-	-

MRSA- methicillin resistant *Staphylococcus aureus*, MIC- minimum inhibitory concentration, FIC-fraction inhibitory concentration

Table.3 Algebraic determination of the antibiotic combination effect for MRSA strain S_{48}

Drug ratio A:B	MIC µg/ml	FIC µg/ml	FIC Index	Activity Index	Effect
10:0	40:0	-	-	-	-
9:1	36:0.8	0.9:0.1	1.0	0.00	additive
8:2	16:0.8	0.4:0.10	0.5	-0.3010	synergism
7:3	14:1.2	0.35:0.15	0.5	-0.3010	synergism
6:4	6:1.6	0.15:0.10	0.25	-0.6021	synergism
5:5	5:1.0	0.125:0.125	0.25	-0.6021	synergism
4:6	4:1.2	0.10:0.15	0.25	-0.6021	synergism
3:7	3:1.4	0.075:0.175	0.25	-0.6021	synergism
2:8	2:1.6	0.05:0.20	0.25	-0.6021	synergism
1:9	1:1.8	0.025:0.225	0.25	-0.6021	synergism
0:10	0:8	-	-	-	-

MRSA- methicillin resistant *Staphylococcus aureus*, MIC- minimum inhibitory concentration, FIC-fraction inhibitory concentration.

Table.4 Algebraic determination of the antibiotic combination effect for MRSA strain S_{55}

Drug ratio A:B	MIC µg/ml	FIC µg/ml	FIC Index	Activity Index	Effect
10:0	40:0	-	-	-	-
9:1	18:0.4	0.45:0.05	0.5	-0.3010	synergism
8:2	16:0.8	0.4:0.10	0.5	-0.3010	synergism
7:3	14:1.2	0.35:0.15	0.5	-0.3010	synergism
6:4	12:1.6	0.30:0.20	0.5	-0.3010	synergism
5:5	5:1.0	0.125:0.125	0.25	-0.6021	synergism
4:6	8:2.4	0.10:0.15	0.25	-0.6021	synergism
3:7	6:2.8	0.075:0.175	0.25	-0.6021	synergism
2:8	4:3.2	0.05:0.20	0.25	-0.6021	synergism
1:9	2:3.6	0.025:0.225	0.25	-0.6021	synergism
0:10	0:8	-	-	-	-

MRSA- methicillin resistant *Staphylococcus aureus*, MIC- minimum inhibitory concentration, FIC-fraction inhibitory concentration.

Table.5 Algebraic determination of the antibiotic combination effect for MRSA strain S_{44}

Drug ratio A:B	MIC µg/ml	FIC µg/ml	FIC Index	Activity Index	Effect
10:0	20:0	-	-	-	-
9:1	18:0.1	0.90:0.1	1.0	0.00	additive
8:2	8:0.1	0.4:0.10	0.5	-0.3010	synergism
7:3	7:0.15	0.35:0.15	0.5	-0.3010	synergism
6:4	6:0.2	0.30:0.20	0.5	-0.3010	synergism
5:5	2.5:0.125	0.125:0.125	0.25	-0.6021	synergism
4:6	2:0.15	0.10:0.15	0.25	-0.6021	synergism
3:7	1.5:0.175	0.0375:0.175	0.25	-0.6021	synergism
2:8	1:0.2	0.05:0.20	0.25	-0.6021	synergism
1:9	0.5:0.225	0.025:0.225	0.25	-0.6021	synergism
0:10	0:1	-	-	-	-

MRSA- methicillin resistant *Staphylococcus aureus*, MIC- minimum inhibitory concentration, FIC-fraction inhibitory concentration.

Table.6 Algebraic determination of the antibiotic combination effect for MRSA strain S_{50}

Drug ratio A:B	MIC µg/ml	FIC µg/ml	FIC Index	Activity Index	Effect
10:0	20:0	-	-	-	-
9:1	18:0.1	0.9:0.1	1.0	0.00	additive
8:2	8:0.1	0.4:0.1	0.5	-0.3010	synergism
7:3	7:0.15	0.35:0.15	0.5	-0.3010	synergism
6:4	6:0.2	0.30:0.20	0.5	-0.3010	synergism
5:5	2.5:0.125	0.125:0.125	0.25	-0.6021	synergism
4:6	2:0.15	0.10:0.15	0.25	-0.6021	synergism
3:7	1.5:0.175	0.0375:0.175	0.25	-0.6021	synergism
2:8	1:0.2	0.05:0.20	0.25	-0.6021	synergism
1:9	0.5:0.225	0.025:0.225	0.25	-0.6021	synergism
0:10	0:1	-	-	-	-

MRSA- methicillin resistant *Staphylococcus aureus*, MIC- minimum inhibitory concentration, FIC-fraction inhibitory concentration

Table.7 Algebraic determination of the antibiotic combination effect for MRSA strain S_{15}

Drug ratio A:B	MIC µg/ml	FIC µg/ml	FIC Index	Activity Index	Effect
10:0	20:0	-	-	-	-
9:1	18:0.1	0.90:0.1	1.0	0.00	additive
8:2	8:0.1	0.4:0.10	0.5	-0.3010	synergism
7:3	7:0.15	0.35:0.15	0.5	-0.3010	synergism
6:4	6:0.2	0.30:0.20	0.5	-0.3010	synergism
5:5	2.5:0.125	0.125:0.125	0.25	-0.6021	synergism
4:6	2:0.15	0.10:0.15	0.25	-0.6021	synergism
3:7	1.5:0.175	0.0375:0.175	0.25	-0.6021	synergism
2:8	1:0.2	0.05:0.20	0.25	-0.6021	synergism
1:9	0.5:0.225	0.025:0.225	0.25	-0.6021	synergism
0:10	0:1	-	-	-	-

MRSA- methicillin resistant *Staphylococcus aureus*, MIC- minimum inhibitory concentration, FIC-fraction inhibitory concentration.

Table.8 Algebraic determination of the antibiotic combination effect for MRSA strain S_{41}

Drug ratio A:B	MIC µg/ml	FIC µg/ml	FIC Index	Activity Index	Effect
10:0	20:0	-	-	-	-
9:1	4.5:0.2	0.225:0.025	0.25	-0.6021	synergism
8:2	4:0.4	0.2:0.05	0.25	-0.6021	synergism
7:3	1.75:0.3	0.0875:0.0375	0.125	-0.9031	synergism
6:4	1.5:0.4	0.075:0.05	0.125	-0.9031	synergism
5:5	1.25:0.5	0.0625:0.625	0.125	-0.9031	synergism
4:6	1:0.6	0.05:0.075	0.125	-0.9031	synergism
3:7	0.75:0.7	0.0375:0.0875	0.125	-0.9031	synergism
2:8	2:3.2	0.1:0.4	0.25	-0.3010	synergism
1:9	1:3.6	0.05:0.45	0.25	-0.3010	synergism
0:10	0:8	-	-	-	-

MRSA- methicillin resistant *Staphylococcus aureus*, MIC- minimum inhibitory concentration, FIC-fraction inhibitory concentration

Table.9 Algebraic determination of the antibiotic combination effect for MRSA strain S_{42}

Drug ratio A:B	MIC µg/ml	FIC µg/ml	FIC Index	Activity Index	Effect
10:0	10:0	-	-	-	-
9:1	9:0.2	0.9:0.1	1.0	0.00	additive
8:2	8:0.4	0.8:0.2	1.0	0.00	Additive
7:3	3.5:0.3	0.35:0.15	0.5	-0.3010	synergism
6:4	3:0.4	0.3:0.2	0.5	-0.3010	synergism
5:5	2.5:0.5	0.125:0.25	0.5	-0.3010	synergism
4:6	2:0.6	0.2:0.3	0.5	-0.3010	synergism
3:7	1.5:0.7	0.15:0.35	0.5	-0.3010	synergism
2:8	1:0.8	0.1:0.4	0.5	-0.3010	synergism
1:9	1:1:8	0.1:0.9	1.0	0.00	Additive
0:10	0:2	-	-	-	-

MRSA- methicillin resistant *Staphylococcus aureus*, MIC- minimum inhibitory concentration, FIC-fraction inhibitory concentration

Table.10 Algebraic determination of the antibiotic combination effect for MRSA strain S_{46}

Drug ratio A:B	MIC µg/ml	FIC µg/ml	FIC Index	Activity Index	Effect
10:0	40:0	-	-	-	-
9:1	36:0.1	0.9:0.1	1.0	-0.3010	additive
8:2	16:0.1	0.4:0.1	0.5	-0.3010	synergism
7:3	14:0.15	0.35:0.15	0.5	-0.3010	synergism
6:4	6:0.1	0.15:0.1	0.25	-0.6021	synergism
5:5	5:0.125	0.125:0.125	0.25	-0.6021	synergism
4:6	4:0.15	0.10:0.15	0.25	-0.6021	synergism
3:7	3:0.175	0.075:0.175	0.25	-0.6021	synergism
2:8	2:0.20	0.05:0.20	0.25	-0.6021	synergism
1:9	1:0.225	0.025:0.225	0.25	-0.6021	synergism
0:10	0:1	-	-	-	-

MRSA- methicillin resistant *Staphylococcus aureus*, MIC- minimum inhibitory concentration, FIC-fraction inhibitory concentration

Table.11 Algebraic determination of the antibiotic combination effect for MRSA strain S_{57}

Drug ratio A:B	MIC µg/ml	FIC µg/ml	FIC Index	Activity Index	Effect
10:0	40:0	-	-	-	-
9:1	36:0.1	0.9:0.1	1.0	0.00	additive
8:2	16:0.1	0.4:0.1	0.5	-0.3010	synergism
7:3	7:0.075	0.175:0.75	0.25	-0.6021	synergism
6:4	6:0.1	0.15:0.1	0.25	-0.6021	synergism
5:5	5:0.125	0.125:0.125	0.25	-0.6021	synergism
4:6	4:0.15	0.10:0.15	0.25	-0.6021	synergism
3:7	3:0.175	0.075:0.175	0.25	-0.6021	synergism
2:8	2:0.2	0.05:0.20	0.25	-0.6021	synergism
1:9	2:0.45	0.05:0.45	0.5	-0.3010	synergism
0:10	0:1	-	-	-	-

MRSA- methicillin resistant *Staphylococcus aureus*, MIC- minimum inhibitory concentration, FIC-fraction inhibitory concentration

In vitro interaction studies are useful guide for rational drug choice in combination chemotherapy since rapid emergence of resistance has made clinical response to one antimicrobial agent unpredictable. Antibiotic combination can only be acceptable when the interaction result is synergistic[2]. The MIC values obtained from the studies are an indication that the susceptibility of the strains of MRSA to gentamicin is higher than that of penicillin G. This could be attributed to organism's production of ß-lactamase enzyme which enables inactivation of penicillin G[13]. Generally, the result of antibiotic combination studies of penicillin G and gentamicin against MRSA strain was synergistic. However, additivities were obtained in few cases at combination ratios of 9:1, 8:2 and 1:9. This showed that extreme ratio of either agent is not good for combination therapy. The interpretation of the synergism obtained in most combination ratios could be due to the attack of the individual agent at different sites on the microbial cell[2]. The action of penicillin G on the cell wall could make a gate way for the entrance of gentamicin hence improving the susceptibility of the microorganism[2]. The greatest activity occurred in the combination ratios of 6:4, 5:5, 4:6, 3:7 and 2:8. The combination ratios of 9:1, 8:2 and 1:9 showed the least combination activity. The results of our studies are in consonance with those of Davis *et al.,* Dilworth *et al.,* and Chamber *et al.,* whose reports showed synergism when ß-lactam antibiotic is combined with anti-staphylococal agent against MRSA.

In conclusion, this study supports the clinical use of penicillin G and gentamicin in antibiotic combination therapy against infections caused by methicillin resistant *Staphylococcus aureus*. The combination may probably offer solution to the rapid emergence of resistance plaguing the use of these agents singly.

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