REPORT

CASE-BY-CASE STUDY USING ANTIBIOTIC-EDTA COMBINATION TO CONTROL *PSEUDOMONAS AERUGINOSA*

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ABSTRACT

Ps. aeruginosa a well-known opportunistic bacterium infects various tissues and organs causing sever problems. In tropical and semitropical countries and because of environmental and sanitizations reasons *Ps. aeruginosa* acquiring more virulent factors, gained multi-resistant genes, adapt resistant mechanisms for antibiotics, drugs, disinfectants and in general for any toxic compound. Six multidrugs resistant strains out of 260 different bacteria isolated from patients at Tanta University hospital, Tanta, Egypt, used in this study. To evaluate the role of divalent cations in antibiotic resistance we used a medium containing Ca^{2+} and Mg^{2+}. Various antibiotics tested with or without the LC_{10} and LC_{50} equivalent amount of EDTA against each strain. We found that after adding EDTA, 70% of the strains turned from resistant to sensitive, especially considering those antibiotics, which inhibit protein synthesis, such, as tetracycline. Simple experiment for testing the effect of Antibiotic-EDTA combination on Rabbits skin artificial ulcer(s) infected by *Ps. aeruginosa* significantly improve the efficiency of using such combination in superficial treatment. We propose using media other than Muller-Hinton agar in antibiotic sensitivity test to select the best antibiotics could be used *in vivo* or in superficial treatment. A combination between a proper amount of EDTA and antibiotics especially protein inhibitors will improve the control of *Ps. aeruginosa.*

Keyword: Antibiotic, EDTA, Ca^{2+}, Mg^{2+}, *Ps. aeruginosa,* protein inhibitor antibiotic.

INTRODUCTION

Ps. aeruginosa mediates acute infections, and known as the most dominant pseudomonades species isolated from clinical sources especially immunocompromised patients (Jones, *et al*., 2003). *Ps. aeruginosa* cause sever problems to patients with diabetes mellitus (Lasisi and Nwaorgu, 2001), AIDS (Monras *et al*., 2002), malignancy (Gronowitz *et al*., 2003), cardiopulmonary resuscitation (Sanchis Minguez *et al*., 2003), transplants (Battaglia *et al*., 2004), liver cirrhosis (Hsu, *et al*., 2004), renal failure (Al-Aloul *et al*., 2005), trauma (Wang *et al*., 2005), intravenous drug abuse (Ross and Shamsuddin, 2004), corticosteroid therapy (Chizuka *et al*., 2005), cytotoxic chemotherapy (Ramphal, 2004), broad spectrum antibiotics (Kumar *et al*., 2004), and immunoglobulin deficiency (Chan *et al*., 2005). *Ps. aeruginosa* founds to be most common isolate in burns (59%) (Agnihotri *et al*., 2004), and invasive instrumentation (Chaiban *et al*., 2005), as well, as in septicaemia (Ali, 2004), endocarditis (Chacko *et al*., 2003), and nosocomial infections (Al-Ghamdi *et al*., 2002).

Ps. aeruginosa have several virulence factors like extracellular toxins (Ahuja *et al*., 2004), proteases (Sarkisova, *et al*., 2005), and exopolysaccharide (Chan *et al*., 2002), which adapt specific host tissues infection. *Ps. aeruginosa* can survive in a number of disinfectants, while continuous sanitation by a single disinfectant could release a resistant strains (Ojima *et al*., 2002). *Ps. aeruginosa* shows inherent multidrug resistance mechanisms by outer membrane low-permeability and some specific multi-drug efflux (Mix), like Mix XY-OprM (Aires *et al.,* 1999). Mutant of *nalB* gene of *Ps. aeruginosa* leads to overexpression of outer membrane protein OprM (49 kDa), with increase in the resistance to quinolones, cephems, penams, meropenem, tetracycline, chloramphenicol and erythromycin (Sanchez *et al*., 2002). A need for new drugs to control *Ps. aeruginosa* was widely reported by many authors whom described that *Ps. aeruginosa* like others pathogenic bacteria gained extra resistances, because plasmid and phage DNA materials mediated resistance to various antibiotics and other antimicrobial drugs (Shahid *et al*., 2003). Divalent cations play an important role in Prokaryotes specially in cell protection or in infection adaptation (Sarkisova *et al*., 2005). Protein signal acting, as a switch for controlling flagella rotation is an important example about the role of divalent cations in Prokaryotic organisms where, a phosphotransfer occurs from CheW to CheY and in which autodephosphorylation of CheW-phosphate can be fulfilled by Co^{2+}, Mg^{2+}, Mn^{2+}, or Zn^{2+}, or a lesser Cd^{2+} (Adamo *et al*., 2004). *Ps. aeruginosa* use divalent cations in many important biological processes

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including infection adaptation (Chen, *et al*., 2005). The role of Ca^{2+} is clear in Cystic Fibrosis patients infected with *Ps. aeruginosa* where, Ca^{2+} ions cause mucoid *Ps. aeruginosa* to have more compact gelatinous appearance (Govan, 1988). EDTA, which chelates Ca^{2+} and other divalent cations, could improve the antibiotic effectiveness *in vivo* (Wood *et al.,* 1980). Magnesium reported, as an important divalent cation for *Ps. aeruginosa* like in protease virulence factor mechanisms (Guina *et al*., 2003). In antibiotic treatment Ca^{2+} and Mg^{2+} can influence bacterial sensitivity to antibiotics, therefore media like Muller-Hinton broth, which lacks Ca^{2+} and Mg^{2+}, can lead to therapeutic failures with strains reported, as sensitive (Christe *et al.,* 1982). Variable effect of EDTA on pathogenic bacteria reported by many authors (Cabo *et al*., 2001), but the sensitivity test and the treatment using EDTA was not established yet. There is a need for a medium representing the human body, which contains most of the divalent cations to substitute Muller-Hinton agar. Aiming to close the gap between the WHO, NCCLS methods and the fact that divalent cations will give wrong result during the sensitivity test, we made our study in sequence to establish the best way for examining the role of divalent cations in presence of EDTA and various antibiotics.

MATERIAL AND METHODS

The present study was carried out from January 2005 to April 2006.

Bacterial strains

The six clinical isolates used in this study are multiresistance to antibiotics and were selected out of 260 bacterial strains and identified by standard criteria, as *Ps. aeruginosa.* The strains were isolated from patients at Tanta University Hospital and represent: strain 1 from pyuria, 2 from septic abdominal wound, 3 from scrotal ulcer, 4 and 5 from periurethral discharge and 6 from breast abscess.

Culture media

Bacterial strains were grown routinely in LB (Luria-Bertani), solid medium at 30 ^{o}C (Sambrook, *et al*. 1989). A defined A-Z (Amara-Zakaria), medium used to control the divalent cations and consisted of $(NH_4)_2SO_4$ (1.3 gl^{-1}), KH_2PO_4 (0.6 gl^{-1}), $MgSO_4$ (0.3 gl^{-1}), $CaCl_2$ (0.02 gl^{-1}), K_2HPO_4 (0.8 gl^{-1}), NaCl (2 gl^{-1}), and glucose (0.2 gl^{-1}). Solid media obtained by adding 1.8% w/v agar (Difco).

Preparation of EDTA

In sterile bidistilled water 2.5 mM EDTA dissolved at pH 8; the clear solution passed through bacterial membrane filter and collected in sterile eppendorf tubes.

Determination of EDTA toxicity

The EDTA toxicity was determined using the following concentration 0, 0.05, 0.5, 2.5 and 5 mM in test tubes containing 5 ml A-Z broth medium, 10 µl of OD_{600}=0.5 (approximately $4x10^5$ CFU) freshly cultivated *Ps. aeruginosa* strains. The six different strains were added to each tube separately and allowed growing for 8 hr at 30^{o}C in 200 rpm shaker incubator. Ten µl taken from each tube and was diluted in 10 ml A-Z broth medium. 50 µl from each tube was spread on A-Z agar medium and incubated overnight at 30 °C. The colonies of each of them were counted and compared to the control, and the inhibition percentage of each concentration determined. EDTA LC_{10} and LC_{50} values were determined by Probit analysis and identified, as mM EDTA, which can inhibit the growth of 10% and 50% of *Ps. aeruginosa.*

Agar disc diffusion methods (Antibiotics-EDTA test)

Conventional agar disc diffusion method was performed, as described in NCCLS documents (NCCLS 1999), to determine the antibiotic sensitivity. The used antibiotics are rifampcin, tetracycline, garamycin, duricef, chloramphenicol, unasyn, erythromycin, nalidixic acid, sulphamethoxazole, ampicillin, and ciprofloxacin were tested on *Ps. aeruginosa* strains 1-6. The treatment was done in absence and presence of LC_{10} and LC_{50} equivalent amounts of EDTA. A-Z medium was used instead of Muller-Hinton agar and the inhibition zone diameter was determined after 24 hr incubation at 30°C.

Table 1: *P* value, heterogeneity factor, $LC_{10 \text{ and } 50}$, Natural response and immunity of EDTA (mM) against *Ps. aeruginosa* strain 1-6.

Ps. aeruginosa Strains no.	Likelihood ratio Chi-square test	t-deviate	Probit analysis		Natural response and immunity
	P	Heterogeneity factor	LC_{10}	LC_{50}	95% limits
1	0,0002877	8,1535127	0,193	2,101	0,000639
2	0,2642521	(*P*>0.050)*	0,706	2,197	0
3	0,0000003	15,3060343	0,421	0,575	0,165
4	0,0000002	15,3060343	0,081	0,378	0
5	0,0200946	3,9073054	0,163	0,500	0
6	0,5382799	(*P*>0.050)*	0,014	0,338	0

*Since heterogeneity is small (*P*>0.050) a normal deviate is used.

Table 2: Effect of different antibiotics (alone and in combination with $LC_{0,\ 10\ and\ 50}$ equivalent amount of EDTA) on different *Ps. aeruginosa* strains.

Antibiotic name	Antibiotic concentration	EDTA conc.	Inhibition Zone diameter mm					
			Strain 1	Strain 2	Strain 3	Strain 4	Strain 5	Strain 6
Tetracyclineᵃ	30 μg ml^{-1}	LC_0	-	-	-	-	6	20
		LC_{10}	20**	20**	18**	12*	15*	-
		LC_{50}	20**	20**	18**	25**	20**	16
Cheloramphenicolᵃ	30 μg ml^{-1}	LC_0	12	20	-	-	20	25
		LC_{10}	30*	22	10*	15*	20	25
		LC_{50}	30*	22	10*	15*	20	30
Garamycinᵃ	10 μg ml^{-1}	LC_0	20	15	20	28	20	-
		LC_{10}	23	20	25	-	20	20*
		LC_{50}	23	25	23	12	25	30**
Erythromycinᵃ	15 μg ml^{-1}	LC_0	12	8	8	10	-	-
		LC_{10}	15	8	-	8	8*	-
		LC_{50}	15	15	-	12	8*	8*
Ampicillinᵇ	10 μg ml^{-1}	LC_0	15	-	12	22	20	-
		LC_{10}	15	-	-	22	20	-
		LC_{50}	15	-	-	22	20	-
Durcefᵇ	30 μg ml^{-1}	LC_0	-	-	-	14	8	-
		LC_{10}	12*	-	8	14	8	8*
		LC_{50}	12*	-	8	14	8	16*
Unasynᵇ	15 μg ml^{-1}	LC_0	-	-	-	-	-	-
		LC_{10}	-	-	-	-	-	-
		LC_{50}	-	-	-	-	20**	8*
Nalidixic acidᶜ	30 μg ml^{-1}	LC_0	10	-	-	14	8	-
		LC_{10}	10	12*	12*	16	8	-
		LC_{50}	10	12*	12*	16	8	-
Ciprofloxacinᶜ	5 μg ml^{-1}	LC_0	25	20	20	22	20	20
		LC_{10}	25	25	0	22	20	20
		LC_{50}	25	25	-	22	20	25
Sulphamethazoleᵈ	25 μg ml^{-1}	LC_0	24	20	-	22	20	26
		LC_{10}	26	20	20*	25	20	26
		LC_{50}	26	20	20*	26	20	30
Rifampicinᵉ	30 μg ml^{-1}	LC_0	-	-	-	-	-	-
		LC_{10}	-	8*	-	-	-	-
		LC_{50}	10*	-	-	10*	8*	8*

The data represents the inhibition diameter, as mm. LC_0 control with antibiotic only. LC_{10} LC_{50} equivalent amount of EDTA. * Significant increase, ** Highly significant increase, - No inhibition data analyzed due to Yates corrected Chi-square. ᵃProtein synthesis inhibitor, ᵇCell wall synthesis inhibitor, ᶜDNA synthesis inhibitor; ᵈFolic acid synthesis analogue and ᵉRNA synthesis inhibition.

Skin experiment

Preparation of the animal skin

The rabbits (3 rabbits for each treatment), were shaved, and residual hair removed (Veet hair removal cream, Reckitt and Colman Pharmaceuticals Co.), for 5 cm^2 area, as in figure 4.

Preparation of artificial ulcer(s)

After complete removal of skin hair, 10 µl of keratolytic solution (October Pharma) consists of salicylic acid (0.2 g ml^{-1}), lactic acid (0.05 g ml^{-1}), and Palidocanol (0.02 gml^{-1}), were spread on the surface of 0.5 cm^2 cut filter paper and tightly attached to the Rabbits skin by the aid of silk tapes and leaved for 20 min or till the skin bleeding.

Infection the ulcer(s) with P. aeruginosa

The skin surface then inoculated by dipping a sterile swab into culture media of *Ps. aeruginosa* strain-6 (OD_{600}=2), the excess inoculum were removed by pressing and rotating the swab firmly against the side of the test tube above the level of the culture media. The swab then streaked allover the surface of the skin-free hair contain the artificial ulcer(s). The process repeated three times each 1hr to be sure that the ulcer(s) were contaminated by *Ps. aeruginosa.*

Preparation of the ointment mixture

Ointment was prepared with the proper combination with antibiotic or antibiotic-EDTA to investigate the effect of adding EDTA to antibiotic in superficial treatment of artificial rabbits skin ulcer(s). Water-Fatty acid (water: hydrated Palm oil, 1:1), prepared, as an ointment and the different antibiotic or antibiotic-EDTA combination (all, as in table 2 in spite of ampicillin and nalidixic acid), were dissolved in the water and mixed with the fatty acid to represent a final concentration same, as in table 2, the mixture were mixed well till it become viscous enough.

Treatment of the ulcer(s) infection

One cm^3 of the ointments containing antibiotics with or without EDTA(LC_{50}) were spread on the surface of the clear hair, which contain the infectious ulcer(s). The control treated only with ointment contains water. The treatment by ointment was continued one time/day, till a complete recovery of the animal skin.

STATISTICAL ANALYSIS

Data was collected and entered the personal computer and the effect of various concentrations of EDTA on *Ps. aeruginosa* was determined by the Probite analysis method using Priprobit ver.1.63 statistical Package software. PriProbit estimates the log-doses by applying a distribution model to the observed quantal data, so a median effective dose (ED_{50}), or LC_{50} where 50% of organisms in population respond can estimate relative potencies (Rho), between preparations. The likelihood ratio Chi-square method was performed to calculate the *P* values, the heterogeneity factors were determined using the t deviate for *P* value < 0.05 and the normal deviate for *P* value > 0.05. The natural response and immunity test was done using 95% limits to evaluate the natural resistant of *Ps. aeruginosa* to the increase in EDTA concentration. Yates corrected Chi-square test where 95% chosen, as the cut-off significant level used to test the effect of adding LC_{10} and LC_{50} equivalent concentrations of EDTA on antibiotics using Statistical Package (SPSS/version 11), software. Cluster analysis was used to determine the average linking among the effect of antibiotics alone and in combination with EDTA on various *Ps. aeruginosa* strains using Hierarchical cluster analysis method and Statistical Package (SPSS/version 11), software. The Chi square for the different between the treatment of skin ulcer(s) with different antibiotics in the presence and absence of EDTA comparing with the control were performed. One-way ANOVA test were performed to analyze the variance between group component and within-group component. The *F*-ratio and *P*-value were calculated.

RESULT

Determination of EDTA LC_{10} and LC_{50}

The inhibition percentage of various EDTA concentrations was determined and the LC_{10} and LC_{50} of EDTA on each strain calculated by Probit analysis methods, as in table 1. Various *P* values have been calculated in which strains 1, 3, 4 and 5 showed a significant sensitivity against EDTA, while strain 2 and 6 did not. Only strain 1 and 3 showed natural response and immunity to the increase in EDTA concentration.

Antibiotic sensitivity test

The inhibition zone diameter of each antibiotic alone, and in presence of LC_{10} and LC_{50} equivalent amounts of mM EDTA were evaluated against *Ps. aeruginosa* strain 1-6, as shown in Table 2. Figure 1 is an example for the increase of the inhibition zone diameter of Germycin after adding EDTA where unasyn and erythromycin did not show any significant increase. The response of *Ps. aeruginosa* strains to various antibiotics in absence of EDTA is variable. All strains were resistant to rifampicin and unasyn and sensitive to nalidixic acid and ciprofloxacin. The other strains ranged from sensitive to resistant, as in table 2. Presence of LC_{10} and LC_{50} equivalent mM EDTA increased the inhibition zone diameter in 70% of the treatments (20% significant and 7.5% highly significant), as in table 2. The highly significant increase in the sensitivity in the presence of EDTA (proved by statistical analysis), was shown with strain 1-5 using tetracycline, 6 with garamycin, 5 with unasyn. nalidixic acid with strains 1, 5, and 6, ampicillin with 1, 2, 3, 4, and 5; durcef with 2, 4, and 5, sulphamethoxazole with 3 and 5, rifampicin with 3, chloramphinicol with 5 and ciprofloxacin with 6 showed no increase in antibiotic inhibition zone diameter after adding EDTA. In rare cases some treatments showed a decrease in antibiotic inhibition zone diameter with EDTA, as erythromycin, ampicillin and ciprofloxacin with strain 3 and garamycin with 4. Some unexpected results showed an increase in antibiotic inhibition zone diameter with LC_{10} equivalent mM EDTA, followed by a decrease with LC_{50} equivalent mM, as in rifampicin with strain 2 and garamycin with 3, while others showed a decrease followed by an increase in inhibition zone diameter, as in erythromycin with 4 and tetracycline with 6. Cluster analysis of various treatments with antibiotic and EDTA, as in table 2, resulted in grouping the protein, DNA and folic acid analogue synthesis inhibitors in one cluster, which contain the highly significant treatments, as shown in figure 2 and table 2. Cluster analysis of the average linkage between *Ps. aeruginosa* strains responding to various treatments, lead to

an interesting result, where strain 1 and 5 come in one group, although coming from different sources, as strain 1 is from pyuria and 5 from periuretheral discharge, but isolated from the same system, the urinary system, as in figure 3.

Skin experiment

The treatment of rabbit(s) ulcer(s) infected by *Ps. aeruginosa* strain 6 using ointment prepared with antibiotic alone and antibiotic-EDTA (LC_{50}) combination (without each of ampicillin and nalidixic acid), shown a clear result as in table 3. The combination between EDTA and antibiotic give a significant improvement over the antibiotic alone as determined by both of Chi-square within individual level and ANOVA between the combination and the antibiotics (each alone), and within the treatment groups at 95.0% confidence level. The *P* value has been illustrated in table 3 for Chi-square analysis and the *F*-ratio and *P*- value for ANOVA at table 4. The protein inhibitor antibiotics were the most effective during the superficial treatment as in table 3, which agree with the data in table 2. The figure 4 shows different stages of superficial treatment of the infected ulcer(s) with unasyn-EDTA combination where the animals have completely recovered within 2 weeks.

Table 3: Days required for ulcer(s) contaminated by *P. aeruginosa* to be recovered after treatment with antibiotics alone and in combination with EDTA LC_{50} equivalent amount for strain 6

Name	no. of days for complete recovery		
	With-EDTA	Without -EDTA	Chi square *P*
Control	16	18	
Tetracycline	8	12	0.0009**
Cheloramphenicol	4	7	0,0000**
Garamycin	4	10	0.0000**
Erythromycin	9	16	0.017*
Durcef	6	16	0.0000**
Unasyn	9	15	0.0139*
Ciprofloxacin	5	7	0.0000**
Sulphamethazole	4	5	0.0000**
Rifampicin	9	13	0.0018**

*Significant **Highly significant

DISCUSSION

This study was designed to be a model to evaluate the interaction between antibiotics and divalent cations during the sensitivity test. *Ps. aeruginosa* was selected because it resists antibiotics, disinfectants, can survive in various habitats, infect different body organs and requires minimum nutrients, which allow the use of defined medium to evaluate the divalent cations in its resistance mechanism. It also causes severe problems for immunocompromised Egyptian patients. Six *Ps. aeruginosa* of 260 different bacterial isolates reported as multiresistant to antibiotics were used in this study to evaluate the role of divalent cations in *Ps. aeruginosa* antibiotic sensitivity. While EDTA has an antimicrobial effect on *Ps. aruginosa,* various concentrations were used to determine its LC_{10} and LC_{50}. Strains 2 and 6 did not show a significant effect with EDTA, which indicates that EDTA alone is not enough to control *Ps. aeruginosa*. To evaluate the role of divalent cations on antibiotic performance, A-Z a new medium was introduced containing Ca^{2+} and Mg^{2+}. EDTA LC_{10} and LC_{50} equivalent amount for each strain was used to evaluate the *in vitro* interaction between the antibiotics, divalent cations, chelating agents and different *Ps. aeruginosa* strains. The antibiotics, which were used in this study represent inhibitors for: protein synthesis (tetracycline, chloramphinicol, garamycin, and erythromycin); DNA synthesis (nalidixic acid and ciprofloxacin); RNA synthesis (rifampicin); cell wall synthesis (ampicillin, durcef and unasyn) and folic acid analogue (Sulphamethoxazol). The results showed that the addition of EDTA to antibiotics *in vitro* increased the sensitivity in 70% of the treated strains, which improve strongly the role of divalent cations in antibiotic resistance and the synergetic effect of EDTA to most antibiotics.

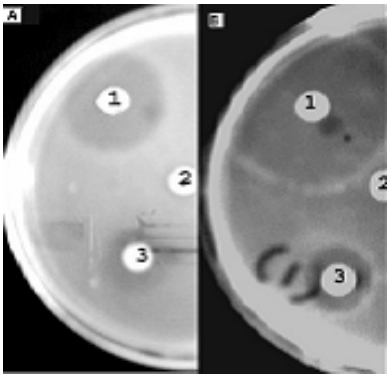

Fig. 1: Increase in inhibition zone diameter after adding EDTA LC_{50} equivalent amount (A) Without EDTA (B) With EDTA, (1) garamycin (2) unasyn and (3) erythromycin.

The response of antibiotics to divalent cations is variable, where tetracycline showed the most significant results in the presence of EDTA and the data was proven using cluster

Table 4: ANOVA test for the data in table 3

Source	Sum of squares	Df	Mean square	*F*-ratio	*P*-value
Between groups	163,567	5	32,7133	7,55	0,0364
Within groups	17,3333	4	4,33333	-	-
Total (Corr.)	180,9	9	-	-	-
Total (Corr.)	-	180,9	9	-	-

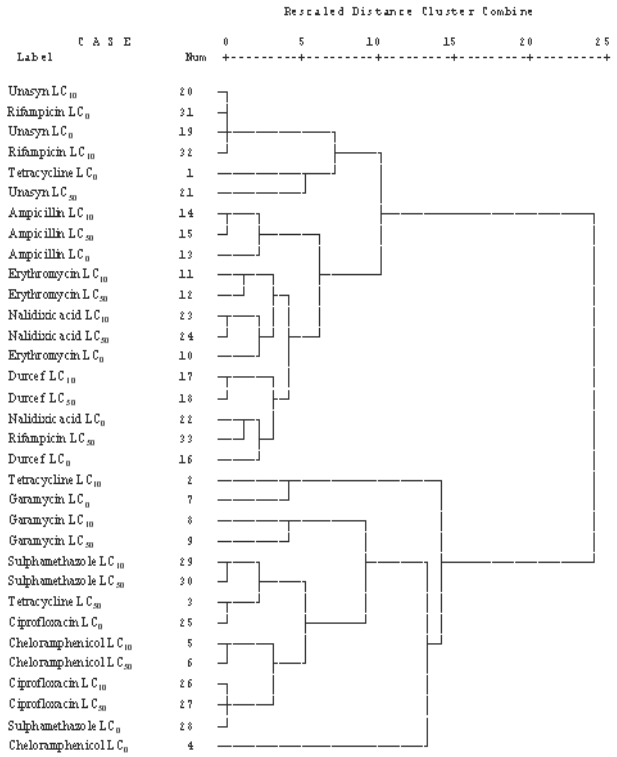

Fig. 2: Hierarchical cluster analyses Dendrogram using average linkage between Antibiotics groups

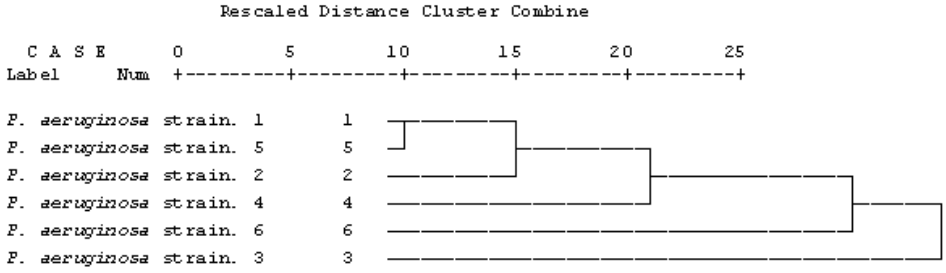

Fig. 3: Hierarchical cluster analysis Dendrogram using average linkage between *Ps. aeruginosa* strains

analysis as in figure 2 and table 2. Cluster analysis show that Protein, DNA inhibitors and Folic acid analogue were the most effective in *Ps. aeruginosa* treatment in presence of EDTA, which highlight the sensitive role of divalent cations in protein and DNA structure and function. The data, which show decrease in *Ps. aeruginosa* sensitivity after adding EDTA, could explain the presence of resistant mechanisms affected with the divalent cation amount, which agrees with the data at tables 1, 2 and cluster analysis. Cluster analysis of the data on strain level proves the presence of a relationship between various infections within different organs in the same system. This study further gives an idea about, which is the best, more fit and stable antibiotic. In this study ciprofloxacin can be used for controlling all these six strains, while the best and stable antibiotic was Sulphamethoxazol for strains 1, 2, 4-6, which show no resistance or change in their sensitivity. We should distinguish between the *in vitro* and the *in vivo* treatment, where divalent cations could not be controlled, like in human blood, in which an improvement in antibiotic performance in the presence of EDTA could give wrong results. On the other hand the antibiotic, which was reported as sensitive with *Ps. aeruginosa* without EDTA and have a stable effect in the presence of divalent cations, will be the best option for *in vivo* treatment. In another word EDTA will give an indicator about what will be happened for the antibiotics in-patient's body where, of course, the performance in the presence of EDTA in petri dish will represent an inhibition in the patient's body as a direct effect of divalent cations. At the same time EDTA will improve the antibiotic performance during superficial treatment or any other where EDTA or EDTA like drugs could be used. For confirming the data in table 2 and specially to prove the effect of protein's inhibitor antibiotics when combined with

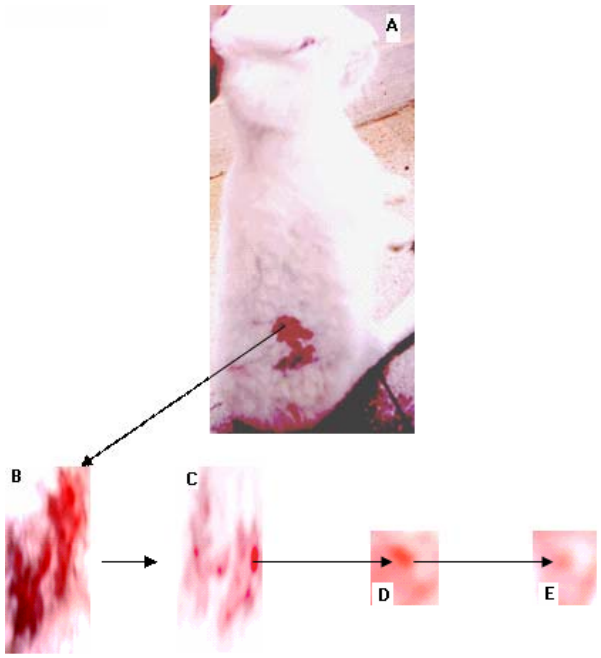

Fig. 4: A Rabbit with artificial ulcer(s); B, C, D and E: 1, 6, 12, 14 (days) treatment with Antibiotic–EDTA (unasyn-EDTA) combination.

EDTA on *Ps. aeruginosa,* an artificial ulcer(s) on Rabbits were done using keratolytic solution followed with infection by strain no. 6, which selected on the base that it shown no significant effect with EDTA (alone) toxicity. The treatment done using simple ointment preparation and the result of various treatments give a clear conclusion about the significant of using EDTA in combination with antibiotics specially protein's inhibitor antibiotics to control *Ps. aeruginosa* and also Adachi *et al*. described that antibiotics with an inhibitory effect on protein synthesis could suppress the production of superantigens (Adachi *et al*., 2002). Case-by-Case study is a new terminology for our experiment to check the role of divalent cations. This study tries to give a new strategy on the control of *Ps. aeruginosa* and could be used with other pathogenic bacteria and open the way for innovations of new drugs more suitable than EDTA for an *in vivo* treatment. In conclusion divalent cations represented in Ca^{2+} and Mg^{2+} play a major role in antibiotic resistant mechanisms. Chelating Ca^{2+} and Mg^{2+} significantly increase the sensitivity of antibiotic. EDTA and other chelating agents effect could be evaluated using their LC_{10} and LC_{50} equivalent amount with various antibiotics. The successful antibiotics after combination with EDTA and other EDTA like drugs could be used in treatment of other pathogen. *Ps. aerginosa* strains from the same origin have the same resistant mechanism even they are different in their phenotypic characteristic, which ease the physician choice and could be used in sensitive bacterial typing. There is a need to substitute Muller-Hinton media in the sensitivity test with media contain divalent cations as described by Christe *et al.* (1982) and confirmed in our study. Innovation of new generations of drugs have antimicrobial, chelating properties and safe for *in vivo* treatment could therefore be a solution for treatment of many pathogens in the future. A combination between EDTA and antimicrobial agents successfully used in treatment of pathogens especially in superficial treatment and can be used in general disinfectants and sanitization purposes.

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