THE SYNERGISTIC EFFECT OF SOME ANTIBIOTICS AGAINST CLINICAL ISOLATES OF *Pseudomonas aeruginosa*

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ABSTRACT

Combination therapy of antibiotics can be used against multi drug resistant bacteria. *In vitro* investigation of two antibiotics combinations regimens were used against multi drug resistant local isolates of *Pseudomonas aeruginosa* that isolated from chronic otitis media patients to determine the usability of combination therapy for the treatment.

The first attempt of combination therapy regimen was tested for synergy between MIC of Rifampicin 4µg/ml with MICs of Tri-methoprim 50µg/ml, Cephlexin 50µg/ml, Tetracyclin 45µg/ml, Gentamicin 4µg/ml, and Erythromycin 40µg/ml, respectively in nutrient broth medium, the results yielded synergistic action in the combination therapy of Rifampicin and Erythromycin, confirmed spectrophotometrically. The second attempt of combination therapy examined against the same isolates on nutrient agar medium between MIC of Ciprofloxacin 20µg/ml and MICs of Rifampicin 4µg/ml, Gentamicin 4µg/ml, Streptomicin 4µg/ml, Tobramicin 3µg/ml respectively, the results were exhibited full synergistic bactericidal activity that took place for four combinations therapy with MIC of Ciprofloxacin in combination with the MICs of other antibiotics.

INTRODUCTION

Pseudomonas aeruginosa is the most common pathogen that causes nosocomial infections in patients with compromised host defense mechanisms **(1)**, which are infections that patients acquire during the course of receiving treatment for other conditions, causes the most severe and life threatening infections with a mortality rate of 50% particularly in hospitalized patients with serious underlying diseases, in intensive care patients, severe burns

patients, cancer and transplant patients who are immunosuppressed **(2)**. The high mortality associated with these infections is due to a combination of weakened host defenses, bacterial resistance to antibiotics, and the production of extracellular bacterial enzymes and toxins **(3)**, adding to its pathogenicity, *P. aeruginosa* has minimal nutritional requirements and can tolerate a wide variety of physical conditions, it has the ability to adapt in many ecological niches, from water and soil to plant and animal tissues **(4)**. The bacterium capable of utilizing a wide range of organic compounds as food sources, thus giving it an exceptional ability to colonize ecological niches where nutrients are limited **(1)**.

P. aeruginosa is notorious for its resistance to antibiotics, it is naturally resistant to many antibiotics due to the low permeability barrier afforded by its Gram-negative outer membrane, the action of multidrug efflux pumps, and its tendency to colonize surfaces in a biofilm form make the cells impervious to therapeutic concentrations antibiotics **(4)**. Since its natural habitat is the soil, living in association with the Bacilli, Actinomycetes and Molds, it has developed resistance to a variety of their naturally-occurring antibiotics, also it maintains antibiotic resistance plasmids, both R-factors and RTFs, and it is able to transfer these genes by horizontal gene transfer, mainly transduction and conjugation **(5)**.

Only a few antibiotics are effective against *P. aeruginosa*, including fluoroquinolones, gentamicin and imipenem, and even these antibiotics are not effective against all strains **(3)**. Monotherapy is recommended for uncomplicated urinary tract infections, while combination therapy is recommended for severe infections, such as bacteraemia and pneumonia, at least in some cases, the advantage of combination therapy remains a matter of debate **(6)**, but, on the other side, combination therapy was considered one way to overcome this problem, both in animal models and in clinical therapy **(7)**, it is generally used to increase bactericidal activity and/or the rate of killing *in vivo*, and to prevent the emergence of drug resistance, it is also used to broaden the antimicrobial spectrum in ill patients while awaiting a bacteriologic diagnosis or because patients have suspected or proven polymicrobial infections **(8)**. The increased clinical response to combination therapy is explained to be due to synergism between the antibiotics used. Synergism of a combination of antibiotics can be expressed as fractional inhibitory concentration indices (FICi) derived from a checker board titration **(9)**. Another way to detect synergism is by performing time kill curve studies **(10)**.

MATERIALS AND METHODS

Samples collection

Two clinical isolates of multi drug resistant *P. aeruginosa* were collected from patients with chronic otitis media, identified by conventional methodology (**11**).

Antibiotics Susceptibility

The susceptibility of isolates were re-examined against 10 types of standard antibiotics that differ in their spectrum and mechanisms of action, by using the Kirby-Bauer method, according to **(12)**. That is Amoxcillin, Cephalixin, Erythromycin, Tetracyclin, Tobramycin, Gentamicin, Streptomycin, Rifampicin, Ciprofloxacin, and Tri-methoprim.

Minimal Inhibitory Concentrations (MICs)

MIC was determined for each antibiotic separately by broth micro dilution assay according to **(13),** sterile stock solutions of antibiotics were prepared by dissolving 1mg of antibiotic (powder/ Amoxcillin, Cephalixin, Erythromycin, Tetracyclin, Streptomycin, Rifampicin, Ciprofloxacin, and Tri-methoprim) in 10ml distilled water, a series of test tubes were prepared containing different antibiotic concentrations ranged from 1µg/ml to 1000µg/ml. From stock solution 40µg/ml of liquid antibiotics such as Tobramycin and Gentamicin , prepared a series of antibiotics concentrations ranged from 1µl to 1000 µl. All antibiotics concentrations were prepared in 3ml of sterile nutrient broth. Each tube was inoculated with 100µl bacterial suspension of an 24hr. broth culture 10^6 colony forming units (CFU)/ml according to McFarland turbidity scale **(14)**, then incubated at 37C° for 24hr. The presence of any bacterial growth in the tubes was indicated by an increase in turbidity. The MIC values of the antibiotics were taken as the concentration at which no measurable growth of bacteria had taken place.

Activity of antibiotics combinations on bacterial growth

The activity of two combinations regimen of the antibiotics was tested on *P. aeruginosa* according to **(15)** using the same method as for MIC determination, so MIC concentration of Rifampicin were combined with MICs of Tri-methoprim, Cephlexin, Tetracyclin, Gentamicin, Erythromycin, respectively in nutrient broth medium, then inoculated with bacterial suspension as the inoculums at concentration of 10^6 CFU/ml, following overnight incubation period at 37C° with antibiotics, test tubes were observed visually for the presence or absence of growth. They were also read spectrophotometrically at 540 nm and optical density (OD) values were recorded to determine the percentage of

bacterial culture survival.

Another attempt of antibiotics combination were tried with MIC concentration of Ciprofloxacin, it was combined respectively with MICs of Rifampicin, Gentamicin, Streptomicin, Tobramicin.

MIC Ciprofloxacin (20µg/ml) + MIC Rifampicin (4µg/ml)

MIC Ciprofloxacin (20µg/ml) + MIC Gentamicin (4µg/ml)

MIC Ciprofloxacin (20µg/ml) + MIC Streptomycin (4µg/ml)

MIC Ciprofloxacin (20µg/ml) + MIC Tobramycin (3µg/ml)

Each combination were mixed with 25ml nutrient agar (with consideration that each ml of nutrient agar medium will have the same concentration of MIC of two combined antibiotics), after the medium was cooled to 45C°, poured in plates, then inoculated with 10^6 CFU/ml of *P. aeruginosa* and incubated at 37C° for 24hr. Antibiotics combination-untreated plates were made as a control plate, Growth results were then recorded **(16)**.

RESULTS AND DISCUSSION

The results of *P. aeruginosa* identification were appeared after 24hr. incubation at 37C° as a growth with blue-green pigmentation due to pyocyanin production on nutrient agar, while it appeared as a pale green colonies due to inability to ferment lactose on MacConkey agar medium, in addition to the fruity odor of aminoacetaphenone and positive reaction to oxidase. Under microscope, the microorganisms appeared Gram negative rods with no particular arrangement.

Isolates of *P. aeruginosa* are identified from clinical specimen by their production of pyocyanin, a blue, water-soluble, non fluorescent, phenazine pigment, in addition to their colonial morphology (**11**). *P. aeruginosa* is the only species of *Pseudomonas* or Gram-negative rods known to excrete pyocyanin (**17**).

In comparison with standard tables of evolution inhibition zones in the WHO report **(18)**, *P. aeruginosa* was showed a differences in their susceptibility to antibiotics under investigation, the results of antibiotics susceptibility and MIC were displayed in Table(1) and Table(2).

Table(1):Inhibition zones diameters for antibiotics susceptibility applied on *P. aeruginosa*

The antibiotics	Concentration(µg/ml)	Inhibition zones diameters(mm)
Rifampicin	10 µg	7mm
Streptomycin	10 µg	8mm
Ciprofloxacin	25 µg	20mm
Gentamycin	10 µg	25mm
Tobramycin	10 µg	29mm
Amoxicillin	10 µg	R
Cephalixin	25 µg	R
Erythromycin	15 µg	R
Tetracyclin	30 µg	R
Tri-methoprim	25 µg	R

R=resistant

Table(2): MIC values of antibiotics applied on *P. aeruginosa*

The antibiotics	MIC values µg/ml
Rifampicin	4 µg/ml
Streptomycin	4 µg/ml
Ciprofloxacin	20 µg/ml
Gentamycin	4 µg/ml
Tobramycin	3 µg/ml
Amoxicillin	35 µg/ml
Cephalixin	50 µg/ml
Erythromycin	40 µg/ml

Tetracyclin	**45 µg/ml**
Tri-methoprim	**50 µg/ml**

The susceptibility patterns of *P. aeruginosa* vary geographically, and susceptibility tests should be done as an adjunct to selection of antimicrobial therapy **(19)**.

Antibiotics combination activity

After turbidimetric measurements at 540 nm, the absorbance values of antibiotics combinations Table (3) were showed the reduction in bacterial growth indicated by decreasing the bacterial growth turbidity in test tubes by visual observation of bacterial growth and by turbidimetric measurements at 540 nm.

Table(3): Combination values at absorbance 540nm

Antibiotic combinations	Absorbance at 540nm	Bacterial turbidity measurements
Rifampicin +Tri-methoprim	**0.141**	**$294*10^6$**
Rifampicin + Cephalixin	**0.108**	**$225*10^6$**
Rifampicin +Tetracyclin	**0.112**	**$233*10^6$**
Rifampicin + Gentamicin	**0.188**	**$392*10^6$**
Rifampicin + Erythromycin	**0.096**	**$200*10^6$**

The absorbance values were revealed a reduction in bacterial turbidity, and the combination of Rifampicin/Erythromycin were showed the maximum synergistic effect of the antibiotics combination, Rifampicin act as an inhibitor of RNA synthesis and function, while Erythromycin inhibit protein synthesis by binding to a subunit of bacterial ribosome (50s)

(20), these results were agree with the study of Tre-Hardy *et al.*, **(20)** which showed that the combination of clarithromycin (belong to Macrolides antibiotics family) with other antibacterial agents offers a successful treatment in eradication of *P. aeruginosa* biofilm, and compatible with work of **(21, 22).** Also the study of **(16)** was present that the combination of Rifampicin with Penicillin or Ampicillin was demonstrated full synergistic bactericidal activity.

The other combination regimen was exhibited a dramatic inhibition was observed on nutrient agar plates of four antibiotic MICs combinations (Ciprofloxacin/ Rifampicin,

Ciprofloxacin/Gentamicin, Ciprofloxacin/Streptomycin, Ciprofloxacin/Tobramycin), nutrient agar plates appear clear and without bacterial growth, in comparison with control plate without antibiotics combination that showed a heavy growth of *P. aeruginosa,* Ciprofloxacin was block DNA synthesis by inhibiting one of the enzymes (DNA gyrase) needed in this process, while Gentamicin, Streptomycin, and Tobramycin considered an inhibitors of protein synthesis by binding to the 30s ribosomal subunit, these results were compatible with the work of **(23, 24, 25)**, that explained the superior activity of Ciprofloxacin against *P. aeruginosa* in combination with Ceftazidime that yielded remarkably activity profile.

Recommendation

Further *in vivo* studies on representative isolates are warranted to support these results, before therapy with Rifampicin or Ciprofloxacin in combination with Erythromycin or other types of antibiotics can be recommended.

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تأثير الفعل التعاوني لبعض المضادات الحياتية ضد عزلات سريرية لجرثومة الزوائف الزنجارية

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الخلاصة

من الممكن أستخدام العلاج المركب ضد البكتريا متعددة المقاومة للمضادات الحياتية. تم في هذا البحث دراسة نظامين لربط المضادات الحياتية ضد عزلات محلية متعددة المقاومة لجرثومة الزوائف الزنجارية المعزولة من مرضى ألتهاب الأذن الوسطى الحاد وذلك لتحديد أمكانية أستخدام العلاج المركب ضدها خارج الجسم الحي. أختبرت المحاولة الأولى في العلاج المركب الفعل التعاوني بين التركيز المثبط الأدنى للمضاد الحياتي ريفامبسين 4مايكروكرام/مل مع التركيز المثبط الأدنى لكل من المضادات الحياتية تراي-مثبريم 50مايكروكرام/مل، كيفلكسين 50مايكروكرام/مل، تتراسايكلين 45مايكروكرام/مل، جنتاميسين 4مايكروكرام/مل والأرثرومايسين 40مايكروكرام/مل على التوالي في وسط المرق المغذي، وأظهرت النتائج الفعل التعاوني للعلاج المركب بين المضادين الحياتيين ريفامبسين والأرثرومايسين.

تم في المحاولة الثانية أختبار أمكانية الربط بين التركيز المثبط الأدنى للمضاد الحياتي سايبروفلوكساسين 20مايكروكرام/مل مع التركيز المثبط الأدنى للمضادات ريفامبسين 4مايكروكرام/مل، جنتاميسين 4مايكروكرام/مل، ستربتومايسين 4مايكروكرام/مل، توبرامايسين 3مايكروكرام/مل على التوالي في وسط الأكار المغذي، وقد أظهرت النتائج الفعل التعاوني ضد الجرثومي التام للعلاج المركب في محاولات الربط الأربعة.

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