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# The Effect of EDTA with Single or Combination of Antibiotics on Pseudomonas aeruginosa Isolates in Vitro

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## Abstract

**Back ground** P. aeruginosa is one of the most common causes of infection in burns and wounds and it is the major cause of death in burn patients. This organism is frequently feared because it causes severe hospital-acquired infections, especially in immunocompromised hosts, and is often antibiotic resistant; complicating the choice of therapy. Thus, there is continuous need for enhancing the antibacterial efficacy of antibiotics against P aeroginosa.

**Objective** This study was conducted to determine the MIC of antibiotics used in combination for resistant isolates of Pseudomonas aeruginosa and measure the effect of EDTA in increasing the inhibition effect of these antibiotics.

**Methods** P. aeruginosa was identified microscopically and biochemically .The swap samples from burns and wounds were collected from patients of AL-YarmooK, Baghdad and Al-Kadhumia Teaching Hospitals. Minimum inhibitory concentration (MIC) was used to evaluate antibiotics effectiveness, while fractional inhibitory concentration (FIC) was used to evaluate the effect of antibiotics combination on pathogenic bacteria (P.aeruginosa). Disk diffusion assay were used to determine the inhibition zone of antibiotic disk (with and without EDTA) against P. aeruginosa.

**Results** Ten isolates were selected according to their pattern of resistance as those showing multi-drug resistance and tested to specify their minimum inhibitory concentration for (amikacin, gentamicin, ceftazidime, and ciprofloxacin). Amikacin had the lowest MIC compared with others. Among combinations, the combination of ß-lactam antibiotics with amikacin was found to be the most effective combination. Results showed that EDTA increases the effect of antibiotic against P. aeruginosa isolates especially when it was combined with aminoglycoside antibiotics.

**Conclusion** Amikacin is the most effective agent against Pseudomonas aeruginosa especially when combined with ceftazidime, more over; EDTA increases the activity of antibiotic against pseudomonas aeruginosa especially when combined with aminoglycoside antibiotics.

**Keywords** P aeruginosa, minimum inhibitory concentration (MIC), Fractional inhibitory concentration (FIC), Ethylenediaminetetraactic acid (EDTA).

## Introduction

Pseudomonas aeruginosa is an important nosocomial pathogen due to its ubiquitous presence wherever there is water. The devastating effect of infection on patients is usually the result of an excessive immune response, and the organism's high resistance to both host defenses and antibacterial agents [(1)].

P. aeruginosa constitute a large group of the normal aerobic intestinal flora. Within the intestine they generally not causing a disease and may even contribute to normal function and nutrition, but these organisms become pathogenic only when they reach tissue outside the intestinal tract, particularly the urinary tract, biliary tract, meninges, lung, eye,

kidney, ear, intestine and damage or burn skin, causing inflammation at these sites only when normal host defense are inadequate, particularly in early infancy, old age, and in terminal stages of other diseases [2, 3].

P. aeruginosa is one of the most common causes of infection in burns and wounds. It could cause burn sepsis through bacterial colonization of the burn site, destruction of the mechanical barrier to tissue invasion and multiple systemic immunological defects related to serious burns. P. aeruginosa is a major cause of death in burn patients [4].

P. aeruginosa has for long been regarded as an antibiotic-resistant organism, its low permeability outer membrane prevents access of many agents to their sites of action [5].The presence of constitutive and enhanceable efflux mechanisms removing a huge range of antimicrobial agents from the cell is considered as important factor of resistance, especially if coupled with enzymatic mechanisms of resistance [6].

P. aeruginosa is frequently resistant to many commonly used antibiotics (e.g. amikacin, gentamicin, ciprofloxacin, ceftazidime , pipracillin, and tetracyclin) by one or more of the following mechanisms: restricted uptake and efflux; drug inactivation and changes in targets [7].

The MIC indicates the minimal concentration of the antibiotic that must be achieved at the site of infection to inhibit the growth of the microorganism being tested. By knowing the MIC and the theoretical levels of the antibiotics that may be achieved in body fluids such as blood and urine, we can select the appropriate antibiotic, the dosage schedule and the route of administration. Generally a margin of safety of ten times the MIC is desirable to ensure successful treatment of the disease [8].

Most infections in humans with normal host defenses system can be treated with a single antimicrobial agent, but there are indications for the use of combinations of antimicrobials for the treatment of infections. The combinations may provide more broad-spectrum coverage than single antibiotics can provide, decrease the emergence of resistance strains, decrease dose-related toxicity by using reduced dose of both drugs, and to obtain enhanced inhibition of microorganisms by antimicrobial drugs [9].

Salts of Ethylenediaminetetraactic acid (EDTA) have long been used as antimicrobial agents, particularly against bacteria. They have also been used as enhancer of other agents, such as: lysozyme, antibiotics, and irradiation, by increasing permeability of bacterial membrane or by removal or destruction of covalently bound lipid components [10].

Its activity appears to be more when used in combination with antibiotics with activity against gram negative than with gram positive bacteria. This is due to the differences in the cell wall structure of the two groups. Gram positive bacteria contain more phospholipids than peptidoglycans in their cell walls in comparison with Gram negative bacteria [11].

Two modes of action of EDTA has been recognized, first EDTA potentiate the effect of antibiotics by binding to the metal ions which compete with aminoglycosides for cell wall receptor that allow them into bacteria, second EDTA disrupt the lipopolysaccharides structure in the outer membrane of gram negative bacteria, through this disruption the membrane becomes more permeable to other agents such as antibiotics [9].

This study was conducted to determine the MIC of antibiotics to be use in combination against resistant isolates of Pseudomonas aeruginosa and measure the effect of EDTA in increasing the inhibitory effect of these antibiotics.

## Methods

**Antibiotic powders**: were obtained from the manufacturers as follows: Amikacin (AL-Razi center for production of diagnostic kits (Iraq)). Ceftazidim "Gulf pharmaceutical industries (UAE)" Gentamicin (AL-Razi) Ciprofloxacin "BAL-pharma (India)".

**Antibiotic Solutions:** Gentamicin, amikacin, and ceftazidime were prepared as stock solution of 10 mg/ml of antibiotic powder in distilled water, sterilized by filtration and store at -20°C [(12)]. Ciprofloxacin solution were prepared as stock solution by dissolving 1g of antibiotic powder in 90 ml sterile distilled water, pH adjusted to 5.0 with 1N HCl then volume completed to 100ml, obtaining a final concentration of 10 mg/ml, sterilized by filtration and stored at -20°C [(13)]. Bacterial isolates of P. aeruginosa (ATCC 27583) were obtained from Department of Biology, College of Science, Baghdad University.

**EDTA Stock Solution (EDTA solution)**: is prepared by adding 186.1g of disodium ethylene diamine tetraacetate. $2H_2O$ to 800 ml of D.W., stirring vigorously on a magnetic stirrer, pH was adjusted to 8.0 with NaOH, dispenses into aliquots and sterilized by autoclave giving a final concentration of (5mM) [(14)]. P. aeruginosa was identified microscopically and biochemically [(15)].

**Sample Collection**: The swap samples from burns and wounds were collected in a sterile tubes containing nutrient broth from patients of AL-YarmooK, Baghdad and Al-Kadhimiya Teaching Hospitals during the period from 1/3/2005 to 15/5/2005.A total of 150 samples were collected and transported to the laboratory within two hrs. of collection.

**Bacterial Isolation:** Burns and wounds samples were cultured by spreading on MacConkey and blood agar plates. Plates were incubated overnight at 37°C. After the incubation, non fermentative colonies which appeared pale on MacConkey agar were selected and streaked on selective media (Citramid agar and king A agar) and incubated at 37°C for 24 hrs to test the pigmentation related to P. aeruginosa. These colonies were subcultured on brain heart infusion agar to obtain pure culture for further identification [(16)].

**Antibiotic Sensitivity by Disk Diffusion Test [(17)]:** After incubation, the diameter of inhibition zone was noted and measured in mm, results were determined according to the National Committee for Laboratory Standard [(18)].

**Determining the Minimum Inhibitory Concentration for Antibiotic Combination**: This test was used to determine the effect of antibiotics combination on pathogenic bacteria (P. aeruginosa)**.** A serial dilution method was used to determine MIC. The result was determined depending on the turbidity of the tube, then the combination whether it's synergistic, additives, antagonistic, or indifferent depending on the fractional inhibitory concentration (FIC), which was determined as follows: (≤0.5) synergism, (0.5 – <1) additive, (1– <2) antagonism, (≥2) indifference, and calculated using the following equation [(19)].

$$\text{FIC} = \frac{\text{MIC for antibiotic in combination}}{\text{MIC for antibiotic alone}}$$

**Determining the Effect of EDTA in Combination with Antibiotics**: Antibiotic disks were soaked in EDTA solution and disk diffusion assay were used to determine the inhibition zone of antibiotic disk (with and without EDTA) against P. aeruginosa according to the National Committee for Clinical Laboratory Standards and the inhibition zones were measured in (mm)

## Results

**Isolation and Identification of P. aeruginosa**: The identification and characterization of the isolates were carried out according to the cultural, morphological and biochemical tests.

**Antibiotic Sensitivity:** The results showed that all isolates of P. aeruginosa were sensitive to amikacin, while percentage of resistant isolates for the remaining antibiotics as fallow: (30%) gentamicin, 72% ceftazidime, 26% ciprofloxacin.

The result showed that isolates No. (A1, A2, A5, A9, A10, A11, A18, A19, A20, A23) had the

highest level of resistance so that they were selected to study the effect of antibiotics combination and inhibitory effect of EDTA against P. aeruginosa.

**Minimum Inhibitory Concentration (MIC):** Ten isolates (which had the highest level of resistance) were tested to determine the MIC of amikacin, gentamicin (which represented the aminoglycoside), Ceftazidime (which represented the β-lactam), and Ciprofloxacin (which represented the flouroquinolons ). Amikacin showed the lowest MIC level for P. aeruginosa which rang from (0.12- 4) µg/ml.While other antibiotics in this study had higher range of MIC as follow: ceftazidime (2-4) µg/ml, ciprofloxacin (2-8) µg/ml and gentamicin (0.5-8) µg/ml (Table 1).

**Table 1. MIC value for four antibiotics (µg/ml) tested against P. aeruginosa isolates.**

| P aeurogenosa Isolates | AK | GN | CIP | CAZ |
|---|---|---|---|---|
| A1 | 4 | 8 | - | 4 |
| A2 | 0.5 | - | 8 | 2 |
| A5 | 0.5 | - | 8 | - |
| A9 | 1 | 1 | 2 | - |
| A10 | 2 | 8 | - | - |
| A11 | 4 | - | - | - |
| A18 | 0.12 | 0.5 | - | - |
| A19 | 0.5 | - | - | - |
| A20 | 2 | - | - | 4 |
| A23 | 0.5 | 0.5 | - | - |

(AK) Amikacin, (CN) Gentamicin, (CIP) Ciprofloxacin, (CAZ) Ceftazidime

**Antibiotics Combination**: The combination between antibiotics for each isolate in this study was based on the selection of antibiotics that have lowest MIC to be used for combination.

Table 2 show the MIC value for antibiotics (amikacin, gentamicin, ceftazidime, ciprofloxacin), before and after combination against P. aeruginosa isolates to determine the effect of antibiotics combination on these isolates, MIC values of antibiotics in combination were found to be lower than MIC values of a single antibiotic.

Table 2 showed that (FIC) values for combination of amikacin with ceftazidime in the isolate No. (1, 2, 11, and 20) was very low in comparison with those (FIC) values of combination of amikacin with gentamicin and ciprofloxacin in the isolate No. (5, 9, 11, 18, 19, and 23).

**Table 2. MIC of antibiotic combinations.**

| P. aeruginosa Isolates | Antibiotics combination | MIC of first antibiotic alone (µg/ml) | MIC of first antibiotic in combination (µg/ml) | MIC of second antibiotic alone (µg/ml) | MIC of second antibiotic in combination (µg/ml) | FIC | Results |
|---|---|---|---|---|---|---|---|
| A1 | AK+CAZ | 4 | 0.25 | 4 | 0.25 | 0.125 | Syn |
| A2 | AK+CAZ | 0.5 | 0.03 | 2 | 0.125 | 0.12 | Syn |
| A5 | AK+CIP | 0.5 | 0.12 | 8 | 2 | 0.49 | Syn |
| A9 | AK+GN | 1 | 0.12 | 1 | 0.12 | 0.24 | Syn |
| A10 | AK+GN | 2 | 0.25 | 8 | 1 | 0.25 | Syn |
| A18 | AK+GN | 0.12 | 0.03 | 0.5 | 0.12 | 0.49 | Syn |
| A20 | AK+CAZ | 2 | 0.125 | 4 | 0.25 | 0.125 | Syn |
| A23 | AK+GN | 0.5 | 0.06 | 0.5 | 0.06 | 0.24 | Syn |

FIC: Fractional Inhibitory Concentration; Ak: Amikacin; Caz: Ceftazidime; Cip: Ciprofloxacin; GN: Gentamicin; Syn: Synergism.

**The Effect of Combination of EDTA-Antibiotic on P. aeruginosa** Table (3) shows synergistic effect of EDTA with other antibiotics of different groups in which the inhibition zone increased after adding the EDTA to the antibiotics. Inhibition zone of isolate No. A10 was found to be increased by (5mm) when EDTA was combined with gentamicin. While inhibition zone increased by (4 mm) when EDTA was combined with ceftazidime for isolate No A2. Adding EDTA to other antibiotics for different isolates show increased inhibition zone by 2-3 mm.

**Table 3. Combination effect of EDTA with antibiotics on p. aeruginosa isolates using disk diffusion assay.**

| Isolateseses | Antibiotics | ZOI[1] without EDTA | ZOI with EDTA mm |
|---|---|---|---|
| **A1** | AK[2] | 22 | 24 |
| | Caz[3] | 19 | 22 |
| **A2** | AK | 23 | 25 |
| | Caz | 20 | 24 |
| **A5** | AK | 24 | 27 |
| | CIP[4] | 18 | 20 |
| **A9** | AK | 21 | 24 |
| | GN[5] | 20 | 22 |
| **A10** | AK | 22 | 25 |
| | GN | 19 | 24 |
| **A18** | AK | 20 | 23 |
| | GN | 18 | 20 |
| **A20** | AK | 20 | 22 |
| | Caz | 20 | 23 |
| **A23** | AK | 23 | 25 |
| | GN | 18 | 21 |

1: Zone of inhibition (mm) 2: Amikacin 3: Ceftazidime 4: Ciprofloxacin 5: Gentamicin

**Discussion**

Pseudomonas aeruginosa is a notoriously difficult organism to control with antibiotics or disinfectants. The emergence of prevalence of antibiotic resistance strains is considered as a major therapeutic problem that can be explained by several hypotheses such as, the influence of excessive and /or inappropriate antibiotic use [(20)].

The results show that all isolates of P. aeruginosa were sensitive to amikacin (100%); this result may be related to the lower random use of this antibiotic by patients. This result was in agreement with that of Startchounski et al who found in a study in Russia that resistance percentage of the isolate to amikacin was (1%) [(21)]. Resistance of P. aeruginosa isolates to gentamicin was found to be as low as (30%) and this result was in agreement with that of Brumfitt and Hamilton who found that resistant to gentamicin was (32%) [(22)]. The results of this study also shows that the resistance percentage to ceftazidime was 72%, and this may be due to the ability of this isolates to produce β-lactamase enzyme which break the β-lactam ring and this results is in agreement with that found by Rice et al [(23)]. P. aeruginosa isolates were found sensitive to fluroquinolones like ciprofloxacin in which (26%) of the isolates was found resistant to this antibiotic and it was in agreement with that of Shawar et al [(24)] who indicate lower than 63% of isolate susceptible to ciprofloxacin, this may belong to the wide use of ciprofloxacin as

therapeutic agent for treatment of diseases caused by P. aeruginosa leading to a low level of susceptibility percentage for this antibiotic.

Amikacin remains the first choice among the tested antibiotics in the present study with lowest MIC for P. aeruginosa which range from (0.12- 4) µg/ml, therefore it is the more preferred in the therapy than other antibiotics and these result agreed with that of Bonfiglios [25] who found that MIC of amikacin against P. aeruginosa (2 µg/ml).While other antibiotics in this study have a higher MIC and as follow: ceftazidime was (2, 4 and 4) µg/ml, and this result was consistent with that found by Rolston et al [26] while gentamicin had a MIC range from (0.5-8) µg/ml, and this may be due to the ability of P. aeruginosa isolate to produce β-lactamase which break the β-lactam ring in the structure of antibiotic. For ciprofloxacin the MIC was ranged (2-8) µg/ml and this agreed with that of Craig [27].

The combination between antibiotics for each isolate in this study was based on the selection of antibiotics that have lowest MIC to be used for combination. MIC values of antibiotics in combination were found lower than MIC values of single antibiotic, revealing a synergistic effect of these combinations and those results are similar to those shown by Hollander et al [28]. Table 2 showed that (FIC) values for combination of amikacin with ceftazidime in the isolate No. (1, 2, 11, and 20) is very low in comparison with those (FIC) values of combination of amikacin with gentamicin, ciprofloxacin in the isolate No. (5, 9, 11, 18, 19, and 23), and this indicates that combination of aminoglycoside with β-lactam antibiotic was more effective than combination of aminoglycoside with other group of antibiotics, and this because aminoglycoside antibiotics exert their effects on protein synthesis of bacterium while β-lactam antibiotics exert their effect on bacterium cell wall and this lead to complete destruction of bacteria [8].

Ethylenediaminetetraactic acid has a more complex inhibition-concentration profile. Synergism between EDTA and other antimicrobials agents have been widely reported against P. aeruginosa [29]. From results obtained in table 3 it was found that EDTA had a synergistic effect on aminoglycoside antibiotics (amikacin and gentamicin) against P. aeruginosa, and these results are in agreement with that obtained by Spark [30] who found that EDTA enhance the activity of aminoglycoside antibiotics by binding to the metal ions which compete with aminoglycoside antibiotics for cell wall receptor that allow antibiotics to enter the bacterial cell. Gotthelf [31] had shown that EDTA is capable of reducing the MIC of ciprofloxacin against P. aeruginosa and this result was in agreement with the result of the current study, while the effect of EDTA on ceftazidime also gives synergistic effect, and this result was found similar to that obtained by Vaara [32], and this because EDTA cause destruction of the outer membrane of the bacterial cell altering the permeability to antibiotics which enter the bacterial cell and exert their effect.

In conclusion, amikacin is the most effective agent against Pseudomonas aeruginosa especially when combined with ceftazidime, moreover, EDTA increases the activity of antibiotic against Pseudomonas aeruginosa especially when combined with aminoglycoside.

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