Evaluation of some Antibiotics in Combination Activity Against Isolates of 
*Staphylococcus aureus* and *Pseudomonas aeruginosa*

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**Abstract**  
The combinations of eight first–line antibiotics were investigated against *S. aureus* and *P. aeruginosa* by the evaluation of fractional inhibitory concentration (FIC) index. Ten isolates of *S. aureus* and ten isolates of *P. aeruginosa* were isolated from clinical samples and the minimum inhibitory concentration (MIC) for each antibiotic was determined. Synergistic interactions were observed in the combinations ampicillin–gentamycin, rifampicin–neomycin and rifampicin–tetracycline against both *S. aureus* and *P. aeruginosa*; and also in ciprofloxacin-tobramycin for *P. aeruginosa*. Other combinations were either additive or indifferent; one antagonistic interaction between chloramphenicol–erythromycin was observed. The results suggest that antibiotic combination is a potential way to achieve synergy when the causal organism is a multi-antibiotic resistance one.

Keywords: Antibiotic resistance, Interactions, Checkerboard and synergy test, *S. aureus*, *P. aeruginosa*.

**Introduction**  
The wide use of antibiotics in the treatment of bacterial infections has led to the emergence and spread of resistant strains. Infections due to *Staphylococcus* are resistant to beta-lactam [1]. While *Enterococcus* strains are resistant to vancomycin, ampicillin, gentamycin and streptomycin [2]. Gram negative pathogens such as *Salmonella* species, *Pseudomonas aeruginosa* and *Klebsiella pneumonia* have become multidrug resistant [3]. Among the various infections caused by bacteria, antibiotic resistant ones are of major concern because of their non-responsiveness to treatment with a single drug regime thus resulting in therapeutic failure [4]. The use of antibiotic combinations has been known since a long time and is often applied when several mechanisms of action and toxicity profile of agents involved can be brought to halt at once [5]. The biocidal (bacteriocidal, fungicidal or virucidal) activity could be best achieved by the combination of two different antibiotics rather than the effect obtained by an individual antibiotic [6]. Antimicrobial synergism occur when two or more antibiotics, in combination exert an inhibitory effect that is greater than the additive effects of the individual antibiotic [7]. The reason to apply more than one antibiotic is to increase the activity of the antibiotic, decrease the side effect of some antibiotics and reduce the dose when situations of resistance development and ineffectiveness of single antibiotic are prevalent in the treatment of inflammatory infections that are life threatening [8].

Combinations of antimicrobials that demonstrate an in vitro synergism against infecting strains are more likely to result in successful therapeutic outcome [9]. Thus there is a need to find new ways to control evolving of drug resistant infections [10].

Among the methods employed in the evaluation of the combination of two antimicrobials potentially exhibiting synergism is the checkerboard or fractional inhibitory concentration (FIC) index. FIC employs a methodology similar to that utilized for the determination of the minimum inhibitory concentration (MIC). The combination is said to have synergistic effect if there is a 2-fold reduction in the MIC for each antimicrobial agent tested alone [11].

The purpose of this study is to investigate antimicrobial activity and evaluate the interaction of various combination of antibiotics against *S. aureus* and *P. aeruginosa* using the FIC index method. It is thought that the results may provide rational basis for clinical use of these combinations against infections caused by these drug resistant organisms.
Materials and Methods

Samples Collection

Samples were collected from wound and post operative infection (POI) of patients hospitalized in various Baghdad hospitals. From these samples, isolates of *S. aureus* and *P. aeruginosa* were included and these were identified depending on their morphological and biochemical tests as compared with the identification scheme described by Holt *et al.* (1994) [12].

Preparation of Bacterial Inoculum

The inoculum of the test organisms were prepared using the colony suspension [13]. Colonies picked from 24 h old culture grown on nutrient agar were used to make suspension of the test organisms in normal saline (NS) to give an optical density of approximately 0.1 at 611 nm. The suspension was then diluted by transferring 1 ml of the bacterial suspension to 9.9 ml of sterile nutrient broth (NB) before use.

The following antibiotics were used in this study. Ampicillin 500 mg (SDI, Iraq), Chloramphenicol 500 mg (Bavaria Pharma, Germany), Ciprofloxacin 500 mg (Remedica, Cyprus), Erythromycin 500 mg (ZetaBoard, India), Gentamicin 500 mg (Morvel, India), Levofloxacin 500 mg (Sandoz, USA), Neomycin 500 mg (Sandoz, USA), Rifampicin 500 mg (Lannett, USA), Tetracycline 500 mg (Actavis, USA), Tobramycin 500 mg (Novaplus, Austria).

Determination of the Minimum Inhibitory Concentrations (MIC) (Tube dilution assay)

To determine the minimum inhibitory concentrations, the antibiotic was dissolved in distilled water (DW) to give stock concentration of 5 μg/ml. Two fold serial dilutions of the antibiotics were made to give concentrations ranging from 0.1 to 5 μg/ml. One hundred micro liter of bacterial inoculum was added to the dilution tubes. The tubes were incubated at 37 °C for 6h under aerobic conditions. The MIC was defined as the lowest concentration of the antibiotic that completely inhibited visible growth of the organism as observed with naked eye [14].

Determination of Interaction between antibiotics

The study of the combined antimicrobial activity of antibiotics was done by broth dilution checkerboard method as described by Mandal *et al.* (2014) [15]. The antibiotics were combined at concentrations ranging from 1/8 x MIC to 2 xMIC, then inoculated with bacterial cultures and incubated for 14 h at 37 °C after which the MIC values were estimated. The fractional inhibitory concentration (FIC) was derived from the lowest concentration of antibiotic combination permitting no visible growth of the test organism in the tube and was calculated for each antimicrobial concentration as follows:

FIC of compound A (FIC A) = MIC of compound A in combination with B / MIC of compound A alone.

FIC of compound B (FIC B) = MIC of compound B in combination with A / MIC of compound B alone.

The fractional inhibitory concentrations of the two compounds in the combination i.e. the FIC index = FIC A + FIC B.

Combination between antibiotics according to accepted criteria [11] as follows: ≤ 0.5, synergy; 0.5 to 1.0, additive; 1.0 to 2.0, indifference; and > 2, antagonism.

Results and Discussion

The tube dilution assay (MIC test) for the inhibitory effect of the antibiotic alone for *S. aureus* and *P. aeruginosa* are shown in Table (1). The results are obtained from ten isolates of each organism. The antibiotics used in this study appeared to vary in the levels of susceptibility to *S. aureus* and *P. aeruginosa*. Most of the antibiotics were effective against *S. aureus* except ampicillin, in comparison with *P. aeruginosa*, which is less susceptible to ampicillin, chloramphenicol, erythromycin, and tetracycline.
**Table (†)**

The MIC values of antibiotics for *S. aureus* and *P. aeruginosa*.

<table>
<thead>
<tr>
<th>Antibiotic</th>
<th><em>S. aureus</em></th>
<th><em>P. aeruginosa</em></th>
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</thead>
<tbody>
<tr>
<td>Ampicillin</td>
<td>32</td>
<td>512</td>
</tr>
<tr>
<td>Chloramphenicol</td>
<td>8</td>
<td>256</td>
</tr>
<tr>
<td>Ciprofloxacin</td>
<td>1</td>
<td>1.5</td>
</tr>
<tr>
<td>Erythromycin</td>
<td>1</td>
<td>1.28</td>
</tr>
<tr>
<td>Gentamicin</td>
<td>8</td>
<td>2</td>
</tr>
<tr>
<td>Levofloxacin</td>
<td>2</td>
<td>4</td>
</tr>
<tr>
<td>Neomycin</td>
<td>16</td>
<td>1.5</td>
</tr>
<tr>
<td>Rifampicillin</td>
<td>0.5</td>
<td>12</td>
</tr>
<tr>
<td>Tetracycline</td>
<td>3</td>
<td>22</td>
</tr>
<tr>
<td>Tobramycin</td>
<td>1</td>
<td>0.5</td>
</tr>
</tbody>
</table>

**Table (‡)**

The Mean FIC index values and standard deviations for the combination of antibiotics against *S. aureus* and *P. aeruginosa*.

<table>
<thead>
<tr>
<th>Combination</th>
<th><em>S. aureus</em></th>
<th><em>P. aeruginosa</em></th>
</tr>
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<tbody>
<tr>
<td></td>
<td>Mean FIC (SD)</td>
<td>Interaction</td>
</tr>
<tr>
<td>Amp – Chl</td>
<td>*,,*8‡</td>
<td>Additive</td>
</tr>
<tr>
<td>Amp – Gen</td>
<td>*,,<em>4</em></td>
<td>Synergy</td>
</tr>
<tr>
<td>Chl – Ery</td>
<td>*,,*3</td>
<td>Antagonism</td>
</tr>
<tr>
<td>Cip – Ery</td>
<td>*,,*4‡</td>
<td>Indifferent</td>
</tr>
<tr>
<td>Cip – Tob</td>
<td>*,,*4</td>
<td>Indifferent</td>
</tr>
<tr>
<td>Gen – Lev</td>
<td>*,,*2</td>
<td>Indifferent</td>
</tr>
<tr>
<td>Rif – Neo</td>
<td>*,,*2‡</td>
<td>Synergy</td>
</tr>
<tr>
<td>Rif – Tet</td>
<td>*,,<em>2</em></td>
<td>Synergy</td>
</tr>
</tbody>
</table>

*Amp = Ampicillin, Chl = Chloramphenicol, Cip = Ciprofloxacin, Ery = Erythromycin*  
*Gen = Gentamicin, Lev = Levofloxacin, Neo = Neomycin, Rif = Rifampicillin*  
*Tet = Tetracycline, Tob = Tobramycin.*

A relationship is suggested between the MIC of an antimicrobial and clinical outcome of infections due to *S. aureus* and *P. aeruginosa* treated with single antimicrobial [17]. In particular, a lower MIC was associated with a faster healing response [18].

It is reasonable to assume that the lower the MIC of an antimicrobial for a given isolate, the more likely it is that the infection will respond to treatment and that the MIC of the antimicrobial can be used to evaluate the potency of a given agent.
In the checkerboard method, the interaction between selected combinations of the eight antibiotics against 11 isolates of each of *S. aureus* and *P. aeruginosa* as estimated by the mean FIC index values and standard deviation (SD) are shown in Table (\(^1\)) for *S. aureus* and *P. aeruginosa*.

Synergistic reactions are seen in the combinations ampicillin–gentamycin mean FIC index 1.4, rifampicin–neomycin mean FIC index 1.34, and rifampicin–tetracycline mean FIC index 1.45 for *S. aureus*, and ampicillin–gentamycin mean FIC index 1.41, ciprofloxacin–tobramycin mean FIC index 1.44, rifampicin–neomycin mean FIC index 1.35, and rifampicin–tetracycline mean FIC index 1.39 for *P. aeruginosa*. Other combinations were either additive or indifferent. Only one combination chloramphenicol–erythromycin was antagonist against *S. aureus*. The combination ampicillin–gentamycin is synergetic against both *S. aureus* and *P. aeruginosa* as a result of ampicillin which is known as an agent of β-lactam block enzyme of transpeptidase needed by the bacteria to make their cell wall, while gentamicin is known to inhibit protein synthesis by binding to the 30S ribosomal subunit. The result of this combination is inline with the work of Kim *et al.* (\(^2\)) \(19\), who demonstrated that ampicillin–gentamycin can act synergistically in inhibiting methicillin-resistant *S. aureus* (MRSA) in *vitro*.

The synergy in the combination ciprofloxacin–tobramycin is explained by the action of ciprofloxacin which is known to block DNA synthesis by inhibiting one of the enzymes (DNA gyrase) needed in this process and the action of tobramycin which is known to work by binding to a site on the bacterial 70S and 30S ribosome by preventing the formation of 50S complex. Our results are compatible with the report of NcNabb *et al.* (\(^3\)) \(1\), that explained the superior activity of ciprofloxacin against *P. aeruginosa* in combination with cefazidime that yield remarkable activity profile.

The combinations rifampicin–neomycin and rifampicin–tetracycline also indicate synergism against both *S. aureus* and *P. aeruginosa*, since rifampicin is commonly used in the treatment of staphylococcal prosthesis or skin associated infection, including chronic wound [\(^4\)]. The chemical structure of rifampicill allows the drug to penetrate the wall into tissue and abscesses, while are poorly penetrated by rest other antistaphylococcal agents [\(^5\)]. However, *S. aureus* can develop rifampicin resistance during a single passage [\(^6\)], and it is therefore always used in combination with other antibiotics to treat bacterial infection [\(^7\)]. In fact, the combination rifampicin - minocycline (tetracycline) has been found to have an efficiency of \(\%\) in rabbit model [\(^8\)]. The synergistic activity of rifampicin – neomycin against *S. aureus* and *P. aeruginosa* is in agreement with the report of Bisdas *et al.*, (\(^9\)) \(26\) that rifampicin - neomycin showed excellent *in vitro* antibiotal activity against both gram-positive and gram-negative pathogens representing an effective candidate for vascular graft impregnation. Fig.(\(^1\)) shows analogy – variance activity of the combined antibiotics against *S. aureus* and *P. aeruginosa* for synergy, additive–indifference and antagonistic reactions.

It is interesting to note that infections with *Staphylococcus* or *Pseudomonas* species are notoriously difficult to treat as both organisms exhibit resistance to multiantibiotic; few new antibiotics are currently in development [\(^7\)]. It has also been shown that combination of antibiotic with non-antibiotic substance can
enhance the efficiency of a number of currently used antibiotics by forming synergistic combinations [18].

Conclusion
The combinations ampicillin–gentamycin, rifampicin–neomycin and rifampicin-tetracycline gave the lowest mean FIC index for S. aureus indicative of synergistic effect in 0.25%. Against P. aeruginosa the combinations ampicillin–gentamycin, ciprofloxacin–tobramycin as well as rifampicin–neomycin and rifampicin-tetracycline also gave lowest mean FIC index indicative of synergy in 0.25%. Only one combination chloramphenicol – erythromycin was consistently antagonistic when used against S. aureus. Other combinations tested were predominately additive or indifferent. An elucidation of the mechanisms of action of these compounds need to be followed by toxicity and in vivo tests to determine the therapeutic applicability of such compounds in combination therapy.

References


كانت أما زيادة التأثير أو عدم اختلاف التأثير، كما لوحظ وجود تفاعل تضادي لحالة واحدة للجمع بين الكلورامفاميكول – ارثراماسين.

ينتشر من هذه الدراسة بان علاج جمع المضادات الحيوية يكون وسيلة ممكنة للوصول إلى التأثير ألتآزيفي. عندما تكون البكتريا السببية مقاومة لعدد من المضادات الحيوية.