Global Academic Journal of Medical Sciences

Available online at www.gajrc.com
DOI: https://doi.org/10.36348/gajms.2025.v07i03.009

OPEN ACCESS

ISSN: 2706-9036 (P)
ISSN: 2707-2533 (O)

Original Research Article

The Effect of Ethylenediaminetetraacetic Acid (EDTA) as a Synergistic Factor with some Biocides on *P.aeruginosa* Isolated from Clinical and Environmental Samples of Hospital

Salwan Abdulmuayn[1*], Aamal Ghazi Mahdi Al-Saadi[2]

[1]University of Al-Qadisiyah, College of Biotechnology
[2] University of Al-Qadisiyah, College of sciences

***Corresponding Author**
Salwan Abdulmuayn
University of Al-Qadisiyah, College of Biotechnology

Article History
Received: 13.05.2025
Accepted: 18.06.2025
Published: 26.06.2025

Abstract: The risk of emergence and spread of microbial pathogens increased due to the overuse of sterilization and disinfection materials with a lack of health supervision. We aimed to determine the extent of sensitivity of *P.aeruginosa* to biocides regularly used in the hospitals, and to study synergistic effect of (EDTA) with the same biocides. Twenty five isolates of *P.aeruginosa* were diagnosed, from 200 samples. (100 clinical and 100 environmental) samples were collected from Al-Diwaniyah Teaching Hospital. Samples were cultivated on blood agar, macconkey agar and cetrimide agar, and bacterial isolates were identified according to Hemolytic lysis of the blood cells in blood agar, unablity to ferment the sucrose and lactose on macconkey agar and the blue greenish pyocyanine stain and odour as fruit on cetrimide agar. Isolates were confirmed using the VITEK2 automated system. A standard broth dilution method was used to determine the MIC concentrations of chlorhexidine digluconate (20%), benzalkonium chloride (50%), formaldehyde (37%), cetrimide (20%), and hydrogen peroxide H2O2 (30 %) at concentrations ranging from 4 µg/ml to 2048 µg/ml. 17% EDTA was used for synergistic test. The results showed that the MIC ranges for the biocides used were as follows: chlorhexidine digluconate (64 - ≥2048 µg/ml) (8-64 µg/ml), formaldehyde (64 -≥1024 µg/ml) (16 - ≥128 µg/ml), cetrimide (256 - ≥2048 µg/ml) (4 - ≥256 µg/ml), benzalkonium chloride (512 - ≥2048 µg/ml) (8 - ≥256 µg/ml), and hydrogen peroxide (≥2048 µg/ml) (4 - ≥256 µg/ml) without and with EDTA respectively. The MBC concentrations were: chlorhexidine digluconate (128-≥2048 µg/ml), benzalkonium chloride (1024-≥2048 µg/ml), formaldehyde (128-≥2048 µg/ml), cetrimide (1024-≥2048 µg/ml) and hydrogen peroxide (≥2048 µg/ml). A combination of biocides and EDTA has been recommended as an effective way to reduce hospital contamination by *P.aeruginosa.*

Keywords: EDTA, Synergistic Factor, Biocides, *P.aeruginosa*, Clinical Samples of Hospital, Environmental Samples of Hospital.

Citation: Salwan Abdulmuayn & Aamal Ghazi Mahdi Al-Saadi (2025). The Effect of Ethylenediaminetetraacetic Acid (EDTA) as a Synergistic Factor with some Biocides on *P.aeruginosa* Isolated from Clinical and Environmental Samples of Hospital. *Glob Acad J Med Sci*; Vol-7, Iss-3 pp- 134-146.

1. INTRODUCTION

Pseudomonas aeruginosa is acknowledged as a unique extensively spreading pathogen that causes about 18 to 63% of contagions universally [1, 2]. It is an important clinical agent, because this bacterium is consider as an opportunistic type that is a source of wide range of acute and chronic diseases in humans. It is caused for numerous infections including, septicemia, necrosis particularly in individuals that are inimmuno [3]. (UTIs) [4], wounds , burn, and infections of skin [5] . Virulence factors in *P.aeruginosa* are one of the essential factors causing pathogenicity [6].

The capability of *P.aeruginosa* to propagate in lowest nutritional supplies and to survive numerous physical environments like disinfectants helps this bacterium to persist in hospital and environmental locations [6, 7]. There are several mechanisms of resistance in *P.aeruginosa* as Outer membrane impermeability , Efflux pumps systems , Alteration target site, Horizontal Evolution and β-Lactamase production [8]. Among these resistance mechanisms to biocides, efflux pumps encoded by qacEΔ1, qacE, qacG and cepA genes play an important role in *P.aeruginosa* resistance to benzalkonium chloride and chlorhexidine [9]. Nowadays, bacterial strains ability to resist disinfectants as well as antibiotics is a main public health concern worldwide [10, 11]. The unsuitable usage of disinfectant is one of the main reasons of nosocomial contagions spreading. Due to the differences between disinfectants as suitable agents for all diverse sterilization requirements, there is a need for new studies to define the affectivity of different disinfectants so the correct disinfectant can be chosen [11-13]. Inappropriate consumption of disinfectants as well as biodegradation lead to biocide attentiveness in clines. Consequently, microorganisms are consecutively affected by non-lethal levels of antiseptics, which assists resistance to sterilizers and to other antimicrobials agents [14-17].

EDTA is known as a metal chelator to fragment the outer lipopolysaccharide layer of Gram-negative bacteria, resulting in increased membrane permeability to disinfectants [18]. For decades, EDTA has been utilized as a great anticoagulant stopping clot development in vitro [19]. It is also utilized widely to treat people with metal poisoning [20]. Nevertheless, EDTA revealed low antimicrobial activity when used alone [21], while it frequently intensify the action of antimicrobial means due to its synergistic effect [22, 23]. The aims of this study are to evaluate the effectiveness of some common used biocides on *P. aeruginosa* isolated from hospital environments by detecting MIC and MBC concentrations and to examine the synergistic influence of EDTA in combination with the same biocides.

2. MATERIALS AND METHODS

2.1 Samples Collection

Two hundred clinical and environment samples were obtained from the Teaching Hospital in Al-Diwaniyah Governorate for the period from July 2023 to December 2023**.** 100 Clinical samples were collected from patients with burns of different degrees, patients with Urinary tract infection (UTI), and Otitis media. In addition 100 hospital environmental samples, were taken from the walls, patients' beds, surgical tools, oxygen masks, the floor, toothpicks, bathrooms, tables, eating utensils, glass, spoons, and from the operation room, duct, pool of patient washing**.** Clinical samples were collected during the morning periods during the presence of the workers and patients in sterile tube without media. Burn and wounds samples were collected from the patients before skin sterilizing early time of morning. The Environmental swabs were taken before the sterilization and cleaning of the hospital in early time of the morning too by sterile tube of Thioglycolate, as mentioned in [24, 25].

2.2 Isolation of *P.aeruginosa*

Samples were taken with sterile cotton swabs and then placed in a tube containing 2 ml of tryptic soy broth. Samples were incubated at 37°C for 24 hours. Samples were cultured with a sterile loop using the streaking method on MacConkey agar, cetrimide agar, and blood agar and then incubated for 24 hours at 37°C.

2.3 Identification of *P. Aeruginosa*

Microscopic Identification Based on Gram's staining, colony phenotype on several media, blood hemolysis, and pigment production, isolates were first identified. Isolates of *P.aeruginosa* were also confirmed by biochemical tests that include Lactose fermentation, Indole test Medium, Oxidase test, Sucrose fermentation, Catalase test (slide test), Blood hemolysis and the VITEK2-automated system.

2.4 Testing of Biocides Susceptibility

The concentrations of biocides considered in this study were chlorhexidine digluconate (20%) (Cercamed, Poland), benzalkonium chloride (50%) (Alpha Schmica, India), formaldehyde (37%) (Panrec Applisime), Spain, cetrimide (20%). (IndiaMart). (India) and hydrogen peroxide H_2O_2 (30%) (Thomas Baker, India). 2048 µg/ml stock solutions of each antimicrobial agents were prepared in a Muller Hinton Broth (MHB). All biocide solutions were sterilized using sterile syringe filters (0.22 µm) before use [26]. To prepare bacterial inoculum, the method of colonies suspensions with MHB was

utilized. Solutions turbidly was compared with the McFarland standard and later suspension absorbance was measured on a spectrophotometer (ThermoFisher scientific, USA) for verification. For this examination, the absorbance range should be in a among 0.08 and 0.13 at 625 nm, which is equivalent to 1×10^8 CFU/mL [27]. By using a standard broth dilution method, the MIC concentrations of each biocide were determined based on culture turbidity and OD_{600} [28, 29]. The biocides concentrations used in a range of 4 µg/ml to 2048 µg /ml. MIC 95(ECOFF) for antimicrobial sensitivity from previous studies was used. Strains with MIC values higher than ECOFF were considered as resistant strains[30]. After determining the MIC of the biocides, 100µl were cultured by a sterile loop from all tubes showing no obvious bacterial growth or no turbidity on Mueller-Hinton agar plates and incubated for 24 hours at 37°C, by observing the agar plates before and after incubation for the presence of or absence of bacteria, plates containing (fewer than 15 colonies) were defined as MBC i.e. 99.9% of the bacterial population was killed at the lowest concentration of the antimicrobial agent [31].

2.5 Synergistic Effect of EDTA with Biocides

Selected values of MIC and MBC for each biocide were tested with the addition of 17% EDTA to determine the synergistic effect using the broth dilution method as specified above. Briefly, the biocides under study were mixed with EDTA one by one (1 mL biocide + 1 mL EDTA 17%) and kept at room temperature for approximately 15 minutes. The dilution series were set and the inoculated tubes were then placed in an incubator at 37°C overnight. MIC and MBC values were then determined for all resistant isolates [32]. For the minimum bactericidal concentration (MBC), determination, 100µL was taken from each tube lacking noticeable turbidity then added to Muller Hinton agar plates and grown at 37°C overnight [33].

2.6 Statistical Analysis

For data collection and statistical analysis, we utilized Microsoft Office Excel 2013 together with GraphPad Prism 9.2.0. Mean ± standard error mean was the format used to show the statistical data. Regarding variables that are regularly distributed, we compared the group means using a one-way ANOVA. A P-value of less than 0.05 was the standard for overall significance.

3. RESULTS

3.1. Isolation and Identification of P. Aeruginosa

200 environmental and clinical samples were collected from the Teaching Hospital in Al-Diwaniyah Governorate. 25 isolates were obtained, 13 clinical isolates (13%) and 12 environmental isolates (12%).

P. aeruginosa isolates were cultured on several media (MacConkey agar, blood agar, Cetrimide agar). The bacterial culture on the MacConkey agar medium, which use to distinguish between lactose fermented and non-lactose fermented bacteria, revealed grew of single small pale colonies of *P.aeruginosa* indicated that bacteria does not ferment the lactose. The growing colonies characters of *P.aeruginosa* on Cetrimide agar, showed growth of mucoid, smooth in shape with flat edges and elevated center, have fruity odor and yellow to green colonies [34]. In blood agar, it show β-hemolysis. The cells appear dark surrounded by a transparent ring, indicating the decomposition of blood cells due to their production of the enzyme Hemolysin. From the observation of the results of the biochemical tests of these isolates, they gave a positive results to the tests of catalase and oxidase, and all isolates were characterized by it is not fermenting to sucrose and lactose. Isolates were further confirmed using the Vitek2 Compact with a 99% percent probability ratio.

3.2. Determination of MIC

Table (1) showed the MIC ranges for various biocides of environmental and clinical isolates as follows: for clinical isolates, chlorhexidine digluconate (64-2048 µg/mL), benzalkonium chloride (512-2048 µg/mL), formaldehyde (64-1024 µg/mL), cetrimide (256-2048 µg/mL), hydrogen peroxide H_2O_2 (≥2048 µg/mL).

While for environmental isolates, chlorhexidine digluconate (64-1024 µg/mL), benzalkonium chloride (512-2048 µg/mL), formaldehyde (512-1024 µg/mL), and cetrimide (1024-2048 µg/mL) and hydrogen peroxide (≥2048 µg/mL).

Table 1: Minimum inhibitory concentration (MIC) (µg/ml) values of biocides against clinical and Environmental Pseudomonas aeruginosa isolates.

Biocide		H_2O_2		chlorhexidine digluconate		Benzalkonium chloride		Formaldehyde		Cetrimide	
		clinical	Environ.	clinical	Environ	clinical	Environ	clinical	Environ	clinical	Enxviron
4 µg/ml											
8 µg/ml											
16 µg/ml											
32 µg/ml								2 / 15.38%			
64 µg/ml				5 /38.46%	7/ 58.33%						
128 µg/ml				5 / 38.46%	2/ 16.66 %			2/ 15.38%			
256 µg/ml					-			4 / 30.76 %		1/ 7.69%	
512 µg/ml				1/ 7.69%	2/ 16.66 %	1/7.69%	1/ 8.33%	4/ 30.76 %	6 / 50%	1/ 7.69%	2 / 16.66%
1024 µg/ml				1/ 7.69%	1/ 8.33 %		1/ 8.33%	1/ 7.69%	6 / 50%	6 / 46.10%	10 / 83.33 %
≥2048 µg/ml		13 / 100%	12 / 100%	1/ 7.69%	-	12 93.3%	10 / 83.33 %		-	5 / 38.46 %	

As shown in table (2) the MIC ranges for various biocides were as follows: chlorhexidine digluconate (64-2048 µg/mL), formaldehyde (64-1024 µg/mL), cetrimide (256-2048 µg/mL), benzalkonium chloride (512-2048 µg/mL), and hydrogen peroxide (≥2048 µg/mL).

Table 2: Minimum inhibitory concentration (MIC) (µg/ml) value of biocides against *Pseudomonas aeruginosa* isolates

Biocide	H_2O_2	chlorhexidine digluconate	Benzalkonium chloride	Formaldehyde	Cetrimide
4 µg/ml	-	-	-	-	-
8 µg/ml	-	-	-	-	-
16 µg/ml	-	-	-	-	-
32 µg/ml	-	-	-	-	-
64 µg/ml	-	12 / (48%)	-	2 / (8%)	-
128 µg/ml	-	7 / (28%)	-	2 / (8%)	-
256 µg/ml	-	-	-	4 / (16%)	1/ (4%)
512 µg/ml	-	3 / (12%)	2 / (8%)	10 / (40%)	1/ (4%)
1024	-	2 / (8%)	1/ (4%)	7 / (28%)	8 / (32%)
≥2048 µg/ml	25 / 100%	1/ 4%	22 / 88%	-	15 / 60%

All the 25 *P.aeruginosa* isolates were subjected to the synergism test of EDTA with H_2O_2 , while isolates exceeding the ECOF values detected in previous studies were selected for this test for the remaining biocides as follows : chlorhexidine digluconate (512 µg/ml) [35], Benzalkonium chloride (1024 µg/ml)[36], Formaldehyde (512 µg/ml)[36], Cetrimide (512 µg/ml) [37]. Based on this (3) isolates obtained in this study were resistant to (chlorhexidine digluconate), (22) isolates to (Benzalkonium chloride), (7) isolates to (Formaldehyde) and (23) isolates to (Cetrimide).

In table (3) the MIC ranges for various biocides after the adding 17% were as follows:

Hydrogen peroxide H_2O_2 (4-256 µg/mL), chlorhexidine digluconate (8-64µg/mL), benzalkonium chloride (8-256 µg/mL), formaldehyde (16-128 µg/mL), and cetrimide (4-256 µg/mL).

Table 3: Minimum inhibitory concentration (MIC) (µl/ml) value of synergism biocides with EDTA against *Pseudomonas aeruginosa* isolates

Biocide	4 µg/ml	8 µg/ml	16 µg/ml	32 µg/ml	64 µg/ml	128 µg/ml	256 µg/ml	512 µg/ml	1024 µg/ml	2048 µg/ml
H_2O_2	4 / (16%)	1/ (4%)	3 / (12%)	3 / (12%)	3 / (12%)	3 / (12%)	8 / (32%)	-	-	-
chlorhexidine digluconate		2 / (8%)	-	-	1/ (4%)	-	-	-	-	-
Benzalkonium chloride		6 / (24%)	3 / (12%)	3 / (12%)	7 / (27%)	2 / (8%)	1 / (4%)	-	-	-
Formaldehyde		-	4/ (16%)	1/ (4%)	-	2 / (8%)	-	-	-	-
Cetrimide	5/ (20%)	2 / (8%)	6 / (24%)	6 / (24%)	2 / (8 %)	1/ (4%)	1/ (4%)	-	-	-

In table (4) Comparisons of MIC mean values of H_2O_2 for the environmental and clinical *P.aeruginosa* isolates with and without EDTA, Significant differences for all the tested isolates were recorded with p values (<0.0001).

Table 4: Comparison of MIC mean values of H_2O_2 for the Environmental and clinical *P.aeruginosa* isolates with and without EDTA

Isolate	EDTA	Mean		Std. Error of Mean.		P value	
		Env.	clinical	Env.	clinical	Env.	clinical
1	a	1.943	1.434	0.019	0.056	<0.0001 ****	<0.0001 ****
	b	0.121	0.110	0.005	0.003		
2	a	1.781	1.043	0.022	0.082	<0.0001 ****	<0.0001 ****
	b	0.118	0.112	0.002	0.001		
3	a	1.870	0.940	0.029	0.039	<0.0001 ****	<0.0001 ****
	b	0.116	0.112	0.003	0.002		
4	a	1.896	0.889	0.031	0.043	<0.0001 ****	<0.0001 ****
	b	0.114	0.114	0.001	0.002		
5	a	1.918	1.044	0.018	0.050	<0.0001 ****	<0.0001 ****
	b	0.119	0.115	0.004	0.002		
6	a	1.890	1.046	0.027	0.054	<0.0001 ****	<0.0001 ****
	b	0.116	0.117	0.003	0.003		
7	a	1.970	1.823	0.021	0.194	<0.0001	<0.0001

	b	0.107	0.116	0.002	0.003	****	****
8	a	1.436	1.415	0.088	0.066	<0.0001 ****	<0.0001 ****
	b	0.116	0.123	0.002	0.005		
9	a	1.859	1.576	0.035	0.134	<0.0001 ****	<0.0001 ****
	b	0.125	0.112	0.010	0.002		
10	a	1.840	1.131	0.044	0.106	<0.0001 ****	<0.0001 ****
	b	0.113	0.115	0.001	0.002		
11	a	1.848	1.293	0.035	0.108	<0.0001 ****	<0.0001 ****
	b	0.114	0.111	0.002	0.001		
12	a	1.934	1.343	0.013	0.155	<0.0001 ****	<0.0001 ****
	b	0.113	0.113	0.001	0.002		
13	a		1.378		0.088		<0.0001 ****
	b		0.113		0.002		

a: without EDTA , b: with EDTA

Table (5) shows comparisons of MIC mean values of benzalkonium Chloride for the environmental and clinical *P.aeruginosa* isolates with and without EDTA. Significant differences for all the tested isolates were recorded except for environmental isolate No (10).

Table 5: Comparison of MIC mean values of benzalkonium chloride for the Environmental and clinical *P.aeruginosa* isolates with and without EDTA

Isolate	EDTA	Mean		Std. Error of Mean.		P value	
		Env.	clinical	Env.	clinical	Env.	clinical
1	a	0.964	0.907	0.163	0.156	<0.0001 ****	0.0028 **
	b	0.140	0.143	0.016	0.014		
2	a	0.958	0.935	0.174	0.133	<0.0001 ****	0.0008 ***
	b	0.115	0.117	0.002	0.002		
3	a	0.970	0.999	0.164	0.119	<0.0001 ****	0.0001 ***
	b	0.113	0.119	0.002	0.003		
4	a	1.344	0.992	0.148	0.157	<0.0001 ****	0.0002 ***
	b	0.118	0.118	0.002	0.002		
5	a	1.130	0.878	0.092	0.141	<0.0001 ****	0.0028 **
	b	0.112	0.113	0.002	0.001		
6	a	0.802	0.839	0.052	0.123	0.0024 **	0.0082 **
	b	0.115	0.120	0.002	0.003		
7	a	0.866	0.895	0.136	0.198	0.0005 ***	0.0022 **
	b	0.120	0.120	0.004	0.003		
8	a		0.873		0.170		0.0035 **
	b		0.118		0.002		
9	a	0.732	0.867	0.162	0.203	0.0135 *	0.0046 **
	b	0.116	0.124	0.003	0.003		
10	a	0.621	0.827	0.162	0.160	0.1266 ns	0.0095 **
	b	0.116	0.115	0.002	0.002		
11	a						
	b						
12	a	0.891	1.179	0.224	0.245	0.0002 ***	<0.0001 ****
	b	0.112	0.115	0.003	0.003		
13	a		0.932		0.198		0.0008 ***
	b		0.115		0.002		

a: without EDTA , b: with EDTA

Table (6) shows that there are significant differences between MIC mean values of chlorhexidine digloconate for the environmental and clinical *P.aeruginosa* isolates before and after synergy with EDTA.

Table 6: Comparison of MIC mean values of chlorhexidine digloconate for the Environmental and clinical *P.aeruginosa* isolates with and without EDTA

Isolate	EDTA	Mean		Std. Error of Mean.		P value	
		Env.	clinical	Env.	clinical	Env.	clinical
6	a	0.7989		0.195		0.0006 ***	
	b	0.02785		0.008415			
7	a		0.982		0.179		0.0002 ***
	a		0.114		0.004		
9	a		0.7406		0.191		0.0093 **
	b		0.116		0.00375		

a: without EDTA , b: with EDTA

Tables (7) also shows that there is a significant difference between using formaldehyde alone and after synergism with the compound EDTA for the environmental and clinical *P.aeruginosa* isolates , except for environmental isolate No. (11), in which it was noted that there is no significant difference between the two values.

Table 7: Comparison of MIC mean values of formaldehyde chloride for the Environmental and clinical *P.aeruginosa* isolates with and without EDTA

Isolate	EDTA	Mean		Std. Error of Mean.		P value	
		Env.	clinical	Env.	clinical	Env.	clinical
3	a	0.6934		0.2121		<0.0001 ****	
	b	0.1091		0.00095			
4	a	0.6861		0.2119		<0.0001 ****	
	b	0.1108		0.0008			
6	a		0.5577		0.1774		0.0006 ***
	b		0.0177		0.00174		
8	a	0.9851		0.2452		<0.0001 ****	
	b	0.114		0.00187			
10	a	0.7017		0.2154		<0.0001 ****	
	b	0.1135		0.00146			
11	a	0.5417		0.2288		0.0814 ns	
	b	0.1122		0.00158			
12	a	0.8991		0.2839		<0.0001 ****	
	b	0.109		0.00153			

a: without EDTA , b: with EDTA

From Tables (8) we noted that there are no significant differences between the values before and after synergy with the compound (EDTA) with cetrimide in most of the clinical isolates when compared with the environmental isolates.

Table 8: Comparison of MIC mean values of cetrimide for the Environmental and clinical *P.aeruginosa* isolates with and without EDTA

Isolate	EDTA	Mean		Std. Error of Mean.		P value	
		Env.	clinical	Env.	clinical	Env.	clinical
1	a	1.021	0.0942	0.1719	0.03443	0.0002 ****	>0.9999 ns
	b	0.1489	0.1121	0.02601	0.001602		
2	a	1.001		0.1918		0.0002 ****	
	b	0.118		0.002314			
3	a	1.036		0.1433		<0.0001 ****	
	b	0.124		0.00458			
4	a	1.112	0.5268	0.1947	0.05029	<0.0001 ****	0.0002 ***
	b	0.1229	0.1328	0.003478	0.01027		
5	a	1.109	0.0759	0.1757	0.02444	<0.0001 ****	>0.9999 ns
	b	0.1222	0.108	0.00403	0.001422		
6	a	0.994	0.0959	0.143	0.0326	0.0002 ****	>0.9999 ns
	b	0.1222	0.1123	0.003872	0.000967		

7	a	0.6774	0.7893	0.08043	0.06675	0.1645 ns	<0.0001 ****
	b	0.1137	0.1151	0.002039	0.002079		
8	a	0.6844	0.1139	0.2207	0.02477	0.1463 ns	>0.9999 ns
	b	0.1128	0.116	0.001659	0.000730		
9	a	1.081	0.1005	0.1694	0.02706	<0.0001 ****	>0.9999 ns
	b	0.1117	0.1147	0.001438	0.001283		
10	a	1.116	0.8244	0.1505	0.1652	<0.0001 ****	<0.0001 ****
	b	0.1297	0.1197	0.006361	0.002385		
11	a	1.15	0.3562	0.1869	0.09466	<0.0001 ****	0.2252 ns
	b	0.1217	0.1149	0.002879	0.001676		
12	a	0.5586	0.255	0.1972	0.09535	0.6102 ns	0.9708 ns
	b	0.1127	0.1134	0.001317	0.001558		
13	a	1.021	0.2615	0.1719	0.1023	0.0002 ****	0.9497 ns
	b	0.1489	0.1121	0.02601	0.00161		

a: without EDTA , b: with EDTA

3.3. Determination of MBC:

Table (9) shows the MBC ranges for various biocides with and without EDTA were as follows:

Without adding of EDTA, the MBC for Hydrogen peroxide H_2O_2 (≥2048 µg/mL), chlorhexidine digluconate (128-2048 µg/mL), benzalkonium chloride (1024-2048 µg/mL), formaldehyde (128-2048 µg/mL), and cetrimide (1024-2048 µg/mL).

While in the adding of EDTA, the MBC were Hydrogen peroxide H_2O_2 (8-2048 µg/mL), chlorhexidine digluconate (16-256 µg/mL), benzalkonium chloride (32-1024 µg/mL), formaldehyde (32-1024 µg/mL), and cetrimide (16-512 µg/mL).

Table 9: Minimum bactericidal concentration (MBC) (µg/ml) value of biocides against *Pseudomonas aeruginosa*

Biocide		4	8	16	32	64	128	256	512	1024	≤2048
H_2O_2	Without EDTA										25 / 100%
	With EDTA		**1/ 4%**	**2 / 8%**	**4 / 16%**	**2 / 8%**	**4 / 16%**	**2 / 8%**	**2 / 8%**	**5 / 20%**	**3/ 12%**
chlorhexidine digluconate	With EDTA						8 / 32%	5/20%	5 / 20%	4 / 16%	3 / 12%
	With EDTA			**1/ 4%**		**1 / 4%**		**1/ 4%**			

Benzalkonium chloride	Without EDTA									1/ 4%	24 / 96%
	With EDTA				**4 / 16%**	**7 / 28%**	**6 / 24%**	**3 / 12%**	**1 / 4%**	**1 / 4%**	
Formaldehyde	Without EDTA						2 / 8%	-	1/ 4%	8 /32%	14 / 56%
	With EDTA				**2 / 8%**	**1 / 4%**	**1/ 4%**	**1 /4%**	**1 / 4%**	**1 / 4%**	
Cetrimide	Without EDTA									1/ 4%	24 / 96%
	With EDTA			**3 / 12%**	**7/ 28%**	**3 / 12%**	**5 / 20%**	**3 / 12%**	**2 / 8%**		

4. DISCUSSION

Major biocide resistance mechanism in Gram-negative bacteria including *P.aeruginosa* is the action of efflux pumps such as the small multidrug resistance family (SMR)[9], although their mechanisms of action are not entirely clear, they generally work on multiple targets within the bacterial cell in a non-selective manner, such as ionic reactions, breaking hydrogen bonds, and chemical reactions (such as oxidants and electrical) [38].

Biocides could act on multiple sites in microorganisms and cause resistance by non-specific means. There are several mechanisms such as efflux pumps, cell wall changes to the reduction of permeability, genetic linkage with both biocide resistance genes and antibiotic resistance genes, the penetration/uptake changes in envelope by passive diffusion, effect on the integrity and morphology of membrane, and effects on diverse key steps of bacterial metabolism. Along with this toxic effect and stress, bacterial cells express some similar defense strategies that can overlap the main functions conferring resistance versus structurally nonrelated molecules [39].

In general, the action of biocides is due to a basic difference in chemistry between antibiotics and biocides. Chemical methods of biocides are not specific to a particular biochemical pathway, but instead can act on multiple structural and functional compartments of bacteria, thereby causing disruption of cell walls, cell membranes, proteins, and nucleic acids. These mechanisms undermine the fundamental drivers of the tertiary and quaternary structures of biological molecules, which explains their significant disruption of bacterial pathways. Therefore, the emergence of biocide resistance is unlikely to be caused by specific changes in the target site or by overproduction of the target site to

overcome the effect of the biocide, as is the case in antibiotic resistance [40].

Today *P.aeruginosa* have developed resistance to multiple antimicrobial agents, with some strains expressing resistance to all antimicrobial compounds. Additionally, these organisms have been reported to contaminate disinfectants in hospitals or other such environments, thereby compromising the ability of the disinfectant to reduce or eliminate bacterial contamination these strains are very common environmental bacteria growing in water, soil, and drainage, and they are critical nosocomial pathogens due to the recent increase in their isolation from various clinical specimens [41].

Chlorhexidine digluconate is an antiseptic and antiseptic and is also considered a preservative. It is a biocide and has a higher antibacterial activity against Gram-positive bacteria compared to Gram-negative bacteria [42]. This biocide is used in mouth disinfection, hand wash, and other health solutions. The antibacterial mechanism of chlorhexidine digluconate lies through the bacterial cell membrane [43]. However, *P.aeruginosa* is intrinsically resistant to this biocide [42].

The mechanism of (BAC) depend of attack the cell wall of *p.aeruginosa* , but the presence of the contents of phospholipids (PL) and fatty and neutral lipids (FNL) in the cell walls act as barrier prevent the crossing of (BAC) to inside of cell, there for the high content of these lipids (PL) and (FNL) due to increase the resistance of *P.aeruginosa* to the Benzalkonium chloride and vice versa. The history of the use of biocides goes back to a history of initial effectiveness, which resulted in the emergence of resistance against these antibacterial. Antimicrobial resistance results from the capture and mobilization of genes that have their origins in multiple environmental sites. These environmental sites may provide the opportunity for the influx of resistance genes for disease-causing bacteria. Some of them do not acquire resistance successfully, and some acquire it from the treatment settings themselves, such as hospitals and the example of *P.aeruginosa* bacteria. These differences and similarities in resistance mechanisms and associated genes result from a complex interaction between gene acquisition and mobilization [44]. The action of Benzalkonium chloride in the cell is Interacts with cell membranes, leading to disruption of membrane integrity and leakage of cellular content, classed as Cationic quaternary ammonium compound. (Pietsch, Heidrich, Nordholt, & Schreiber, 2021)

While hydrogen peroxide (H_2O_2) is a topical biocide used to clean and remove chronic wound infections. Biofilms in clinical wounds may hinder the optimal efficacy of these biocides. Among the various biocides, H_2O_2 is generated as part of normal cellular inflammatory responses and is noteworthy for its potential properties in removing biofilms in wounds and stimulating wound healing [45]. One limitation of using H_2O_2 on wounds is that it is rapidly oxidized/reduced in wound environments, losing its activity over time. Therefore, continuous generation and delivery of H_2O_2 to wounds to reduce biofilms can be considered for optimal antibacterial effects [46].

Our results indicated that MIC & MBC values were become lower with synergist with (EDTA).

One possible reason is that EDTA has been documented to strengthen the action of antimicrobial agents through attaching to metals that contest with these agents for receptors on cell wall allowing them into bacterial cells. EDTA also interrupt the lipopolysaccharides organization of the gram negative bacterial outer membrane making it more penetrable to antibacterial agents[47]. Furthermore, EDTA has a straight bactericidal consequence on bacteria biofilm. EDTA is able to stop and decrease the threat of creation and establishment of bacterial biofilm because of its capacity to sequester cations. So, the mixture of biocides with EDTA clues to synergistic properties then could be valuable in avoiding appearance of resilient strains, decreasing nosocomial contagions, thus increase therapy effectiveness [23-48]. (Ríos-Castillo, González-Rivas, & Rodríguez-Jerez, 2017).

CONCLUSION

Combination of EDTA with the biocides [chlorhexidine digluconate (20%), benzalkonium chloride (50%), Formaldehyde (37%), cetrimide (20%), and Hydrogen peroxide H2O2 (30%)] could be an effective way to diminish infections by *P. aeruginosa.*

RECOMMENDATION

The synergism of biocides with EDAT will increase the effectiveness of compound against the microorganisms because it consider new form , takes long period to get the resistance of it or adaptive will be during more of mutations in gene of microorganisms.

REFERENCES

1. Kang, C.-I., et al., *Pseudomonas aeruginosa bacteremia: risk factors for mortality and influence of delayed receipt of effective antimicrobial therapy on clinical outcome.* Clinical infectious diseases, 2003. 37(6): p. 745-751.

2. Odumosu, B.T., B.A. Adeniyi, and R. Chandra, *Analysis of integrons and associated gene cassettes in clinical isolates of multidrug resistant Pseudomonas aeruginosafrom Southwest Nigeria.* Annals of clinical microbiology and antimicrobials, 2013. 12(1): p. 1-7.
3. Chen, W., et al., *CRISPR/Cas9-based genome editing in Pseudomonas aeruginosa and cytidine deaminase-mediated base editing in Pseudomonas species.* IScience, 2018. 6: p. 222-231.
4. Adhikari, S., et al., *Prevalence and antibiograms of uropathogens from the suspected cases of urinary tract infections in Bharatpur Hospital, Nepal.* Journal of College of Medical Sciences-Nepal, 2019. 15(4): p. 260-266.
5. Nagoba, B., et al., *Treatment of skin and soft tissue infections caused by Pseudomonas aeruginosa—A review of our experiences with citric acid over the past 20 years.* Wound Medicine, 2017. 19: p. 5-9.
6. Zeb, A., et al., *Antibiotic susceptibility pattrens of Pseudomonas aeruginosa in tertiary care hospital.* J Entomol Zool Stud, 2017. 20: p. 50.
7. Moradali, M.F., S. Ghods, and B.H. Rehm, *Pseudomonas aeruginosa lifestyle: a paradigm for adaptation, survival, and persistence.* Frontiers in cellular and infection microbiology, 2017. 7: p. 39.
8. Hasoon, Z.T.A.A., *Molecular Study of Flouroquinolon Genes among Multidrug Resistant Pseudomonas aeruginosa Isolates in Babylon Province*, in *Department ofBiology*. 2021, University of Babylon: Iraq.
9. Subedi, D., A.K. Vijay, and M. Willcox, *Study of disinfectant resistance genes in ocular isolates of Pseudomonas aeruginosa.* Antibiotics, 2018. 7(4): p. 88.
10. Elbadawi, H.S., et al., *Detection and characterization of carbapenem resistant Gram-negative bacilli isolates recovered from hospitalized patients at Soba University Hospital, Sudan.* BMC Microbiol, 2021. 21(1): p. 136.
11. de Abreu, P.M., et al., *Persistence of microbial communities including Pseudomonas aeruginosa in a hospital environment: a potential health hazard.* BMC Microbiol, 2014. 14: p. 118.
12. Firesbhat, A., et al., *Bacterial profile of high-touch surfaces, leftover drugs and antiseptics together with their antimicrobial susceptibility patterns at University of Gondar Comprehensive Specialized Hospital, Northwest Ethiopia.* BMC Microbiol, 2021. 21(1): p. 309.
13. Rutala, W.A. and D.J. Weber, *Guideline for disinfection and sterilization in healthcare facilities, 2008. Update: May 2019.* 2019.
14. Tezel, U. and S.G. Pavlostathis, *Quaternary ammonium disinfectants: microbial adaptation, degradation and ecology.* Curr Opin Biotechnol, 2015. 33: p. 296-304.
15. Gadea, R., et al., *Effects of exposure to quaternary-ammonium-based biocides on antimicrobial susceptibility and tolerance to physical stresses in bacteria from organic foods.* Food Microbiol, 2017. 63: p. 58-71.
16. Molina-González, D., et al., *Effect of sub-lethal concentrations of biocides on the susceptibility to antibiotics of multi-drug resistant Salmonella enterica strains.* Food Control, 2014. 40: p. 329-334.
17. Capita, R., et al., *Exposure of Escherichia coli ATCC 12806 to sublethal concentrations of food-grade biocides influences its ability to form biofilm, resistance to antimicrobials, and ultrastructure.* Appl Environ Microbiol, 2014. 80(4): p. 1268-80.
18. Liu, F., et al., *Tetrasodium EDTA Is Effective at Eradicating Biofilms Formed by Clinically Relevant Microorganisms from Patients' Central Venous Catheters.* 2018. 3(6).
19. Lam, N.Y., et al., *EDTA is a better anticoagulant than heparin or citrate for delayed blood processing for plasma DNA analysis.* Clinical chemistry, 2004. 50(1): p. 256-257.
20. Finnegan, S. and S.L. Percival, *EDTA: An Antimicrobial and Antibiofilm Agent for Use in Wound Care.* Adv Wound Care (New Rochelle), 2015. 4(7): p. 415-421.
21. Hogan, S., et al., *In Vitro Approach for Identification of the Most Effective Agents for Antimicrobial Lock Therapy in the Treatment of Intravascular Catheter-Related Infections Caused by Staphylococcus aureus.* Antimicrob Agents Chemother, 2016. 60(5): p. 2923-31.
22. Brown, M.R. and R.M. Richards, *Effect of ethylenediamine tetraacetate on the resistance of Pseudomonas aeruginosa to antibacterial agents.* Nature, 1965. 207(5004): p. 1391-3.
23. Lambert, R.J., G.W. Hanlon, and S.P. Denyer, *The synergistic effect of EDTA/antimicrobial combinations on Pseudomonas aeruginosa.* J Appl Microbiol, 2004. 96(2): p. 244-53.
24. Muslim, H.B. and A.G.M. Al-Saadi, *The Prevalence of some Heavy Metal Resistance Genes in Pseudomonas aeruginosa Isolated from Hospital Environments.* IOP Conference Series: Earth and Environmental Science, 2023. 1262(2): p. 022006.
25. Popęda, M., E. Płuciennik, and A.K. Bednarek, *[Proteins in cancer multidrug resistance].* Postepy Hig Med Dosw (Online), 2014. 68: p. 616-32.
26. Tomasz Swebocki, A.B., Aleksandra Maria Kocot , magdalena.plotka , rabah.boukherroub *Minimum Inhibitory Concentration (MIC) and Minimum Bactericidal Concentration (MBC) Assays Using Broth Microdilution Method.*2023.
27. Wiegand, I., K. Hilpert, and R.E. Hancock, *Agar and broth dilution methods to determine the minimal inhibitory concentration (MIC) of*

antimicrobial substances. Nature protocols, 2008. 3(2): p. 163-175.
28. Tankeshwar, A., *Broth Dilution Method for MIC Determination. Bacteriology. microbe online.* 2022.
29. Horváth, G., et al., *Chapter 12 - Sensitivity of ESBL-Producing Gram-Negative Bacteria to Essential Oils, Plant Extracts, and Their Isolated Compounds*, in *Antibiotic Resistance*, K. Kon and M. Rai, Editors. 2016, Academic Press. p. 239-269.
30. Namaki, M., et al., *Prevalence of resistance genes to biocides in antibiotic-resistant Pseudomonas aeruginosa clinical isolates.* Molecular Biology Reports, 2022. 49(3): p. 2149-2155.
31. Parvekar, P., et al., *The minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) of silver nanoparticles against Staphylococcus aureus.* Biomater Investig Dent, 2020. 7(1): p. 105-109.
32. Lebeaux, D., et al., *In vitro activity of gentamicin, vancomycin or amikacin combined with EDTA or l-arginine as lock therapy against a wide spectrum of biofilm-forming clinical strains isolated from catheter-related infections.* J Antimicrob Chemother, 2015. 70(6): p. 1704-12.
33. Dapgh, A., et al., *Study of Some Disinfectants Efficacy on Aeromonas Hydrophila Recovered from Local Animal and Water Sources.* 2019: p. 41-47.
34. Al-Bayati, S.S., et al., *Isolation and identification of Pseudomonas aeruginosa from clinical samples.* Biochemical and Cellular Archives, 2021. 21: p. 3931-3935.
35. Ekizoğlu, M., et al., *An investigation of the bactericidal activity of chlorhexidine digluconateagainst multidrug-resistant hospital isolates.* Turk J Med Sci, 2016. 46(3): p. 903-9.
36. Namaki, M., et al., *Prevalence of resistance genes to biocides in antibiotic-resistant Pseudomonas aeruginosa clinical isolates.* 2022. 49(3): p. 2149-2155.
37. Vijayakumar, R., et al., *Determination of Minimum inhibitory concentrations of Common Biocides to Multidrug-Resistant Gram-negative bacteria.* Applied Medical Research, 2016. 2: p. 56-62.
38. Prevention(CDC), C.f.D.C.a., *Chemical Disinfectants-Guideline for Disinfection and Sterilization in Healthcare Facilities. Available online: https://www.cdc.gov / infection control /guidelines /disinfection /disinfection - methods/ chemical.html.* 2023.
39. Goudarzi, M. and M. Navidinia, *Overview perspective of bacterial strategies of resistance to biocides and antibiotics.* Archives of Clinical Infectious Diseases, 2019. 14(2).
40. Capriotti, K. and J.A. Capriotti, *Topical iodophor preparations: chemistry, microbiology, and clinical utility.* Dermatol Online J, 2012. 18(11): p. 1.
41. Vijayakumar, R., et al., *Determination of minimum inhibitory concentrations of common biocides to multidrug-resistant gram-negative bacteria.* Appl Med Res, 2016. 2(3): p. 56.
42. Thomas, L., et al., *Development of resistance to chlorhexidine diacetate in Pseudomonas aeruginosa and the effect of a "residual" concentration.* J Hosp Infect, 2000. 46(4): p. 297-303.
43. Chapman, J., *Biocide resistance mechanisms.* International Biodeterioration & Biodegradation, 2003. 51: p. 133-138.
44. Wright, G.D., *Environmental and clinical antibiotic resistomes, same only different.* Curr Opin Microbiol, 2019. 51: p. 57-63.
45. Schreml, S., et al., *A new star on the H2O2rizon of wound healing?* Experimental dermatology, 2011. 20(3): p. 229-231.
46. Raval, Y.S., et al., *In vitro antibacterial activity of hydrogen peroxide and hypochlorous acid, including that generated by electrochemical scaffolds.* Antimicrobial Agents and Chemotherapy, 2021. 65(5): p. 10.1128/aac. 01966-20.
47. Lambert, R.J., et al., *Theory of antimicrobial combinations: biocide mixtures - synergy or addition?* J Appl Microbiol, 2003. 94(4): p. 747-59.
48. Bakht, M., et al., *Phenotype and genetic determination of resistance to common disinfectants among biofilm-producing and non-producing Pseudomonas aeruginosa strains from clinical specimens in Iran.* BMC microbiology, 2022. 22(1): p. 124.