Journal of Al-Nahrain University Science **Vol.١٧ (٤), December, ٢٠١٤, pp.١٧٤-١٧٩**

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

Rita N. N. Rammo
Department of Biology, College of Science, University of Kirkuk, Iraq.
E-mail: rita_rammo@yahoo.com.

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 chloramphinicol–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 [١]. While *Enterococcus* strains are resistant to vancomycin, ampicillin, gentamycin and streptomycin [٢]. Gram negative pathogens such as *Salmonella* species, *Pseudomonas aeruginosa* and *Klebsiella pneumonia* have become multidrug resistant [٣]. 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 [٤]. 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 [٥]. 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 [٦]. 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 [٧]. 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 [٨].

Combinations of antimicrobials that demonstrate an *in vitro* synergism against infecting strains are more likely to result in successful therapeutic outcome [٩]. Thus there is a need to find new ways to control evolving of drug resistant infections [١٠].

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 ٤-fold reduction in the MIC for each antimicrobial agent tested alone [١١].

The purpose of this study is to investigate antimicrobial activity and evaluate the interaction of various combination of antibiotics against *S. aurous* 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 ١٠ isolates of *S. aureus* and ١٠ isolates *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*. (١٩٩٤) [١٢].

Preparation of Bacterial Inoculum

The inoculum of the test organisms were prepared using the colony suspension [١٣]. Colonies picked from ٢٤ 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 ٠,١ at ٦٠٠ nm. The suspension was then diluted ١:١٠٠ by transfer ٠,١ ml of the bacterial suspension to ٩,٩ ml of sterile nutrient broth (NB) before use.

The following antibiotics were used in this study. Ampicillin ٥٠٠ mg (SDI, Iraq), Chloramphinicol ٥٠٠ mg (Bavaria Pharma, Germany), Ciprofloxacin ٥٠٠ mg (Remedica, Cyprus), Erythromycin ٢٥٠ mg (ZetaBoard, India), Gentamicin ٢٠٠ mg (Morvel, India), Levofloxacin ٥٠٠ mg(Sandoz, USA), Neomycin ٥٠٠ mg (Sandoz, USA), Rifampicin ٥٠٠ mg (Lannett, USA), Tetracycline ٢٥٠ mg (Actavis, USA), Tobramycin ٥٠٠ 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 ٥١٢ µg/ml. Two fold serial dilutions of the antibiotics were made to give concentrations ranging from ٠,٥ to ٥١٢ µg/ml. One hundred micro liter of bacterial inoculum was added to the dilution tubes. The tubes were incubated at ٣٧ ºC for ٢٤h 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 [١٤].

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*. (٢٠٠٤) [١٥]. The antibiotics were combined at concentrations ranging from ١/٨ x MIC to ٢xMIC, then inoculated with bacterial cultures and incubated for ٢٤h at ٣٧ ºC 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 sum of 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 [١٦] as follows: ≤ ٠,٥, synergy; ٠,٥ to ١,٠, additive; ١,٠ to ٤,٠, indifference; and > ٤, 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 (١). 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, chloramphinicol, erythromycin, and tetracycline.

Table (١)
The MIC values of antibiotics for S. aureus and P. aeruginosa.

Antibiotic	MIC values µg/ml	
	S. aureus	P. aeruginosa
Ampicillin	٣٢	٥١٢
Chloramphinicol	٨	٢٥٦
Ciprofloxacin	١	٠,٥
Erythromycin	١	١٢٨
Gentamicin	٨	٢
Levofloxacin	٢	٤
Neomycin	١	٤
Rifampicillin	٠,٥	١٦
Tetracycline	٢	٣٢
Tobramycin	١	٠,٥

Table (٢)
The Mean FIC index values and standard deviations for the combination of antibiotics against S. aureus and P. aeruginosa.

Combination	S. aureus		P. aeruginosa	
	Mean FIC (SD)	Interaction	Mean FIC (SD)	Interaction
Amp – Chl	٠,٨٤ (٠,١٣)	Additive	٠,٧٩ (٠,١١)	Additive
Amp – Gen	٠,٤٠ (٠,٠٧)	Synergy	٠,٤١ (٠,٠٥)	Synergy
Chl – Ery	٥,٣٥ (٠,٢٣)	Antagonism	١,١٤ (٠,٢٩)	Indifferent
Cip – Ery	١,٨٩ (٠,١٣)	Indifferent	١,٤١ (٠,١٢)	Indifferent
Cip – Tob	٢,٠١ (٠,١٦)	Indifferent	٠,٤٤ (٠,٠٩)	Synergy
Gen – Lev	١,٣٢ (٠,١١)	Indifferent	١,٢٥ (٠,١٠)	Indifferent
Rif – Neo	٠,٣٤ (٠,٠٩)	Synergy	٠,٣٥ (٠,٠٧)	Synergy
Rif – Tet	٠,٤٥ (٠,٠٤)	Synergy	٠,٣٩ (٠,٠٦)	Synergy

Amp = Ampicillin, Chl = Chloramphinicol, Cip = Ciprofloxacin, Ery = Erythromycin Gen = Gentamycin, Lev = Levofoxcin, Neo = Neomycin, Rif = Rifampicin 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 [١٧]. In particular, a lower MIC was associated with a faster healing response [١٨]. 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 ١٠ 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 (٢) for *S. aureus* and *P. aeruginosa*.

Synergistic reactions are seen in the combinations ampicillin–gentamycin mean FIC index ٠,٤, rifampicin–neomycin mean FIC index ٠,٣٤ and rifampicin–tetracycline mean FIC index ٠,٤٥ for *S. aureus*, and ampicillin–gentamycin mean FIC index ٠,٤١, ciprofloxacin – tobramycin mean FIC index ٠,٤٤, rifampicin–neomycin mean FIC index ٠,٣٥ and rifampicin–tetracycline mean FIC index ٠,٣٩ for *P. aeruginosa*. Other combinations were either additive or indifferent. Only one combination chloramphinicol–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 ٣٠s ribosomal subunit. The result of this combination is inline with the work of Kim *et al*. (٢٠٠٩) [١٩], who demonstrated that ampicillin– gentamycin can act synergistically in inhibiting methicillin – resistant *S. aurues* (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 ٣٠s and ٥٠s ribosome by preventing the formation of ٧٠s complex. Our results are compatible with the report of NcNabb *et al*., (٢٠٠٠) [٢٠] that explained the superior activity of ciprofloxacin against *P. aeruginosa* in combination with ceflazidime 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 staphellococcual prosthesis or skin associated infection, including chronic wound [٢١]. The chemical structure of rifampicillin allows the drug to penetrate the wall into tissue and abscesses, while are poorly penetrated by rest other antistaphylococcal agents [٢٢]. However, *S. aureus* can develop rifampicin resistance during a single passage [٢٣], and it is therefore always used in combination with other antibiotics to treat bacterial infection [٢٤]. In fact, the combination rifampicin - minocycline (tetracycline) has been found to have an efficiency of ٧٠٪ in rabbit model [٢٥]. The synergistic activity of rifampicin – neomycin against *S. aureus* and *P. aeruginosa* is in agreement with the report of Bisdas *et al*., (٢٠١٢) [٢٦] that rifampicin - neomycin showed excellent *in vitro* antibiotical activity against both gram-positive and gram-negative pathogens representing an effective candidate for vascular graft impregnation. Fig.(١) shows analogy – variance activity of the combined antibiotics against *S. aureus* and *P. aeruginosa* for synergy, additive–indifference and antagonistic reactions.

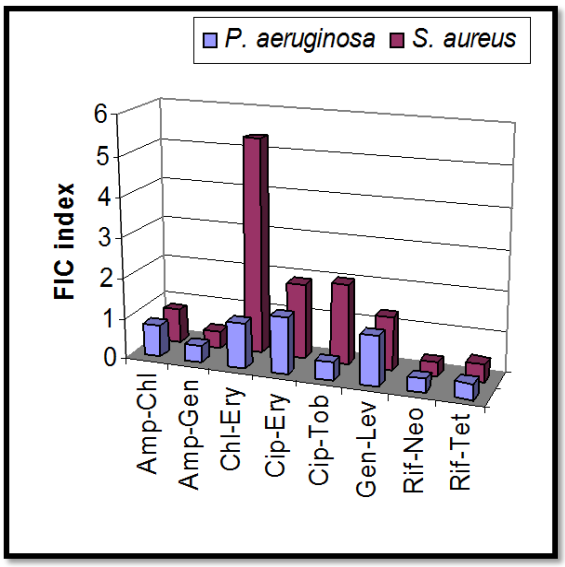

Fig.(١) Interaction of combined antibiotics in terms of FIC index against S. aureus and P. aeruginosa.

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 [٢٧]. 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 [٢٨].

Conclusion

The combinations ampicillin–gentamycin, rifampicin–neomycin and rifampicin–tetracycline gave the lowest mean FIC index for *S. aureus* indicative of synergitical effect in ٨٥٪. Against *P. aeruginosa* the combinations ampicillin – gentamycin, ciprofloxacin–tobramycin as well as rifampici–neomycin and rifampicin–tetracycline also gave lowest mean FIC index indicative of synergy in ٨٠٪. Only one combination chloramphinicol – 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

[١] Cook, N.; "Methicillin resistant *Staphylococcus aureus* the burn patient"; Burns, ٢٤: ٩١-٩٨, ١٩٩٨.

[٢] Montecalvo, M.A.; Horowitz, H.; Gedris, C.; Carbonaro, C.; Tenover, F.C.; Issah, A.; Cook, D.; and Wormser, G.P. ; "Outbreak of vancomycin-, ampicillin-, and aminoglycoside - resistant *Enterococcus faecium* bacteremia in an adult oncology unit"; Antimic. Agent Chemother., ٣٨(٦): ١٣٦٣-١٣٦٧, ١٩٩٤.

[٣] Fluit, A.C.; Schmitz, F.J.; Verhoef, J.; "Multi-resistance to antimicrobial agents for the ten most frequently isolated bacterial pathogens"; Int. J. Antimic. Agents, ١٨: ١٤٧-١٦٠, ٢٠٠١.

[٤] Ibezim, E.C.; Esimone,C.O.; OKorie, O.; Onyishi, I.V.; Nnamani, P.O.; Brown, S.A.; and Obodo, C.E.; "A study of the in vitro interaction of cotrimoxazole and ampicillin using the checkerboard method"; Afr. J. Biotechnol., ٥: ١٢٨٤-١٢٨٨, ٢٠٠٦.

[٥] de Souza, D.; Mehta, D.; and Leavitt, R.W.; "Bactericidal activity of combinations of silver-water dispersion TM with ١٩ antibiotics agents seven microbial strains"; Curr. Sci., ٩١: ٩٢٦-٩٢٩, ٢٠٠٦.

[٦] Williams, L.V.; and Baltimore, W.; "Antibiotics in laboratory medicine: Laborotory methods used to assess the activity of antimicrobial combinations"; ٣: ٤٣٤-٤٤٤, ١٩٩١.

[٧] Rani, A.; Jafn, S.; Dureja, P.; Kumar, R.; and Kumar, A.; "Synergistic interaction between synthetic and natural products: a promising tool for the development of environmentally safe potent antimicrobial agents"; World Appl. Sci. ٥(special issue for Environment): ٥٩-٦٣, ٢٠٠٩.

[٨] Casey, J.R. and Pichichero, M.E.; "Metaanalysis of short course antibiotic treatment for a streptococcal tonsillopharyngitis group"; The Pedeatric Infectious Disease Journal, ٢٤ (١٠): ٩٠٩-٩١٧, ٢٠٠٥.

[٩] Kamatou, G.P.; Vilijoen, A.M.; van Vuuren, S.F.; and van Zyl, R.L.; "*In vitro* evidence of antimicrobial synergy between *Salvia chamelaeagnes* and *Leonotis leonurus*"; S. Afr. J. Bot., ٧٢: ٦٣٤-٦٣٦, ٢٠٠٦.

[١٠] Ccdric, J.; Navas, D.; Batard, E.; Miegerille, A. F.; Mabecque, V. L.; Kergueris, M. F.; Bugnon, D.; Potel, G.; and Caillon, J. ; "*In vitro* and *in vivo* synergistic activities of linezolid combined with subinhibitory concentrations of imipenem against methicillin-resistant *Staphylococcus aurues* Antimicrobial Agents"; Chemother., ٤٩: ٤٥-٥١, ٢٠٠٥.

[١١] Vishwanatha, T.; Divyahree, B. C.; Spoorthi, N. J.; Reena, V.; Aishwarya, S.; Siddhalingeshwara, K. G.; and Venugopal, N.; "Evaluation of *in vitro* synergy between ampicillin and kanamycin against *Staphylococcus aureus*"; J. Drug. Deli. Therap., ٢(٤): ١٤٤-١٤٦, ٢٠١٢.

[١٢] Holt, J. G.; Krieg, N. R.; Sneath, P. H.; Staley, J. T.; and Williams, S. T.; "Bergy's Manual of Determination Bacteriology"; ٩th ed. Williams and Wilkins, Baltimor, USA, ١٩٩٤.

[١٣] Sibanda, J. and OKoh, A. I.; "*In vitro* evaluation of the interactions between acetone extracts of *Garcinia kola* seeds and some antibiotics"; Afr. J. Biotechnol., ٧(١١): ١٦٧٢-١٦٧٨, ٢٠٠٨.

[١٤] Orhan, G.; Bayram, A.; Zer, Y. and Balci, I.; "Synergy tests by E-test and checkerboard methods of antimicrobial combinations against *Brucella melitensis*"; J. Clin. Microbiol., ٤٣: ١٤٠-١٤٣, ٢٠٠٤.

treatment of a local outbreak"; J. Am. Accd. Dermatol., ٥٠: ٨٥٤-٨٥٨, ٢٠٠٤.
[٢٣] Eng, R.H.K.; Sharon, M.; Buccin, F.J.; and Cherabin, C.E.; "Difference in ability of cell-wall antibiotics to suppress emergence of rifimpicillin resistance in *Staphylococcus aureus*"; J. Antimicrob. Chemother., ١٥: ٢٠١-٢٠٧, ١٩٨٥.
[٢٤] Campbell, E.A.; Korzhera, N.; Mustaer, A.; Murakami, K.; Nair, S.; Goldfarb, A.; and Darst, S.A.; "Structure mechanism for rifampicillin inhibition of bacteria of RNA polymerase"; Cell., ١٠٤: ٩٠١-٩١٢; ٢٠٠١.
[٢٥] Zeheriya, U.I.; Rabih, O.D.; Glenn, C.L.; Thomas, B.; "Efficiency of antibiotic alone for orthopaedic device related infections"; Clin. Ortho. Res., ٣٣٢: ١٨٤-١٨٩, ١٩٩٦.
[٢٦] Bisdas, T.; Beckmann, E.; Marsch, G.; Burgwitz, K.; Wilhelim, M.; Kuehn, C.; Haverich, A.; and Teebken, O.E. ; "Prevention of vascular graft infections: in vitro comparison between daptomycin, rifampicillin and nebacetin"; Eur. J. Vasc. Endovas. Sur., ٤٣(٤): ٤٤٨-٤٥٦, ٢٠١٢.
[٢٧] Mendoza, N.; and Tyring, S.K.; "Emerging drugs for complicated skin and skin structure infections"; Expert Opin. Emerg. Drugs, ١٥: ٥-٢٠, ٢٠١٠.
[٢٨] Ejim, L.; Farha, M.A.; Falconer, S.B.; Wildenhain, J.; Coombes, B.K.; Tyers, M.; Brown, E.D.; and Wright, G.D.; "Combinations of antibiotics and nonantibiotic drugs enhance antimicrobial efficiency"; Nat. Chem. Biol., ٧: ٣٤٨-٣٥٠, ٢٠١١.

[١٥] Mandal, S.; Mandal, M.D.; and Pal, N.K.; "Evaluation of combination effect of ciprofloxacin and cefazolin against *Salmonella enterica* serovar *typhi* isolates by *in vitro* methods"; Calicul Med. J., ٢(٢): e٢, ٢٠٠٤.
[١٦] Chung, P.Y.; Navaratnam, P.; and Chung, L.Y.; "Synergistic antimicrobial activity between pentacyclic triterpenoids and antibiotics against *Staphylococcus aureus* strains"; Ann. Clin. Microbiol. Antimicrob., ١٠: ٢٥-٣١, ٢٠١١.
[١٧] Kaye, S. B.; Neal, T.; Nichdson, S.; Szkurlat, J.; Bamber, S.; Baddon, A. C.; Anderson, S.; Seddon, K.; Dwyer, N.; Lovering, A.M.; and Smith, G.; "Concentration and bioavailability of ciprofloxacin and teicoplanin in the cornea"; Invest. Ophtbalmol. Vis. Sci., ٥٠: ٣١٧٦-٣١٨٤, ٢٠٠٩.
[١٨] Sueke, H.; Kaye, S. B.; Neal, T.; Hall, A.; Tuft, S.; and Parry, C. M.; "Minimum inhibitory concentrations of standard and novel antimicrobials for isolates from bacterial keratitis"; Invert. Ophtamol. Vis. Sci., ٥١(٨): ٤١-٥١-٤١٥٥, ٢٠١٠.
[١٩] Kim, E.S.; Jeong, S.I.; Kim, J.H.; Park, C.; Kim, S.M.; Kim, J.K.; Lee, K.M.; Lee, S.H.; So, H.; and Park, R.; "Synergistic effects of the combination of ٢٠-hydroxyecdysone with ampicillin and gentamicin against *Staphylococcus aureus*"; J. Microbiol. Biotechnol., ١٩(١٢): ١٥٧٦-١٥٨١, ٢٠٠٩.
[٢٠] NcNabb, J.; Quintiliani, R.; Nightingle, C.H.; and Nicolau, D.P.; "Comparison of the bactericidal activity of trovfloxacin and cefepime, against *Pseudomonas aeruginosa*"; Chemother., ٤٦:٣٨٣-٣٨٩, ٢٠٠٠.
[٢١] Villar, M.; Marimon, J.M.; Garcia-Areenzana, J.M.; Adela, G.; Ferrandiz, M.J.; Perzer-Trallero, E.; "Epidemiological and molecular aspects of rifampicillin-resistant samples"; J. Antimicrobial. Chemother., ٦٦(٥): ٩٩٧-١٠٠٠, ٢٠١١.
[٢٢] Lyer, S.; and Jones, D.H.; "Community-acquired methicillin resistance *Staphylococcus aurues* skin infections a retrospective analysis of clinical presentation and

الخلاصة

درست فعالية جمع ثمان مضادات حيوية من الصف الأول ضد بكتريا *Staphylococcus aureus* و *Pseudomonas aeruginosa* من خلال تقييم دليل التركيز التثبيطي ألكسري FIC. عزلت ١٠ عزلات لكل من *S. aureus* و *P. aeruginosa* من نماذج سريرية وتم تحديد التركيز التثبيطي الأدنى MIC لكل مضاد حيوي. لوحظ وجود تفاعل تآزري لجمع المضادات الحيوية نوع امبسلين – جنتامايسين، رفامبسلين – نيومايسين و رفامبسلين – تتراسايكلين ضد كلا *S. aureus* و *P. aeruginosa* وكذلك للجمع بين سبروفلوكساسين – توبرامايسين لـ *P. aeruginosa*. أما للجمع الأخرى فقد

عندما تكون البكتريا المسببة مقاومة لعدد من المضادات الحيوية.

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

يستنتج من هذه الدراسة بان علاج جمع المضادات الحيوية يكون وسيلة ممكنة للوصول إلى التأثير ألتآزري