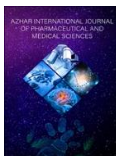

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LasR and NdvB genes of *Pseudomonas aeruginosa* posed resistance against different antibiotic concentration and develop anti-biofilm properties against certain disinfectants level

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Abstract: Antibiotic resistance has become one of the most common problems that threaten the world and increases the mortality rate. *Pseudomonas aeruginosa* is reported to be one of commonest multi-drug resistant (MDR), which is responsible for 10 to 15% of nosocomial infection worldwide and high death rates ranging from 18 to 61%. We investigate the anti-biofilm activity of different disinfectants on MDR *Pseudomonas aeruginosa*. Two hundred two water environmental isolates were collected from different hospitals in Cairo, Egypt. Microbact™ Gram-negative system used for identification. That is showed 41.3% of isolates were *Pseudomonas aeruginosa*. Antibiotic susceptibility test revealed that 34.7% of the isolates were MDR. Biofilm production was determined by Congo red assay (CRA) and Microtitre plate (MTP) method. CRA showed 89% as biofilm producers. MTP method showed 87% were biofilm-forming. MIC of Carbapenems was determined by the broth macrodilution method. It showed that 50% of isolates were resistant. (MIC) Minimum Inhibitory Concentration of 7 disinfectants against 10-selected MDR strong biofilm *Pseudomonas isolates* was determined using the broth microdilution method. It showed that the most effective disinfectants with the lowest MICs were the Sodium hypochlorite 5% and Povidone-Iodine 10%. The Real-time PCR was done on lasR and ndvb genes for the selected isolate (E20) before and after exposure to both effective disinfectants. The sample E20 showed a significant down regulation for lasR and ndvb genes with both effective disinfectants. Our study showed that Povidone-iodine 10 % at appropriate concentrations at less than 30 minutes has significant anti-biofilm activity against MDR *Pseudomonas aeruginosa*.

Keywords: *Pseudomonas aeruginosa;* biofilm; antibiotic sensitivity; Sodium hypochlorite; Povidone-iodine; Real-time RT-PCR.

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1. INTRODUCTION

Nosocomial infections are a main health obstacle in the world, mainly in the developing countries [(1)]. *Pseudomonas aeruginosa (P. aeruginosa)* is gram-negative organism that causes hospital acquired infections globally [(2)]. Biofilm that is formed by *P.aeruginosa* increases health problems and death rate [(3)]. Biofilms are a group of microorganisms in an organic matrix that adheres to inert or living surfaces. The extracellular matrix appears to promote attachment and confer resistance by inhibiting antibody coating, phagocytosis, and intracellular leukocyte killing [(4)]. Biofilms are a highly organized and structured bacterial community whose formation is closely linked to the quorum sensing system [(5)]. Bacterial biofilms are responsible for about 80% [(6)] of all chronic human infections and 65% of all microbial infections [(7)]. However, biofilm-growing pathogens are much more resistant to antibacterial agents than plankton cells [(8)]. Antibiotic resistance is the ability of bacteria to grow in the anti-reproductive or bactericidal conditions of antibiotics. Antimicrobial resistance is the ability of

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a bacteria against the antagonizing effect of an antibacterial agent upon reproduction prevention or bactericidal. The development of resistance to antibiotics in bacteria often develop as a result of unnecessary and inappropriate use of antibiotic [9]. Bacterial resistance can be genotypically confirmed by demonstrating the existence of specific antibacterial resistance genes or resistance mechanisms using genetic methods [10]. MDR *P.aeruginosa*, which grows on biofilms, may be ten thousand times more resistant and resistant to antibiotics than its floating counterparts [11]. Carbapenem antibiotics are available and routinely prescribed by physicians in the (ICU) intensive care unit. In this case, the regional policy of mixing these 3 drugs (imipenem, meropenem, and doripenem) are used [12]. Carbapenem resistance can be due to the production of enzymes such as AmpC and metallolactamase, overexpression of excretion pumps, porin deficiency, or altered target sites [13]. Disinfectants are broad-spectrum antimicrobials used to prevent the transmission of organisms from infected surfaces and medical elements to patients [7]. Sodium hypochlorite disinfectant kills microbial cells in the biofilm by invading proteins in the biofilm matrix and inhibiting the main functional enzymes of the microbes [7]. Povidone iodine is known as a preservative and disinfectant with broad-spectrum antibacterial activity [14]. Povidone iodine has anti-biofilm activity because it can penetrate biofilms. Related lack of resistance, low cytotoxicity, destruction of bacterial cytoplasm and nuclear structure, and damage to bacterial cell walls [15].

2. METHODS

2.1. Sample Collection, Growth Conditions and Identification

During the period from May 2017 to March 2018, allover 202 environmental isolates were collected from water samples (tanks, filters & water circulations) from governmental hospitals in Cairo, Egypt. Samples were gathered using clean swabs and were grown on cetrimide agar media and were incubated aerobically at 37°C for 24 hours. The isolates were identified by their green color on agar; they are Gram-negative rods, motile, oxidase-positive and citrate-positive and by using the Microbact™ Gram-negative system for identification. The Microbact system was implemented in compliance with the manufacturer's protocol (Oxoid, UK). *P. aeruginosa* (ATCC 12924) standard strain was used as a positive control.

2.2. Antibiotic Susceptibility Testing

Antibiotic susceptibility of 75 *pseudomonas* isolates was determined by using Kirby Bauer Disk diffusion method [16] according to CLSI, Sterilized Mueller-Hinton agar (MHA) plates were uniformly inoculated with the standardized bacterial suspension using sterile cotton swabs, and then the plates were allowed to dry for 5 minutes then incubated at 37°C for 24 h. After incubation, the antibiotic inhibition zone diameters were measured. Antibiotics were selected according to **Table (S1).** And the same procedure was applied after exposure to the selected disinfectants.

2.3. In vitro Biofilm Formation Study

Isolates were screened parallel to *p. aeruginosa* (ATCC 12924) for biofilm formation by CRA and MTP methods.

2.3.1. Congo Red Agar Method (CRA)

Consisted of (BHI) brain heart infusion (37 gram), agar (10 gram), sucrose (5 gram), & Congo red stain (0.8 gm) / litter distilled water [17]. The dyes were prepared in sterilized technique. When the dye cooled, it was added to sterile BHI agar. Then the samples were streaked in the plates and incubated overnight at 37°C. Then the bacterial colony color is identified [18]. The black colonies are positive while the red colonies are negative [19].

2.3.2. Microtitre Plate Assay (MTP)

Formation of biofilm in 96 well microtitre plates containing Luria Bertani (LB) broth media. Briefly, the overnight broth cultures of the isolated bacterial strains were calibrated in to 0.5 McFarland turbidity standard diluted to 1:100 in LB broth, and inoculated into a microtitre plate (200 µL per well). The microtitre plate was incubated overnight at 37 ºC. Negative control wells were also included. Following incubation, cells were dumped out by turning the plate over and washed with 200-µL sterile phosphate buffer (pH- 7.2) three times. 200 µL of 0.1% of crystal violet (CV) was added to each well for 15 min at room temperature. Soaking in distilled water washed the excessive dye away. Then 200 µL of 33% acetic acid was added to each well for 10–15 min to solubilize the CV. The plate was covered and incubated at room temperature for about 30 minutes. Then, the assessment of biofilm production was categorized according to the criteria of *Stepanović* [20] as follows: ODc was defined as

three standard deviations (SDc) above the mean OD of the negative control. Absorbance was recorded in a microplate reader at 595 nm using acetic acid as a blank. Each growing strain was tested three times, and the mean was obtained. The biofilm forming ability was categorized into four classes based on OD595 values of the isolates and control (OD control) as follows: OD ≤ OD control: Not a biofilm producer, OD control < OD ≤ 2ODcontrol: Weak biofilm producer, 2ODcontrol < OD ≤ 4ODcontrol: Moderate biofilm producer, 4ODcontrol < OD: Strong biofilm producer [21].

2.4. Disinfectants

Different types of disinfectants were used as in table (**S1**).

2.4.1. Determination of MICs of Disinfectants

The bacterial lethal concentration of the tested disinfectants were determined through the serial dilution method [22]. The inoculum was adjusted to 0.5 McFarland standards. A reference strain of *Pseudomonas aeruginosa* (ATCC 12924) was used for quality control and all disinfectants were freshly prepared prior to testing. Briefly, for each disinfectant, a series of 12 test tubes were prepared using one mL sterile (TSB) Tryptone Soya broth medium in each tube, with the exception of tube 1. The two -fold serial dilutions were used with positive and negative controls. One millimeter of *Pseudomonas aeruginosa* suspension was added to all flasks except flask number 11. Incubation at optimum temperature was designed for 24 and 48 hours. The MIC is the concentration of the tube containing the highly diluted tube where bacterial growth did not occur.

2.5. Contact Time (killing time assay)

The procedure was performed with minor changes [23]. To 0.5 McFarland bacterial suspensions, 4.75 ml of disinfectant and various dilutions of deionized water were added. Deionized water is used as a positive control as it contains only the organism & free of disinfectant. All tubes were incubated at 37°C for 0.5, 1, 5, 10, 15, 20, 30, 45, 60, 90 and 120 minutes. After each time exposure, take a loop full of suspension containing disinfectant and control, subculture on nutrient agar medium, 24 hr. After incubation at 37°C, colonies were counted. The endpoint was calculated as the minimum exposure time to various dilutions of the inoculated disinfectant, indicating the absence of viable bacteria.

2.6. Determination of MICs of Carbapenems

The MIC can be identified by the least concentration of antibiotic that can kill the organism [24] .

2.6.1. Broth macrodilution method:

The MIC was determined according to **CLSI guidelines (2017)**. Broth macrodilution is one of the main methods of antibiotic susceptibility testing. In this method, a two-fold dilution of the antimicrobial agent is prepared in (MHB) Muller hinton broth dispensed into a tube with a minimum volume of 2 mL. Then, each tube is inoculated with a microbial inoculum prepared in the same medium after dilution of standardized microbial suspension adjusted to 0.5 McFarland scale. After well mixing, the inoculated tubes are incubated at 37ºC for 24 hours.

MICs for carbapenems by broth macrodilution method was performed after exposure to both disinfectant Sodium hypochlorite 5% and Povidone-iodine 10% at a specific contact time as we calculated.

2.7. Molecular Identification of Selected *Pseudomonas* isolate:

All genetic tests were performed by sigma Scientific Service Technical Support.
Sigma Scientific Services Co, Head Scientific Office: Lebon building- La Cite mall - El Hossary - 6 of October, Cairo – Egypt.

Isolate (E 20), which produces the strongest antimicrobial resistance and the strongest biofilm, was identified by molecular techniques 16S rRNA amplification and sequencing: The culture medium of the isolate was centrifuged for 15 minutes. Then the Pellet was used for bacterial DNA extraction.

2.8. Real Time PCR for Quantification of Genes Expression

It was used to detect the expression of biofilm-forming-specific antibiotic-resistant genes in environmental sample E20 in the existence and removal of selected disinfectants. Real-time PCR was conducted using SYBR green kits (SensiFAST SYBR No-ROX Kit, Meridian Life science, UK). The primers were shown in **Table (1)**.

Table 1. List of primers sequences used for Quantitative Real-time PCR.

Gene	Primer direction	Sequence 5´- 3 ´	Amplicon size (bp)	Reference
16S RNA	Forward	CCTACGGGAGGCAGCAG	193	[25]
	Reverse	ATTACCGCGGCTGCTGGCA		
LasR	Forward	CTGTGGATGCTCAAGGACTAC	133	[26]
	Reverse	AACTGGTCTTGCCGATGG		
ndvB	Forward	GGCCTGAACATCTTCTTCACC	157	[27]
	Reverse	GATCTTGCCGACCTTGAAGAC		

2.9. Statistical Analysis

All experiments were performed as triplicates and the results are expressed as the mean values and standard deviations. The difference between untreated and treated bacteria with chemicals was analyzed using one-way ANOVA and Tukey´s posthoc test by GraphPad Prism 8 program and only results at $P < 0.05$ were considered significant.

3. RESULTS

3.1. Bacterial Isolation and Identification

A total of 202 water isolates collected from 7 governmental hospitals in Cairo, Egypt. Seventy-five isolates were Positive *pseudomonas* spp. All isolates were detected by Microbact™ Gram-negative system that is a standardized micro-substrate system designed to simulate conventional biochemical substrates used for the identification of *Enterobacteriaceae* and common miscellaneous Gram-negative bacilli (MGNB) [28]. It revealed that out of 75 isolates there were 31 isolates (41.33%) *P. aeruginosa*, 20 isolates (26.67%) *Pseudomonas putida,* 16 isolates (21.33%) *Pseudomonas stutzeri,* 8 isolates (10.67%) *Pseudomonas fluorescence,* as shown in **table (2)**.

Table 2. Distribution of isolated *Pseudomonas* spp.

Distribution of isolated *Pseudomonas* spp.	
Pseudomonas spp.	No of isolates and percent
Pseudomonas aeruginosa	31 (41.33%)
Pseudomonas putida	20 (26.67%)
Pseudomonas stutzeri	16 (21.33%)
Pseudomonas fluorescence,	8 (10.67%)

3.2. Antibiotic susceptibility testing

The results are shown in **table (3)**, **Figure (1),** 26 (34.7%) of the isolates were MDR. *Pseudomonas* isolates showed relatively high resistance against Minocycline and Cefepime with 80% and 48% respectively. On the other hand, these isolates showed sensitivity to Colistin, Tobramycin and Polymyxin-B with 86.67%, 82.66% and 84% respectively.

Table 3. Susceptibility of the tested *Pseudomonas* spp. isolates to different antibiotics.

Antibiotics	Sensitive S (%)	Resistant R (%)	Intermediate I (%)
Amikacin	56 (74.67)	15 (20)	4 (5.33)
Aztreonam	47 (62.67)	10 (13.33)	18 (24)
Polymyxin-B	63 (84)	12 (16)	0
Cefepime	30 (40)	36 (48)	9 (12)
Ceftazidime	39 (52)	25 (33.3)	11 (14.7)
Ciprofloxacin	50 (66.7)	22 (29.3)	3(4)
Colistin	65 (86.67)	10 (13.33)	0
Gentamicin	58 (77.33)	14 (18.67)	3(4)
Imipenem	50 (66.7)	22 (29.3)	3(4)
Levofloxacin	54 (72)	11 (14.67)	10 (13.3)
Minocycline	5 (6.7)	60 (80)	10 (13.3)
Meropenem	60 (80)	25 (17.33)	2 (2.67)
Piperacillin/Tazobactam	41 (54.67)	11 (14.67)	23 (30.66)
Ticarcillin/Clavulanic acid	15 (20)	28 (37.33)	32 (42.67)
Tobramycin	62 (82.66)	11 (14.67)	2 (2.67)

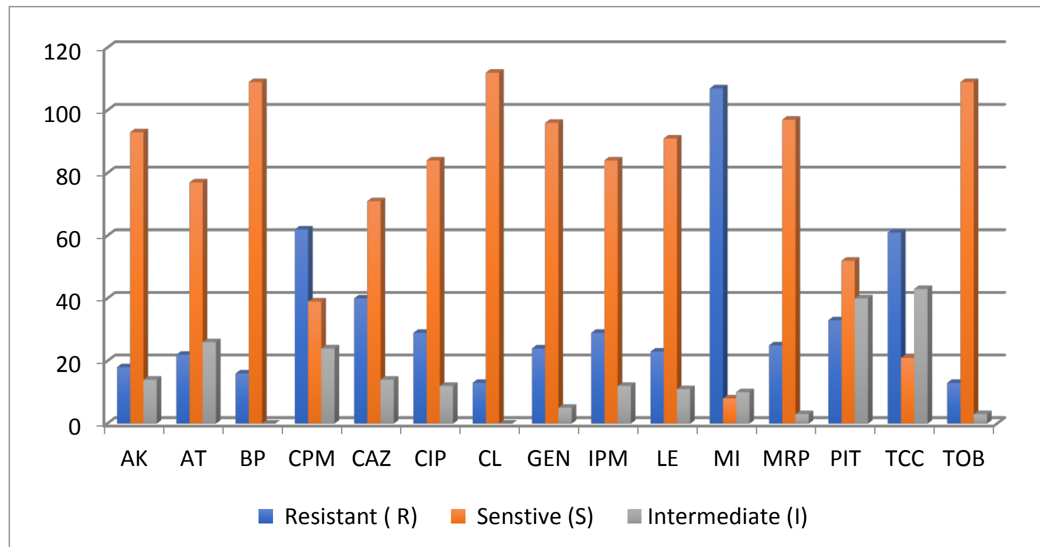


Figure 1. The antibiotic susceptibility of *Pseudomonas* spp. to antibiotics.
(R) Resistant; (I) Intermediate; (S) Sensitive; AK (Amikacin), AT (Aztreonam), BP (Polymyxin-B), CPM (Cefepime), CAZ (Ceftazidime), CIP (Ciprofloxacin), CL (Colistin), GEN (Gentamicin), IPM (Imipenem), MRP (Meropenem), LE (Levofloxacin), PIT (Piperacillin / Tazobactam), TCC (Ticarcillin / Clavulanic acid), TOP (Tobramycin).

3.3. Biofilm Production

3.3.1. Congo-Red Assay

Sixty-seven isolates (89%) biofilm formation and only 8 isolates (11%) as non-biofilm formation.

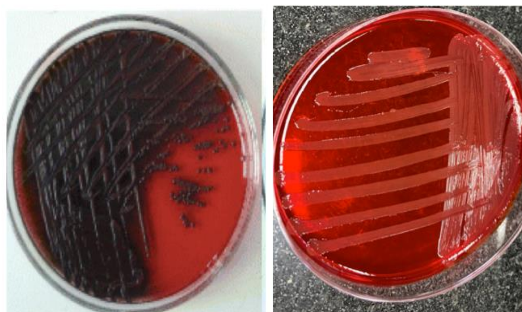


Figure 2. Plates showing biofilm formation on Congo red agar (A): strong biofilm forming *Pseudomonas* isolate while (B) non-biofilm forming *Pseudomonas* isolate.

3.3.2. Microtiter Plate (MTP) / Crystal Violet Assay

Seventy-five isolates were classified into 15 strong isolates (20%), 26 moderate isolates (35%), 24 weak isolates (32%) and 10 isolates (13%) couldn't form any detectable biofilm **(Figure 3).**

3.4. MICs of disinfectants against selected MDR strong biofilm producer isolates

All isolates were sensitive for formalin and resistant to Chlorhexidine, cetrimide (Savlon). MIC% for Sodium hypochlorite 5% ranging from 0.3215 to 0.625 %, Povidone-Iodine 10% ranging from 0.625% to 1.25%, Phenol ranging from 1.25 to 2.5 %, Ethyl alcohol ranging from 4.375 to 8.75%, and Chloroxylenol ranging from 0.3 to 0.6% as in **Table (4).**

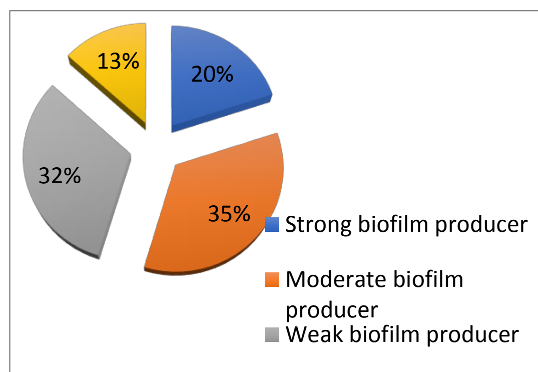


Figure 3. A pie chart showing percent of biofilm formation among *Pseudomonas* isolates by MTP method.

Table 4. MICs of disinfectants against MDR strong biofilm producer isolates.

Isolate`s code No	MICs of disinfectant (%)						
	Sodium hypochlorite	Povidone-Iodine	Phenol	Ethyl alcohol	Choroxylenol	Formalin	Savlon
ATCC	0.625	1.25	2.5	8.75	0.6	S	R
E 20	0.625	0.625%	1.25	R	0.6	S	R
E 40	0.625	1.25	1.25	4.375	0.6	S	R
E 45	0.625	1.25	1.25	8.75	0.3	S	R
E 58	0.3215	1.25	1.25	4.375	0.3	S	R
E 64	0.3215	1.25	1.25	4.375	0.3	S	R
E 72	0.625	1.25	1.25	4.375	0.3	S	R
E 74	0.625	1.25	2.5	8.75	0.6	S	R
E 76	0.3215	1.25	1.25	4.375	0.3	S	R
E 78	0.3215	1.25	1.25	4.375	0.3	S	R

3.5. Contact Time

MDR strong biofilm isolates were tested, and experiments for contact time using for the lowest MICs disinfectants that were sodium hypochlorite 5% and Povidone-iodine 10% on ten isolates **table (5).** Each bacterial isolate was completely eradicated by sodium hypochlorite 5% and Povidone-iodine 10% at specific time points tested. So, the selected isolate showing MDR strong biofilm producers and had the lowest contact time with the most two effective disinfectants was E 20.

3.6. MIC of Carbapenems

MIC of Carbapenems on ten MDR strong biofilm *pseudomonas* isolates showed by the naked eye that 5 (50%) of samples were resistant to both, 7 (70%) samples were resistant to Imipenem, and 5 (50%) isolates were resistant to Meropenem **as** in **table (6)**.

3.7. Antibiotic Susceptibility Changes After Exposure to Sodium Hypochlorite 5 % and Povidone-iodine 10 %

Susceptibility of *P. aeruginosa* (E20) isolate after exposure **table (7).** after exposure to Povidone-iodine 10% it converted from resistant to sensitive with these antibiotics Levofloxacin, Amikacin, Meropenem, Imipenem, Gentamicin, Tobramycin, Ciprofloxacin, and Ceftazidime. While after exposure to Sodium hypochlorite 5% converted from sensitive to resistant with Polymyxin-B and Colistin.

3.8. Determination of Biofilm Formation After Exposure to Sodium hypochlorite 5 % and Povidone-iodine 10%

There is no detectable biofilm formation by Congo red method **figure (4)** of the selected *pseudomonas* isolate E20 after exposure to both effective disinfectants at a specific contact time.

Table 5. Contact time of disinfectants on selected *Pseudomonas* isolates.

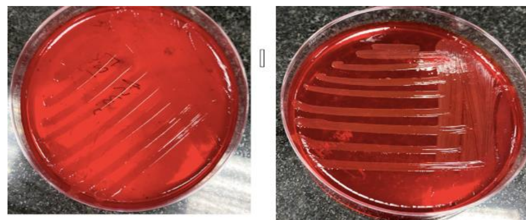
Isolate`s code No	Contact time (Minutes)	Contact time (Minutes)
	Sodium hypochlorite 5%	Povidone-iodine 10%
ATCC	45	60
E 20	10	30
E 40	45	90
E 45	90	60
E 58	30	120
E 64	20	90
E 72	10	90
E 74	60	45
E 76	40	40
E 78	60	90

Table 6. MICs of Carbapenems.

Isolate`s code No	**Imipenem (µg/ mL)**		**Meropenem (µg/ mL)**	
E 20	Turbid	R	R	16
ATCC	16	R	S	< 0.25
E 40	64	R	R	32
E 45	256	R	S	< 0.25
E 58	< 0.25	S	S	< 0.25
E 64	< 0.25	S	S	< 0.25
E 72	128	R	R	16
E 74	32	R	R	32
E 76	< 0.25	S	S	2
E 78	64	R	R	64

Table 7. Antibiotic Susceptibility of sample E 20 after exposure to both effective disinfectants.

After exposure to Povidone iodine10%, converted from resistant to be sensitive	After exposure to Sodium hypochlorite 5%, converted from sensitive to be resistant
Levofloxacin	Polymyxin-B
Amikacin	Colistin
Meropenem	" NA"
Imipenem	" NA"



(A) **(B)**

Figure 4. Biofilm formation on CRA after exposure to (A) Sodium hypochlorite 5% (B) Povidone-iodine 10%.

3.9. Determination of MIC of Carbapenems after exposure to Sodium hypochlorite 5% and Povidone-iodine 10%

MICs of Imipenem and Meropenem after exposure to Sodium hypochlorite 5% and Povidone-iodine 10% showing **table (8)** MICs of Meropenem for the isolate E20 after exposure to Sodium hypochlorite 5% and Povidone-iodine 10% converted to be sensitive.

Table 8. Determination of MIC of Carbapenems against Sodium hypochlorite and Povidone-iodine.

	Before exposure		After adding Sodium hypochlorite		After adding Povidone-iodine	
	Antibiotics (µg/ml)					
Isolate	**IPM**	**MRP**	**IPM**	**MRP**	**IPM**	**MRP**
E 20	R	R	S	S	S	S

IPM: Imipenem, MRP: Meropenem, (R) Resistant; (S) Sensitive.

3.10. Molecular Identification of Selected *Pseudomonas* Isolate

The 16S rRNA gene sequences were compared with the NCBI GenBank database by the Blast tool in order to identify the isolate E 20 with accession number MZ436810 For E 20.

3.11. Effect of Sodium hypochlorite 5% and Povidone-iodine 10% on Genes of Biofilm Formation (*lasR*) and Antibiotics Resistance (*ndvb*) of *P. aeruginosa* E20

The expression of *lasR* and *ndvB* in *P. aeruginosa* isolates were investigated using real-time PCR referring to the housekeeping gene (16S rRNA). The reaction was performed using MJ Mini (Biorad, Singapore).

For the environmental *P. aeruginosa* PAO1 (E 20), the expression levels of *lasR*, and *ndvb* genes in

Sodium hypochlorite 5% treated environmental *P. aeruginosa PAO1* (E 20) was downregulated by 66.8%, 55.6%, respectively, compared to untreated control **figure (5)**. The expression level of *lasR*, and *ndvb* genes in Povidone-iodine 10% treated environmental *P. aeruginosa* PAO1 (E 20) was downregulated by 97.8%, and 99.6%, respectively, relative to the housekeeping gene *16S RNA* of the same strain under the same conditions, compared to untreated control.

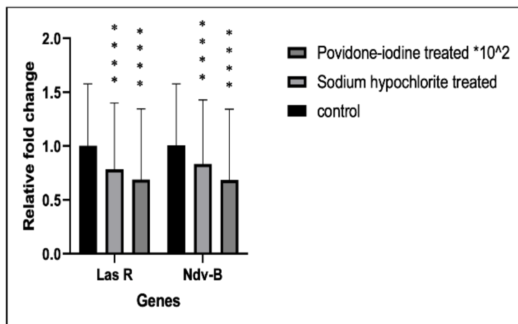


Figure 5. Effect of Sodium hypochlorite and Povidone-iodine on genes of the organism.

4. DISCUSSION

Hospital acquired infections are one of the biggest obstacles in the world. This is an important cause of high health problems, mortality rate, and financial problems globally [(29)]. *Pseudomonas* is one of main causes of hospital-acquired infections that threaten the lives of many patients each year [(30)]. Another factor that contributes to the etiology of *Pseudomonas aeruginosa* is its ability to form biofilms when adhered to surfaces [(31)]. Biofilms are a group of microbes gathered in a matrix of self-generated and foreign bodies that float or adhere to the surface. The extracellular matrix of biofilms is composed of secreted substances that function to gather bacterial cells [(32)]. The matrix helps in stability, protection, and resistance to antibiotics [(18)].

Treatment of *pseudomonas* infections has come to be a brilliant undertaking because of the capacity of this bacterium to face up to some of the presently to be had antibiotics from carbapenem to the third-era cephalosporin. Conventionally used antibiotics are lively in opposition to planktonic cells that reason acute infection; however frequently fail to absolutely get rid of biofilms main to chronic infections [(33)].

The purpose of this study was to determine the expression levels of the *Pseudomonas aeruginosa lasR* gene (biofilm formation) and *ndvb* gene (antibiotic sensitivity). After being exposed to disinfectants. Two hundred two environmental samples were collected from water samples (tanks and filters) from a governmental hospital in Cairo, Egypt. Bacterial isolates were isolated and identified using standard methods.

In the present study, we found that the Microbact™ Gram-negative system revealed that out of 75 isolates showed 31/75 (41.33%) *P. aeruginosa*. In agreement with our results, the study by Al Najim *et al* reported that 20/55 (36.36%) isolates belonged to *P. aeruginosa* [(34)]. Similar results were reported by Ghane *et al* who stated that the most prevalent of *Pseudomonas* spp. found were related to *P. aeruginosa*, followed by *P. stutzeri*, *P. putida* and *P. fluorescens* [(35)].

Environmental isolates were tested for susceptibility to various antibiotics using the disc diffusion method by measuring the diameter of the inhibition zone. The results obtained showed that 26 (34.7%) of the isolates had MDR. *Pseudomonas* isolates showed relatively high resistance to minocycline, cefepime and ceftazidime with 80%, 48% and 33%, respectively. Consistent with other study, there was a high resistance rate to cefepime [(36)]. The results of the current study are in similarity with previously published results where there was an increase in the resistance ratio of Ceftazideme (73%) to cefepime (61%) [(37, 38)]. Also consistent with the assessment of resistance to cefepime (39 %) and the identification of resistance to tobramycin (79%) and amikacin (65%) [(39)]. But this difference is because the ongoing development of multi drug resistant strains around the world. Meanwhile, this study showed that colistin, tobramycin, and polymyxin B were the most effective antibiotics against *Pseudomonas aeruginosa* at 86%, 82%, and 84%, respectively. Colistin and tobramycin are often considered first-line drugs for eradicating early-stage *Pseudomonas aeruginosa infections*, and tobramycin is also considered first-line drug for chronic treatment [(40)]. Mustafa and his colleagues agreed with our results that colistin is the most effective antibiotic against *Pseudomonas aeruginosa* is isolated with 92% susceptibility, followed by tobramycin, meropenem, and imipenem at 72%, 63%, and 48%, respectively [(41)].

In this study, the results obtained showed that 26 (34%) of isolates had MDR. The results are

consistent with the observations of 51 % and 58 % of *Pseudomonas aeruginosa* MDR strains [42-43]. The reasons behind such resistance are the inappropriate or irrational use of antibiotics, a mutation in the genome of *P. aeruginosa*, and environmental conditions of the specific area [44]. The biofilm associated with the *Pseudomonas* isolate was determined using two different methods, CRA and MTP. The MTP method was considered the gold standard for this study and was compared with data from CRM [45]. Therefore, our isolates were classified according to their ability to form biofilms. Our current study has shown that at least 87% of the isolates tested are biofilm producers. 15 (20%) are strong biofilm producers, 26 (35%) are medium biofilm producers, 24 (32%) are weak biofilm producers, and 10 isolates (13%) couldn't form any detectable biofilm.

Several studies have reported different rates of biofilm production with *Pseudomonas aeruginosa* isolates. A study conducted in Egypt reported that biofilm formation was detected in 91% of *Pseudomonas aeruginosa* isolates. (25 %) Strong, (40 %) Medium, (25 %) Weak, (8 %) non-biofilm producer [46]. Another study conducted in Egypt found that of the fifty *P. aeroginosa* isolates investigated, thirty-eight (76 %) were biofilm-forming, with a strong 30 %, depending on the strength of the biofilm. It was divided into medium 22% and weak 24% formed [47]. Consistent with our results, the study reported that biofilm phenotypes accounted for 83% (n = 67) and were classified into the following categories: 33% (n =27) produced moderate biofilms. 33% (n = 27) produced weak biofilms, while 16% percent (n = 13) of isolates were identified as non-biofilm producers [48].

In this study, the biofilm-forming ability of *Pseudomonas* isolates was investigated primarily using the Congo red assay. The Congo red method detected 67 isolates (89%) as biofilm producers and 8 isolates (11%) as non-biofilm producers. In this review, it was not possible to make a speculative distinction between high, medium and low biofilm-forming isolates. Another study Showed the potential for biofilm formation in 23 (44%) samples based on the Congo red agar test, and 49 (94%) samples varied using the microplate assay, reported showing levels of biofilm formation **[44]**. The highest percentage (40%) that produces a medium biofilm was *P. aeruginosa* followed by Strong biofilm producer (36%). All (n = 20) *P. aeruginosa* MDR isolates showed biofilm formation by MPA.

Carbapenem antibiotics are often reserved for the treatment of severe nosocomial infections caused by *Pseudomonas aeruginosa.* These hospital-acquired infections are often resistant to other classes of antibiotics [49]. Of the 5 isolates (50%) are resistant to both imipenem and meropenem, 7 isolates (70%) are resistant to imipenem, and 5 isolates (50%) are resistant to meropenem. The MIC of the Carbapenem was measured in 10 MDR strong biofilm *Pseudomonas* isolates using the broth macrodilution method, as recommended by CLSI 2017, 7 (70%) isolates were resistant to imipenem and 5 (50%) isolates were resistant to meropenem. Consistent with our findings Steward *et al*., reported 88 (77%) and 66 (57%) MICs of 114 *pseudomonas* strains tested against imipenem and meropenem, respectively [50], were the same for microdilution method and agar dilution. Also MIC observed in *P. aeruginosa* isolates was 43% to meropenem and 56% to imipenem [51]. The MICs of meropenem were lower than those of other beta-lactam agents may be explained in part by the greater stability of meropenem as compared with imipenem or ceftazidime against *pseudomonas* beta-lactamases [52-53]

Disinfectants can be used to disinfect environmental surfaces such as walls, floors and tables. These medical devices should be disinfected with instrument disinfectants [54]. In this study, all isolates were formalin-sensitive and resistant to chlorhexidine, cetrimide (Savlon). Sodium hypochlorite 5% MIC% is 0.3215 to 0.625%, povidone iodine 10% is 0.625% to 1.25%, phenol is 1.25 to 2.5%, ethyl alcohol is 4.375 to 8.75%, and chloroxylenol is 0.3 to 0.6%. In this study; MDR-resistant biofilm samples sub cultured at contact time were calculated with minor modifications [23]. Sodium hypochlorite 5% and Povidone iodine 10% were selected for their high potency in 10 isolates. Each bacterial isolate was completely eradicated by both effective disinfectants at a specific contact time. The lowest contact time for both disinfectants with isolate E20. Povidone iodine 10% was shown to be effective against *P.* aeruginosa; it is consistent with the high efficacy and broad spectrum of this disinfectant found [55], in killing *P. aeruginosa* isolated from wounds and burns. Studies conducted by Mitiku and Husain in Nigeria [56] and Ethiopia [57], respectively, have

reported similar results. Low dilution of Povidone iodine was effective against 100% of the drug-sensitive group and 76% of the MDR group. Clinical MDR *pseudomonas* showed a statistically significant MIC for Povidone iodine. The results of sodium hypochlorite disinfectant are consistent with the importance of using sodium hypochlorite (5.25%) as a powerful oxidant, which is a widespread disinfectant against bacteria [(58)]. In addition, the disinfectant susceptibility test of the study found that user dilution of sodium hypochlorite (0.5% concentration) had a fatal effect on 91% of *pseudomonas* that is isolated. MDR *Pseudomonas aeruginosa* showed a statistically significant MIC for Povidone iodine [(56)]. Contrary to our results, *Pseudomonas aeruginosa* is reported to be resistant to formalin and sensitive to savlon in all hospitals [(59-60)]. The study showed a 25% increase in disinfectant concentration, resulting in weaker early suppression of both disinfectants [(61)]. Ethyl alcohol as the weakest disinfectant compared to cetrimide C and Betadine [(62)]. Both susceptible and MDR isolates of *Pseudomonas aeruginosa* were inhibited by certain concentrations of Povidone iodine, savlon, phenol, and formalin [(36)]. But MDR *Pseudomonas aeruginosa* showed a statistically significant MIC for Povidone iodine and savlon ($p<0.05$). No significant difference was found in the MIC of formalin or phenol. A low formalin concentration of 0.018% effectively suppressed the growth of all susceptible *Pseudomonas aeruginosa* and MDR more than 60%. On the other hand, both *Pseudomonas aeruginosa* groups were inhibited at phenol concentrations below 1.25%. Both *Pseudomonas* aeruginosa groups had MIC values that exceeded user-defined sodium hypochlorite concentrations. MDR *P. aeruginosa* had statistically significant MICs to sodium hypochlorite.

In this study, some isolates were converted from susceptible to resistant and others showed opposition after exposure to both potent disinfectants. Microbial resistance to disinfectants could be mainly attributed to the accumulation of disinfectants the development of efflux mechanisms, and, rarely, changes in cells affected by mutations [(63)]. The continues use of disinfectants can lead to the formation of some nosocomial MDR microbes [(57)]. The study done by Mc Cay investigated the theory of adaptation to disinfectants that could develop antibiotic resistance [(64)]. Also, the development of resistance of *Pseudomonas aeruginosa* to antibiotics. This is due to the accommodation of organism with disinfectant [(65)].

In the current study, there is no detectable biofilm formation by the Congo-Red method of selected *Pseudomonas* isolates after exposure to both effective disinfectants at specific contact times.

A study by (Olszewska et al) showed great efficacy of chlorinated sanitizers against *Pseudomonas*. Mainly when other studies demonstrated that biofilm bacteria of *pseudomonas* are less affected by chlorine. The increase in resistance of *pseudomonas* biofilms cannot be explained by reduction of biocides in biofilms [(67)]. Lineback *et al*. illustrated that Sodium hypochlorite disinfectants were effective against *P. aeruginosa* biofilm, as it destroys both the matrix of biofilm and the bacteria cells [(7)]. The resistance of *Pseudomonas* biofilms to disinfectants was reported by many studies [(68-69)]. Therefore, further research is needed to elucidate the nature and physiology of the cell-forming community regarding the prevalence and composition of various materials, especially biofilms. Aboushleib *et al*. observed a positive correlation between biofilm formation and the presence of the *lasR* and *rhlI* genes, confirming the important role of QS in the pathogenicity of *Pseudomonas aeruginosa* [(47)]. Reducing pathogenicity with QS inhibitors is a strong action plan for infection, as the majority of virulence factors are known to be controlled by QS. Inhibition of QS can be achieved by interfering with acyl homoserine lactone (AHL) signaling, by inhibiting the propagation of AHL signaling, or by inhibiting AHL signaling due to binding to signal receptors [(71)].

This study investigated the effect of disinfectants on the expression of both the biofilm-forming gene (*lasR*) and the antibiotic resistance gene (*ndvb*). Treated with 5% sodium hypochlorite (test) and treated with 10% povidone iodine (test). The housekeeping gene 16SRNA was used as a normalizer. For the environment*al Pseudomonas aeruginosa* PAO1 (E20), the expression levels of the *lasR* and *ndvb* genes in sodium hypochlorite 5% were downregulated by 66.8% and 55,6%, respectively, compared to the untreated control. Expression levels of the *lasR* and *ndvb* genes at povidone iodine 10% treatment was downregulated by 97.8%, and 99.6%, respectively, compared to the untreated control, under the Same conditions. The downregulation of these *lasR* and

ndvb genes is due to the downregulation of the virulence factors of *Pseudomonas aeruginosa* PAO1 (pyocyanin, elastase, and protease) and inhibits bacterial attachment to the polypropylene surface [(72)]. Inhibition of *Pseudomonas* aeruginosa pyocyanin production reduces biofilm production in a dose-dependent manner, presumably by disrupting the Qs signaling system [(73)]. In agreement with our results, the downregulation of *lasR* and *ndvb* genes with Povidone iodine 10% is due to povidone a synthetic polymer that is a carrier of iodine (iodophor). A peculiar chemical property of this iodophor is that the concentration of free iodine, the active antimicrobial element, increases with the dilution of Povidone, due to weakening of the chemical bonding between iodine and povidone [(74)]

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5. CONCLUSIONS

P. aeruginosa is well known organism as a nosocomial pathogen environmental isolate. Resistance of *Pseudomonas* species to disinfectants was reported in several studies. Our results demonstrated that the tested isolates were sensitive to Colistin, and Tobramycin compared to other tested antibiotics. Five percent sodium hypochlorite and ten percent Povidone iodine are the most effective disinfectants against *Pseudomonas isolates* as they have antibacterial and anti-biofilm activity. It was approved that these disinfectants exhibited the anti-biofilm properties through the down regulating of the biofilm-forming gene (lasR) and the antibiotic resistance gene (ndvb). We concluded that these disinfectants are promising agent for eradication of environmental MDR *P. aeruginosa* especially in pharmaceutical industry.

Supplementary Materials:

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