Offline Controlling of *Pseudomonas aeruginosa* Resistant to protein Inhibitor Antibiotics Using Combination of EDTA and Na – citrate or Disinfectant (s) *

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Abstract: *Pseudomonas aeruginosa* recognized as opportunistic pathogen causes severe infections for hospitalized patients, survive in and resist many antimicrobial agents like antibiotics and disinfectants. The aim of this study is to evaluate the role of EDTA in improving the sensitivity of resistant *P. aeruginosa* strains to disinfectants and Na-citrate. The strains used in this study were selected in house from Tanta University hospital, Egypt and tested for the synergistic effect of EDTA with Na-citrate or disinfectant (s). The results showed a significant effect of EDTA in improving *P. aeruginosa* sensitivity. In conclusion, we proposed that using EDTA in combination with different sanitization compounds and antimicrobial agents especially in hospitals aiming to control the spreading of infections.

Key words: Antimicrobial, Sensitivity, Preservative, Divalent cations

P. aeruginosa have a number of virulence factors like extracellular toxins[1], proteases[2,3], haemolysins[4,5], and exopolysaccharide[6,7], which adapt the infection of specific host tissues[8], causing severe problems. *P. aeruginosa* can survive in a number of disinfectants[9,10], while continuous sanitation by single disinfectant could release a resistant[11]. *P. aeruginosa* could resist organomercurials such as phenylmercuric acetate[12], mercurochrome[13], fluorescein[14], mercuric acetate[15], thimerosal[16], chlorhexidine[17], cetrimide[18], antibiotics[19~21]. *P. aeruginosa* reported as the most dominate pseudomonades species was isolated from clinical sources in many countries such as USA[8]. *P. aeruginosa* exhibited innate multidrug resistance caused by outer membrane low-permeability and a number of specific multidrug efflux (Mex) system, like Mex XY-OprM[22]. *P. aeruginosa* mutant *nalB* gene caused overexpression of outer membrane protein, OprM (49 kD), leading to increase in antibiotics resistance[23]. Root *et al.* suggested that the use of EDTA alone might be effective in eradicating catheter-associated biofilms[24]. Percival *et al.*,[25] described the tetrasodium EDTA as a novel catheter lock solution against biofilm where *P. aeruginosa*, *Klebsiella pneumoniae*, *Escherichia coli*, *Staphylococcus epidermidis*, and *Candida albicans* were used to evaluate an experimental protocol designed as a model for treatment of a catheter colonized by biofilm microorganisms. This study aimed at the evaluation of the performance of disinfectant (s) and Na-citrate on *P. aeruginosa* strains, when combined with EDTA. The experimental design was

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to be reliable, simple and would give clear results for the improvement in the antimicrobial activity.

1 Material and methods

1.1 Bacterial strains and culture media

Bacterial strains used in this study were grown routinely in LB solid medium (Luria-Bertani), at 30℃[26]. A defined A-Z (Amara-Zakaria) medium was used to control the divalent cations and consists of $(NH_4)_2SO_4$ (1.3 g/L), KH_2PO_4 (0.6 g/L), $MgSO_4 7H_2O$ (0.3 g/L), $CaCl_2 2H_2O$ (0.02 g/L), K_2HPO_4 (0.8 g/L), NaCl (0.2 g/L) and glucose (2 g/L). Solid media were obtained by the addition of agar (Difco 1.8% w/v).

1.2 Broth dilution susceptibility test (without EDTA)

Broth dilution was performed using A-Z media for screening *P. aeruginosa* resistant strains, isolated from Tanta University hospital. The strains were grown in A-Z broth media till OD_{600} = 0.9, (Approximately $5x10^8$ CFU) at 30 °C using 200 r/min shaking incubator. 10 L of each strain were inoculated to 5 mL test tube containing A-Z media and 0.05, 0.50 and 1.50 L/mL of each disinfectants (Cetrimide 3% w/v - Chlorhexidine gluconate 3% w/v), disinfectant 2 (Chloroxylenal 4.8% w/v), disinfectant 3 (Cetrimide 1.5 % w/v - Dichloroxylenol 1.87 % w/v) and 0.2, 2 and 6 mmol/L Na-citrate respectively while control contained A-Z media only. The % of inhibition of each treatment was determined by taking 10 L from each tube to 10 mL A-Z media, then 20 L from each dilution tube was taken and spread on A-Z agar plates. The results were compared with control and the most resistant strains were selected.

1.3 Antibiotic sensitivity test

Three isolates which successfully grew in presence of disinfectant (s) or Na-citrate were further tested for their resistance to antibiotics rifampcin, tetracycline, garamycin, duricef, chloramphenicol, unasyn, erythromycin, nalidixic acid, sulphamethazole, ampicillin and ciprofloxacin using standard antibiotic disk methods[27].

1.4 Preparation of EDTA stock solution

Stock solution of 0.25 mmol/L EDTA was prepared in distilled water at pH 8. The clear solution was passed through bacterial membrane filter (pore size = 0.22 m) and collected in sterile eppendorf tubes.

1.5 Determination of EDTA LC_{50}

The EDTA toxicity was determined using 0, 0.05, 0.5, 2.5 and 5.0 mmol/L in 5 mL test tube containing A-Z broth medium and 10 L of OD_{600} = 0.5 (approximately $4x10^5$ CFU) freshly cultivated *P. aeruginosa* strains at 30℃ using 200 r/min shaking incubator. *P. aeruginosa* strains was allowed to grow for 8 h at 30 ℃ using 200 r/min shaker incubator. 10 μL taken from each tube was added to 10 mL A-Z broth medium, then 50 μL was spread on A-Z agar plates. The plates then were incubated overnight at 30 ℃ and the colonies of each of them were counted and compared with the control. The percentage of inhibition for each concentration was determined and the LC_{50} was calculated using Probit analysis.

1.6 Broth dilution susceptibility test (with EDTA)

Broth dilution was performed using A-Z broth media. Inoculums of *P. aeruginosa* strains were grown in A-Z broth media till OD_{600} = 0.9, (Approximately 5 x 10^8 CFU), at 30℃ using 200 r/min shaker incubator. 10 L from each strain was inoculated to each test tube containing 5 mL A-Z broth media. 1.5 L/mL of disinfectant (s) and 6 mmol/L Na-citrate was added to each test tube. Another set of tubes containing the same amount as above, in addition LC_{50} equivalent mmol/L of EDTA were incubated at 30℃ using 200 r/min in shaking incubator. Plate count was preformed as described above and the % of inhibition was calculated by comparing with the control, which contained only A-Z medium and *P. aeruginosa* strains.

1.7 Statistical analysis

Statistical analysis for data in table 1 was done using Statistic Package (SPSS/version 11) software. Chi-squire (Z-test) was used for evaluate the result of the broth dilution methods and 5% was chosen as the cutoff level of significant.

2 Result

2.1 Broth dilution susceptibility test (without EDTA)

The experiment results leading to isolation of three *P. aeruginosa* resistance strains were shown in figure 1 and table 1.

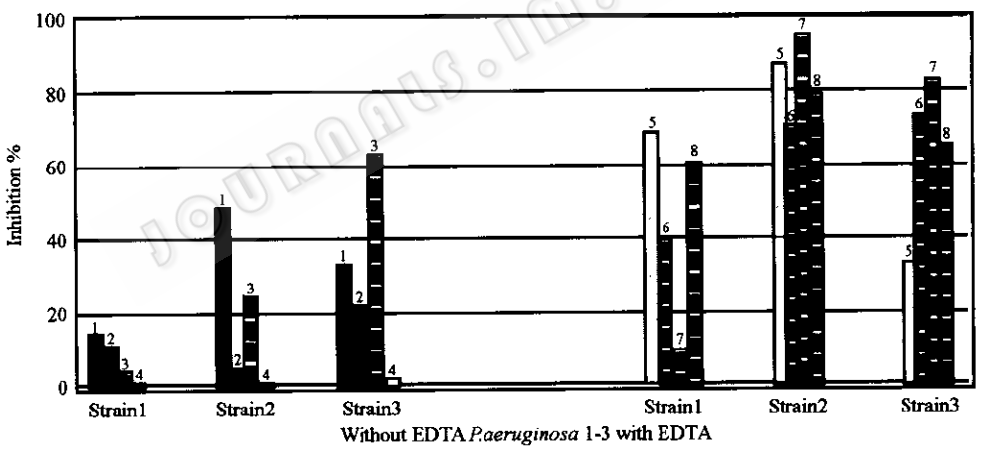

Figure 1 The effect of disinfectant 1-3 and Na-citrate on the sensitivity of *P. aeruginosa* with or without EDTA

1 Cetrimide 1.5% and dichloroxylenol 1.87 % w/v, 2 Cetrimide 3% w/v and chlorohexidine gluconate 3% w/v, 3 Chloroxelenol 4.8% w/v, 4 Na-citrate, 5 Cetrimide 1.5% and dichloroxylenol 1.87% w/v-EDTA, 6 Cetrimide 3% w/v and chlorohexidine gluconate 3% w/v-EDTA, 7 Chloroxelenol 4.8% w/v-EDTA, 8 Na-citrate-EDTA

Table 1 Effect of EDTA on Na citrate and disinfectant on different type of *P. aeruginosa* strains

Strains (No.)	Name of the disinfectant (w/v)	Disinfectant alone	EDTA and disinfectant	*P*
1	Cetrimide 1.5% – dichloroxylenol 1.87%	14	68	0.001 *
1	Na citrate	1	61	0.0001 *
1	Cetrimide 3% – chlorhexidine gluconate 3%	11	39	0.0025 *

1	Chloroxelenol 4.8%	10	4	>0.05 N.S.
2	Cetrimide 1.5% - Dichloroxylenol 1.87	49	87	0.0103 *
2	Na citrate	1	79	0.00001 *
2	Cetrimide 3% - Chlorhexidine gluconate 3%	5	71	0.0001 *
2	Chloroxelenol 4.8%	25	95	0.0025 *
3	Cetrimide 1.5% - Dichloroxylenol 1.87%	33	33	>0.05 N.S.
3	Na citrate	2	65	0.0001 *
3	Cetrimide 3% - Chlorhexidine gluconate 3%	22	73	0.0032 *
3	Chloroxylenol 4.8%	63	83	0.041 *

* Significant ; Data calculated as % of inhibition regarding to the control, Data were analyzed by using Z-test

2.2 Antibiotic sensitivity test

The three strains were evaluated using antibiotic disk methods according to NCCLS document[27]. According to the inhibition zone diameter the antibiotic sensitivity was classified as r (resistant), i (intermediate) and s (sensitive). The strains were completely resistant to tetracycline, chloramphenicol, erythromycin, durcef, unasyn and rifampicin. The profile of antibiotic sensiticity test with ampicillin, nalidixic acid, ciprofloxacin and sulphamethazol were variable as shown in table 2.

Table 2 Antibiotic resistant profile for *P. aeruginosa* strain 1, 2 and 3

Antibiotic name	Antibiotic concentration (g/mL)	*P. aeruginosa* sensitivity		
		Strain 1	Strain 2	Strain 3
Tetracycline	30	r*	r	r
Chloramphenicol	30	r	r	r
Erythromycin	15	r	r	r
Ampicillin	10	s*	i*	s
Durcef	30	r	r	r
Unasyn	15	r	r	r
Nalidixic acid	30	r	r	i
Ciprofloxacin	5	s	i	s
Sulphamethazole	25	s	r	s
Rifampicin	30	r	r	r

s* sensitive, i* intermediate and r* resistant

2.3 Determination of EDTA LC_{50}

The LC_{50} of EDTA was determined by probit analysis methods. For strain1 was 2, 93 mmol/L EDTA, strain 2 was 0.72 mmol/L and for strain 3 was 0, 895 mmol/L as shown in figure 2.

2.4 Broth dilution susceptibility test (with EDTA)

The effect of various concentration of disinfectant (s) or Na-citrate in combination with the LC_{50} equivalent mmol/L amount of EDTA was shown in figure 1. All treatment showed the increase in the inhibition % after combination of EDTA with disinfectant (s) or Na-citrate except chloroxylenol 4.8% w/v against *P. aeruginosa* strain1 and cetrimide 1.5% w/v-dichloroxylenol 1.87% against strain 3.

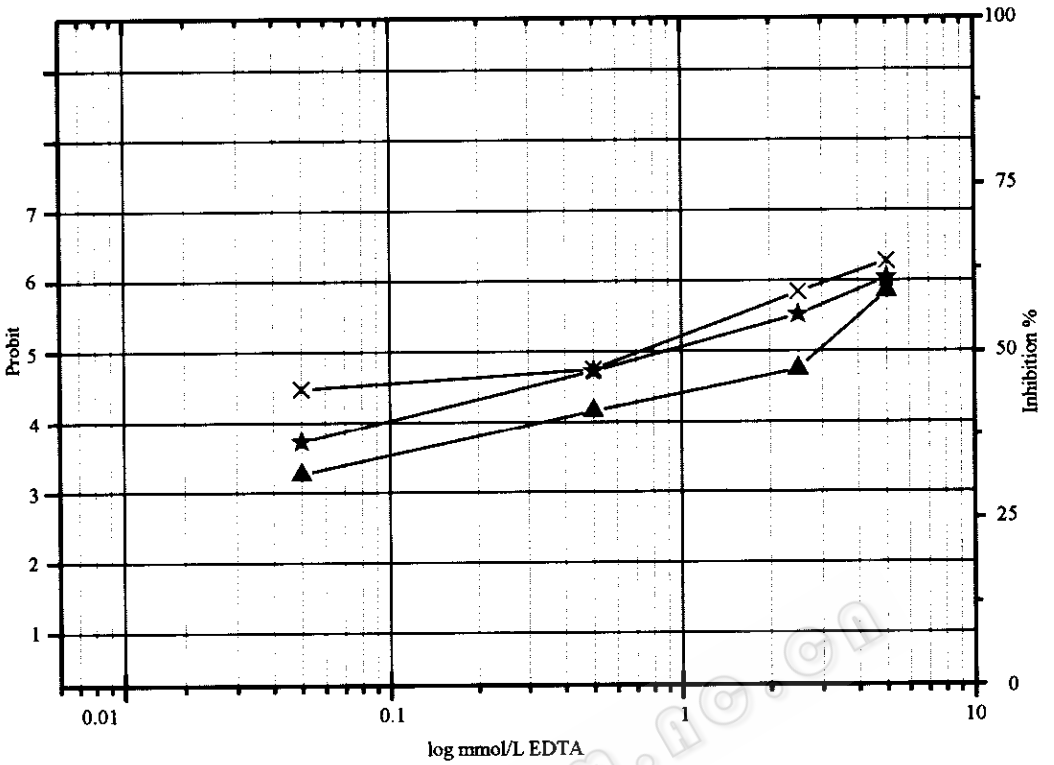

Figure 2 LC_{50} of EDTA against *P. aeruginosa* strain 1-3

2.5 Discussion

Most of the cell activities are performed by proteins which are in a fold form in their natural environment. The binding to ions giving rise to cross-linkage will be helpful to the folding of heavily charged proteins. Ca^{2+}, Mg^{2+} and other divalent cations are well known for their important role in folding, refolding, signaling, enzyme activity, bioenergetics, structure stability, antigen, etc[6,7,16,20,22,23]. In case of *P. aeruginosa*, Ca^{2+} is an example about the role of divalent cations which involved in alginate-biofilm formation and stability. Dragging out the divalent cations either from the cells or from the cells surrounded environment will lead to blocking of many essential biological functions and could further result in the death of microbes[6,7,25]. In this study we screened for *P. aeruginosa* multiresistant strains to antibiotics, Na-citrate and disinfectants resulting in isolation of three strains from Tanta University and identified by standard criteria as *P. aeruginosa*. The strains successfully grew on Na-citrate, disinfectant (s) and completely resistant to protein synthesis inhibitor antibiotics: tetracycline, chloramphenicol, erythromycine, cell wall synthesis inhibitors antibiotics: eurcef, unasyn and RNA synthesis inhibitors antibiotic: refampicin. The strains were grown on defined media containing Ca^{2+} and Mg^{2+} to improve the roles of EDTA. While EDTA is a chelating agent to divalent cations it should suppress most of *P. aeruginosa* enzymatic functions which could be used by one or another used in protection and degradation of antimicrobial agents. Suppressing the *P. aeruginosa* resistant system, which depends on divalent cations, by using EDTA make *P. aeruginosa* an open target to any antimicrobial gents, is it clear in our study? Using the LC_{50} equivalent

mmol/L of EDTA omitted any result related to the EDTA toxicity the role of. Na-citrate which is a week antimicrobial agent has been clearly improved by adding EDTA as in figure 1 and table 1 which indicating the role of divalent cations in protecting microbes against antimicrobial agent. The statistical analysis of data in table 1 proves the significant effect of adding EDTA to both of disinfectant (s) or Na-citrate.

The offline strategy is a sanitization method for controlling *P. aeruginosa* antibiotics resistance strains before they infect susceptible patients where the antibiotics treatment in this case will not be efficient but the protection against the speeding of the infections still available.

While using EDTA *in vivo* could not be easily established for many biological aspects, such as its toxicity, so controlling pathogens, which have powerful protection mechanisms such as alginate production in *P. aeruginosa*, will be improved *in vitro* by using EDTA[25]. Without Ca^{2+}, which involved in the rigidity of alginate, *P. aeruginosa* could not perform biofilm. In conclusion we recommended to add EDTA to disinfectant (s) and Na-citrate for increase the sensitivity of *P. aeruginosa* and other microbs.

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