Phenotypic and Genotypic Determination of Resistance to Common Disinfectants Among Strains of *Pseudomonas Aeruginosa* Producing and Non-Producing Biofilm Isolated From Iran

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Research Article

Keywords: Hospital disinfectants, disinfectants, Pseudomonas aeruginosa, Clinical isolates, sub-inhibitory concentrations, Antibiogram

Posted Date: October 1st, 2021

DOI: https://doi.org/10.21203/rs.3.rs-918248/v1

Abstract

Background: One of the most important reasons for human mortality worldwide is Hospital-acquired infections, which can be controlled by efficient use of proper disinfectants for the Hospital settings. The main aim of the present survey was to assess the susceptibility of *Pseudomonas aeruginosa* producing and non-producing biofilm isolated to the five commonly used Hospital disinfectants, and evaluation of the synergistic effect of selective disinfectants and Ethylene-diamine-tetra acetic acid (EDTA), and the effect of exposure to sub-inhibitory concentrations of Sodium hypochlorite on antimicrobial susceptibility test.

Results: The results showed that Sodium hypochlorite 5%, and Ethanol 70% is the most and less potent disinfectants against *Pseudomonas aeruginosa*, respectively. Clearly, the addition of EDTA increased the efficacy of selected disinfectants significantly. The changes in the antibiotic-resistance profiles after exposure to sub-inhibitory concentrations of disinfectants were observed for different classes of antibiotics. As well as near the all isolates harbored efflux pump genes and produced biofilm.

Conclusion: For disinfection of Hospital surfaces and instruments, the mixture of disinfectant and EDTA were the most suitable selection in this study. In our study, it was clear that exposure to sub-inhibitory concentrations of disinfectants results in resistance to antibiotics. Also, strong and intermediate biofilm formers belonged to MDR/XDR strains.

Background

Today, resistance to antibiotics and disinfectants in various bacterial strains is a major public health problem in the world, with increasing growth worldwide. Reports of this have generally been based on the detection of antibiotic resistance in bacteria associated with nosocomial infections, but in recent years with the identification of multidrug-resistant (MDR), strains in different countries, ways to spread and spread the relevant genes are important (1).

One of the most important factors in the spread of nosocomial infections is the incorrect use of disinfectants. These infections infect all medical devices, and equipment needed by patients, thereby result in Increasing the duration of Hospitalization. One of the basic principles of disinfection is the use of effective and safe disinfectant solutions to minimize damage to equipment and personnel. Also considering the fact that none of the disinfectants for all different needs disinfection are not suitable, and also because healthcare environments choice of material significance depends on the size of the problem, research is needed to determine the antiseptic effects of different disinfectants in order to select the appropriate disinfectant (2).

Improper use of disinfectants, dilution in the environment after discharge, and biodegradation result in biocide concentration gradients. As a result, microorganisms are alternately exposed to non-lethal concentrations of disinfectants. Recent studies have shown that, when bacteria are exposed to non-lethal concentrations of disinfectants, it facilitates resistance to disinfectants and may also lead to resistance to other antimicrobials, such as antibiotics (3, 4). While it has been shown that improper administration of antibiotics to humans and inappropriate use of antibiotics in animal feed has been a major factor in increasing antibiotic resistance in recent years. there is a growing concern that the widespread use of disinfectants has also been involved in antibiotics resistance (3, 5).

Pseudomonas aeruginosa is a non-fermentative gram negative bacilli. The bacterium is known to be an important human pathogen, especially for high-risk patients, including burn wounds, immunocompromised patients, and cystic fibrosis (6, 7). This bacterium is one of the most important bacteria in the clinic because of its ability that leads to severe infections and results in high mortality rates (8, 9). It is a member of the ESKAPE species described by the American Association of Infectious Diseases as a human pathogen Related to the emergence of clinical antibiotic Resistance (10). Also, *Pseudomonas aeruginosa* is one of the most important bacteria that the World Health Organization (WHO) named as antibiotic-resistant “priority pathogens” and have acquired and expanded different kinds of resistance mechanisms (11–13).

Multi-drug-resistant or Extensively drug-resistant (MDR, XDR) strains, in the nosocomial base, is a global threat to health care systems and vulnerable patients, and it has been reported to cause a large number of Hospital incidence in high-risk patients such as patients admitted to intensive care units (ICUs) (14).

Several factors cause resistance to disinfectants, and different bacteria have different mechanisms of resistance to disinfectants, and antibacterial agents. Among these, multidrug resistance proteins (SMR) are small proteins that are located in the inner layer of the cytoplasmic membrane and are divided into three groups: SUG, SMP, and PSMR. And they cause resistance to the biocides, and a number of antibiotics. The genes *qacE* and *qacEΔ1* are located in the SMP subgroup and have been identified on plasmids and integrons of many gram-negative and gram-positive bacteria resistant to drugs. *SUG* genes are also located on the plasmid. The SMR family includes proton-dependent efflux pumps (15). Also, one of the main resistance mechanisms in *Pseudomonas aeruginosa* is Biofilm formation (16). One of the purposes of the current study is to determine the correlation between antibiotic/detergent resistance and biofilm formation in *Pseudomonas aeruginosa*. treatment of *Pseudomonas aeruginosa* infections is challenging, due to the acquired and intrinsic resistance of *Pseudomonas aeruginosa* against a wide range of antibacterial agents (17, 18), biofilm formation, enzyme production suppression, and overexpression of efflux pumps, known as resistance mechanisms of this microorganism (19).

The present study identified phenotypic and genotypic resistance to common disinfectants in two steps: mixed or without Ethylene-diamine-tetra-acetic acid (EDTA). The disinfectants examined in the current survey are Sodium hypochlorite 5%, Ethanol alcohol 70%, Sayasept- HP 2%, Chlorhexidine 2%, Dettol 4.8% among *Pseudomonas aeruginosa*, strains producing and non-producing biofilm isolated from educational Hospitals in Iran.

Result

A total of 120 *Pseudomonas aeruginosa* strains related to nosocomial infections were collected. Out of 120 obtained isolates, 67 (55.8%) from urine, 11 (9.2%) of them BAL, and 28 (23.3%) ,14 (11.7%) of them were from blood, and wound respectively.

Antimicrobial susceptibilities

Base on susceptibility testing results, 46(38.3%) and 19(15.8%) isolated strains were categorized as MDR and XDR strain respectively (Table 2).

The highest resistance rate was against Cefoxitin, and Ampicillin/Sulbactam (100%) followed by Imipenem (45.8%), levofloxacin (33.3%). The highest susceptibility rate was related to Colistin 116(96.7%),

Piperacillin/Tazobactam 94(78.3%), Piperacillin 93 (77.5%), Tobramycin 93 (77.5%), and Gentamicin 91 (75.8%) respectively.

Table 1 shows the antibiotic susceptibility patterns of the 120 isolates of *Pseudomonas aeruginosa*. Comparative diagram of the results of antibiotics susceptibility tests before and after exposure to sodium hypochlorite was performed in Fig. 2.

Table 1 Antibiotic Susceptibility of clinical *Pseudomonas aeruginosa* isolates before and after treatment with Sodium hypochlorite.

	sensitive		intermediate		resistance	
	before	**after**	**before**	**after**	**before**	**after**
Antibiotic	N/%	N/%	N/%	N/%	N/%	N/%
Meropenem	78(65%)	65(54.2%)	8(6.7%)	15(12.5%)	34(28.3%)	40(33.3%)
Ciprofloxacin	71(59.2%)	59(49.2%)	10(8.3%)	17(14.2%)	39(32.5%)	44(36.7%)
Tobramycin	93(77.5%)	83(69.2%)	3(2.5%)	9(7.5%)	24(20%)	28(23.3%)
Gentamicin	91(75.8%)	81(67.5%)	6(5%)	11(9.2%)	23(19.2%)	28(23.3%)
Cefepime	85(70.8%)	76(63.3%)	14(11.7%)	18(15%)	21(17.5%)	26(21.7%)
Imipenem	25(20.8%)	16(13.3%)	40(33.3%)	40(33.3%)	55(45.8%)	64(53.3%)
Amikacin	89(74.2%)	82(68.3%)	11(9.2%)	12(10%)	20(16.7%)	26(21.7%)
Ceftazidime	66(55%)	60(50%)	26(217%)	28(23.3%)	28(23.3%)	32(26.7%)
Levofloxacin	50(41.7%)	44(36.7%)	30(25%)	33(27.5%)	40(33.3%)	43(35.8%)
Colistin	116(96.7%)	116(96.7%)	0(0%)	0(0%)	4(3.3%)	4(3.3%)
Piperacillin	93(77.5%)	93(77.5%)	14(11.7%)	14(11.7%)	13(10.8%)	13(10.8%)
Piperacillin/Tazobacta	94(78.3%)	94(78.3%)	14(11.7%)	14(11.7%)	12(10%)	12(10%)
Cefoxitin	0(0%)	0(0%)	0(0%)	0(0%)	120(100%)	120(100%)
Ampicillin Sulbactam	0(0%)	0(0%)	0(0%)	0(0%)	120(100%)	120(100%)
AST1	MDR=46(38.3%)		XDR=19(15.8%)			
AST2	MDR=58(48.3%)		XDR=23(19.2%)			

Note: AST1: AST before exposing to the Sodium hypochlorite, AST2: AST after exposing to the Sodium hypochlorite

The effect of exposure to sub-inhibitory concentrations of disinfectants on antimicrobial susceptibility test

Another important resistance link was revealed in the current study. The changes in the antibiotic-resistance profiles after exposure to sub-inhibitory concentrations of Sodium hypochlorite were observed for different classes of antibiotics (Table 2). Most of *Pseudomonas aeruginosa* strains showed increased resistances to

different kind of classes of antibiotics, With the respect of resistance Meropenem 13(10.8%), Ciprofloxacin 12(10%), tobramycin and Gentamicin 10(8.3%), Imipenem and Cefepime 9(7.5%), Amikacin 7(5.9%), Ceftazidime and Levofloxacin 6(5%) showed the most changes. As a result, the rate of MDR 12(10%) and XDR 4(3.4%) increased.

Determination of Minimal Inhibitory Concentration (MIC) and Minimal Bactericidal Concentration (MBC) of disinfectants

In this study the most effective disinfectants were Sodium hypochlorite 5%, Chloroxylenol (Dettol) 4.8%, Sayasept- HP 2%, Chlorhexidine 2%, and Ethanol 70%, respectively. Moreover, the disinfectant susceptibility testing in this study confirmed that at 1/32 concentration of Sodium hypochlorite and Dettol showed lethal effect (MBC) on 120(100%) of the *Pseudomonas aeruginosa* isolates. On the other side the less effective detergent was Ethanol 70% that 59(49.2%) of isolates did not show lethal effect on 1/16 concentration of Ethanol 70% (Table 2).

Table 2
The number of isolates with MICs and MBCs

Biocides	1/8	1/16	1/32	1/64	1/128	1/256	1/512
MIC Ethanol 70%	12(10%)	65(54.2%)	30(25%)	11(9.2%)	2(1.7%)		
MIC Ethanol 70%+EDTA	1(0.8%)	7(5.8%)	32(26.7%)	68(56.7%)	7(5.8%)	4(3.3%)	1(0.8%)
MBC Ethanol 70%	59(49.2%)	34(28.3%)	25(20.8%)	1(0.8%)	1(0.8%)		
MBC Ethanol 70%+EDTA	3(2.5%)	25(20.8%)	51(42.5%)	34(28.3%)	6(5%)	1(0.8%)	
MIC Sodium hypochlorite 5%	-	-	-	15(12.5%)	50(41.7%)	30(25%)	25(20.8%)
MIC Sodium hypochlorite 5%+EDTA				6(5%)	12(10%)	24(20%)	78(65%)
MBC Sodium hypochlorite 5%				33(27.5%)	56(46.7%)	30(25%)	1(0.8%)
MBC Sodium hypochlorite 5%+EDTA				14(11.7%)	24(20%)	59(49.2%)	23(19.2%)
MIC Dettol 4.8%				42(35%)	39(32.5%)	39(32.5%)	
MIC Dettol 4.8%+EDTA				5(4.2%)	26(21.7%)	44(36.7%)	45(37.5%)
MBC Dettol 4.8%				65(54.2%)	42(35%)	13(10.8%)	
MBC Dettol 4.8%+EDTA				32(26.7%)	43(35.8%)	37(30.8%)	8(6.7%)
MIC Sayasept-HP 2%				50(41.7%)	38(31.7%)	30(25%)	2(1.7%)
MIC Sayasept- HP 2%+EDTA				13(10.8%)	29(24.2%)	36(30%)	42(35%)
MBC Sayasept- HP 5%			2(1.7%)	63(52.5%)	45(37.5%)	10(8.3%)	
MBC Sayasept- HP 5%+EDTA				24(20%)	47(39.2%)	41(34.2%)	8(6.7%)

Biocides	1/8	1/16	1/32	1/64	1/128	1/256	1/512
MIC Chlorhexidine 2%				71(59.2%)	36(30%)	13(10.8%)	
MIC Chlorhexidine 2%+EDTA				27(22.5%)	33(27.5%)	43(35.8%)	17(14.2%)
MBC Chlorhexidine 2%			44(36.7%)	64(53.3%)	12(10%)		
MBC Chlorhexidine 2%+EDTA			16(13.3%)	56(46.7%)	48(40%)		

Synergistic effect of selective disinfectants and Ethylene-diamine-tetra acetic acid (EDTA)

Adding EDTA increased the efficiency of all disinfectants included in this survey. At the concentrations used, antiseptics without EDTA were efficient at higher concentrations in comparison with mixed EDTA and, when combined with EDTA, a reduction of concentration was observed in MBC and MIC, the effects of EDTA and antiseptics were additive. Ethanol 70%, Sodium hypochlorite 5%, Sayasept- HP 2%, respectively gave the best results when combined with EDTA, although its antibacterial efficiency at the concentration used in Chloroxylenol (Dettol) 4.8%, and Chlorhexidine 2% is lower than that of other antiseptics (Table3). Comparative diagram of MBC disinfectants at different concentrations before and after mixing with EDTA was performed in Fig. 1.

Biofilm assessment

In this part, 117(97.5%) of clinical *Pseudomonas aeruginosa* isolates were found to develop biofilm and 45 (37.5%), 51(42.5%), and 21(17.5%) isolates were strong, intermediate, and week biofilm formers respectively, compared to the reference strain. 37(30.8%) and 28(23.3%) of strong and intermediate biofilm formers belong to MDR/XDR strains. Also in the case of strong biofilm-producing isolates, higher concentrations of disinfectants were used to kill the isolates (MBC). The results of the present study showed that higher concentrations of disinfectants are required to kill strong and moderate biofilm-producing isolates (Fig. 3).

Detection of efflux pump genes (qac-E, qacE-Δ1, sug-E1**) by PCR technique**

Genomic detection of *qacE*, *qacΔE1*, and *sug-E1* showed that 111(92.5%), 21 (17.5%), and 114(95%) out of 120 *Pseudomonas aeruginosa* isolates harbor the *qacE*, *qacΔE1*, and *sug-E1* genes, respectively. Among the isolates carrying *qacEΔ1* gene, 16 isolates were MDR / XDR.

Discussion

Since 1980, nosocomial infections, specially caused by *Pseudomonas aeruginosa* were classified as a big issue in Hospital, as a result of this problem, medical costs for health care systems have been high(20). Many studies has shown that efficacy of disinfectants and antibiotics are gradually reduced(21). There are several reasons of prevalence of resistance of disinfectants: inaccurate concentration, Inappropriate usage, and insufficient training to prepare and storage of Hospital disinfectants are among them(22). Compared to many antimicrobial

resistance surveys about antibiotics, the number of global researches regard to resistance to biocides is yet very low. Due to clinical importance of *Pseudomonas aeruginosa*, the efficacy of five Hospital disinfectants was assessed against clinical isolates of *Pseudomonas aeruginosa*. Structural factors, bacterial efflux pumps, biofilm, toxins and enzymes are the main reasons of resistance to disinfectants and antibiotics in *pseudomonas* genus(23). Sodium hypochlorite 5%, showed the strongest bactericidal activity against isolated *Pseudomonas aeruginosa* followed by Dettol 4.8%, Sayasept- HP 2%, Chlorhexidine 2%, and Ethanol 70%.

In our study, the effectiveness of sodium hypochlorite in the concentration of active ingredient 0.078 was determined as 100% MBC of the studied isolates, which is more effective than all disinfectants in the present study. These results are consistent with another study performed on *Pseudomonas aeruginosa* and 0.5% sodium hypochlorite isolates. Sodium hypochlorite has also been shown to be more effective than ethanol and salvone (1.5 ﷺ v / v chlorhexidine + 15 ﷺ w / v cetrimide) in the study, and the results of our study show similar results(24). Also, the results of a study conducted in Brazil showed that sodium hypochlorite is more effective than ammonium tetravalent compounds against bacteria. In our study, sodium hypochlorite was more effective disinfectant than Sayaspet, which is a fifth generation of ammonium tetravalent compounds(25). The results of other studies on the active ingredient sodium hypochlorite confirm the results of our work (26–28).

Currently, EDTA has been approved as an antimicrobial agent to reduce the risk of bacterial biofilm formation and colonization. Another goal of the current study is to determine the synergistic effect of EDTA in combination with five other non-antibiotic antimicrobials. In the current survey the addition of EDTA increased the efficacy of selected disinfectants significantly. In a study reported that 4% tetra sodium EDTA is able to eradicate pre-formed biofilms of clinical isolates(29). some surveys revealed that combined antibiotics are more effective compared to single antibiotics(30). The efficacies of disinfectants currently are being investigated in order to decrease the rate of emerging resistance among clinically isolated bacteria. For decades, EDTA has been known as a potentiating and sensitizing agent. Several studies showed that EDTA biofilm disrupting is due to its ability to cations sequestering (Mg^{2+}, Ca^{2+} and Fe^{3+}), as a result increase the effect of other antimicrobial agents (31–33). Combination of antibiotics and other antimicrobial agents with disodium EDTA has been broadly studied (31, 34, 35). A study conducted on Candida and methicillin-resistant S. aureus (MRSA) on catheters, in this study a combination of ethanol (25 %), EDTA (30 mg/mL), and minocycline (3 mg/mL), eradicated pre-formed biofilms, synergistically(31). Another survey performed on commonly pathogens involved in canine otitis specially *Pseudomonas*, revealed that Combination of Tris-EDTA with Chlorhexidine 0.15% has excellent synergistic activity against all isolates (36). These trials propose that the combination of Chlorhexidine or ethanol with EDTA does not compromise the activity of one another. However, standard EDTA or disodium EDTA is not a potent and practical antimicrobial agent even when used at high level concentrations, and is not able to kill bacteria. On the other side, some studies showed that tetra-sodium EDTA has broad-spectrum antimicrobial activity on its own(29, 33). It was reported, tetra-sodium EDTA (40 mg/ mL) decreased biofilm colonization by *Pseudomonas aeruginosa, S. epidermidis, Klebsiella pneumoniae, C. albicans* and *E. coli* on catheter segments(33). In another survey killing ability of 4% tetra-sodium EDTA against clinically relevant pathogens was reported (29). Also There are many studies on Ethanol for its antimicrobial activity alone and combined with other antimicrobial agents, that indicated combination with other biocides increase the potent of Ethanol (32, 37, 38).

The role of biocides in bacterial antibiotics resistance is worrying since several changes in European law and claims of a risk of biocide use were promoted. All of the studies in this subject are significant to attain a better conception of the interaction between bacteria and biocides, and to make a proper risk assessment on the use of

disinfectants and emerging resistance and cross-resistance in bacteria(39, 40). In this study, there was a significant difference between the results of antibiogram before and after exposure to sodium hypochlorite in most antibiotics. our results showed that 16 isolates were isolated from antibiotic-sensitive isolates, of these, 12 isolates became MDR isolates, and 4 isolates became XDR isolates. There are many studies that have emphasized the associations of biocide and antibiotic resistance in bacterial isolates. For instance, in 2016, Lloyd et al showed that in mercury contaminated place, the rate of resistance to antibiotics was higher in mercury-resistant bacteria in comparison to mercury-sensitive isolates(41). It is worth bearing in mind that, the 'in-use' concentration of disinfectants, in most times are 1,000 times greater than of their MIC, to gain a rapid killing of bacteria. Biocide at high level concentration usually interacts with several targets in the bacterial cell. For this reason, bacteria hardly become resistant via adaptation or other mechanisms. However, bacterial usually exposed to sub-inhibitory concentration of biocides. It has been shown that, bacteria exposing to sub-inhibitory concentrations of biocides result in increasing resistance to biocides and antibiotics in bacteria (42–45). In a study conducted on 2017, bacteria harboring biocide resistance genes were more probable to harbor an antibiotic resistance gene in comparison with bacteria lacking biocide resistance genes (46). In this study, we analyzed the correlation between biocides and antibiotics and positive connection were detected between sub-inhibitory biocides exposing and antibiotics resistance. Such relationships were extensively reported and often involved the up- regulation of efflux pumps (47).

Usage of disinfectant in Hospitals must be intently measured and re-evaluated due to the selection pressure effect of antimicrobials on the advent of resistant bacteria which could be spread to the Hospitalized patients. For this reason, resistance is inducible after exposure to sub-inhibitory level of disinfectant (sodium hypochlorite), which result in an increase in the isolates resistant to some antibiotics. It is noteworthy that evaluate some disinfectants and assess correlations with antibiotics resistant which should be taken into account for disinfection practices. It is not correct to consider bacteria that grow in low concentrations of disinfectants as resistant to biocides. This must be determined as 'increasing MIC value' or reducing susceptibility and as a result it is important to evaluate the bactericidal concentration instead of the inhibitory concentration of disinfectants (48). It should be noted that, results of different surveys and the methods employed must also be considered. However, opposed results show that there is not any correlation between antibiotic resistance and exposing to sub-lethal concentration of biocides (49), and it is not completely obvious that there is a correlation between biocide resistance and antibiotic resistance, and surveys still continue in this subject(50). In conclusion, it is clear that biocide concentration is a significant element in the bacterial resistance induction. if the disinfectant prepare at low concentrations, and if the diluted disinfectant is kept in a long time than manufacturer instruction, then bacteria are exposed to low concentration of biocide (48).

A vital key used by *Pseudomonas aeruginosa* to survive in harsh environment such as exposure to antibiotics agents is biofilm formation (51). The National Institute of Heart, Blood, and Lung reported that up to 80% of all infections caused by bacterial are related to biofilm formation (52). The results of our study showed that 117 (97.5%) isolates formed biofilm, which was similar to other studies (53). Antibiotic resistance has increased by biofilm formation, and resulted in using a higher concentrations of antibiotics in MDR *Pseudomonas aeruginosa* isolates infections (54). In a study, it was indicated that the rate of *Pseudomonas aeruginosa* isolates biofilm formation from Iranian patients varied from 48.5–99.5%. Generally, the biofilm formation ratio was reported as 87.6%. As well, 27.4%, 30.2%, and 47.7% of *Pseudomonas aeruginosa* isolates were weak, moderate, and strong biofilm producers, respectively (16). Accordingly, our data were in line with the results published in studies where

40–100% of *Pseudomonas aeruginosa* isolates produce biofilm (55, 56). Karami et al, reported 73% of both clinical and environmental isolates were biofilm producers (57). Also, other studies reveals the importance of biofilm formation by *Pseudomonas aeruginosa* (58, 59). In line with our study it was reported that 58.6% of MDR isolates produce strong biofilm. These results revealed a significant correlation between biofilm formation and MDR isolates (60). It should be noted that, In contrast to these findings, some studies from different parts of the world indicated a lower prevalence of biofilm formation, and as a result, there was no correlation between antibiotic resistance and biofilm producing (61–63). This issue possibly linked to other resistance mechanisms (efflux pumps, purines, chromosomal mutation, and plasmid acquisition) involved in antibacterial resistance (64).

Another aim of the present study was to investigate the prevalence of *qacEΔ1*, *qacE* and *sug-E1* genes, and their relationship with resistance to antibiotics and biocides in *Pseudomonas aeruginosa*. In the current study, the prevalence of efflux pump genes was very high, and due to the high prevalence of *qacE* and *sug-E1* genes, no association was found between these genes and resistance to disinfectants and antibiotics. Of the 21 isolates carrying the *qacEΔ1* gene, 16 isolates were MDR / XDR, indicating an association between this gene and antibiotic resistance. In a study conducted in Egypt, the *qacEΔ1* gene was identified in 57.8% of multidrug-resistant isolates, and 21.4% in susceptible strains, which confirms the results of our study (65). In our study, 95% prevalence of *sug-E1* gene was reported, which is similar to the results of a study conducted in Australia, While the prevalence of *qacEΔ1* gene was 46.2%, it was higher than the prevalence reported in our study (66).

Conclusions

The present study showed that exposure to sub-inhibitory diluents of disinfectants increases resistance to antibiotics. EDTA also had a significant additive effect in increasing the lethality and inhibitory power of disinfectants. This study also showed that sodium hypochlorite has high lethality and inhibitory power and ethanol alcohol has low lethality against isolates of this study. Most isolates in this study produced strong and moderate biofilm.

Materials And Methods

Bacterial Strains

A cross-sectional study was performed from April 2019 to July, 2020, by approval of the Ethics Committee of Qazvin Medical University (IR.QUMS.REC.1398.156). A total of 120 samples of *Pseudomonas aeruginosa* were collected from clinical specimens of Hospitalized patients. Isolates from Urine, CSF, Blood cultures, Tracheal aspirates, and various catheter tips were included in this study. All isolates were cultured on the MacConkey agar medium; standard laboratory methods were performed to identify *Pseudomonas aeruginosa* strain; all isolates were confirmed by PCR method. For further investigation, All *Pseudomonas aeruginosa* isolates cultured in trypticase soy broth (Merck, Darmstadt, Germany) were supplemented with 15% glycerol and were stored at −20°C.

Antibiotic susceptibility testing

Antibiotic susceptibility test (AST) was done base on the Clinical and Laboratory Standard Institute CLSI 2020 guideline (CLSI 2020). Antibiotics included in our study were as follow: Piperacillin (30), Piperacillin/Tazobactam

(PTZ, 100 µg/10 µg), Ceftazidime (CAZ, 30 µg), Levofoxacin (LEV, 5 µg), Amikacin (AN, 30 µg), Imipenem (IMI, 10 µg), Gentamicin (GM, 10 µg), Meropenem (MEM, 10 µg), Tobramycin (TOB, 10 µg), Ciprofoxacin (CIP, 5 µg), Cefepim (CPM, 30 µg). Cefoxitin (30 µg), Ampicillin/Sulbactam (10 µg /10 µg). *Pseudomonas aeruginosa* (ATCC 27853) and *Escherichia coli* (ATCC 25922) was used as control. The MICs for Colistin was determined using the MIC method. colistin susceptibility were interpreted according to the CLSI 2020 clinical breakpoints (67). Extended drug resistance (XDR) in *Pseudomonas aeruginosa* is categorized as non-susceptibility to at least one antibacterial agent in all but two or fewer antimicrobial categories in contrast, and multi-drug resistance (MDR) is determined as non-susceptibility to at least one antibacterial agent in three or more categories (10, 11).

The effect of exposure to sub-inhibitory concentrations of disinfectants on antimicrobial susceptibility test

For this purpose, antibiogram was done after exposing to sub-inhibitory concentrations of Sodium hypochlorite, and compared with before exposing. For this goal, antibiogram test was performed for bacteria that had grown in the highest concentration of Sodium hypochlorite. The reason for choosing sodium hypochlorite to study the effect of sub inhibitory on *Pseudomonas aeruginosa* was that, it is widely used in most countries in inappropriate concentrations.

Molecular Method for detection of resistance detergent genes

Detection of resistance detergent genes performed by PCR method and following primers in Table 3.

Table 3
The lists of primers were used in this study

Gene	Product size (bp)	Sequence	Reference
qac-E	206	F: TGCGTTCCTGGATCTATCTG R: GACGATGCCAATGCCTTC	In study
qacE-Δ1	202	F: TTGTTATCGCAATAGTTG R: AATGGCTGTAATTATGAC	In study
sug-E1	196	F: CCGTTGGTCTGAAATACAC R: ATGGATTCGCCGAACAGG	In study
ZnbT	372	F: GCCAGTTGCGAGTAGATGTC R: CCGTGGAGTGAACCTGAATC	In study

Disinfectants susceptibility testing

Five different disinfectants were studied, that have been used in Iranian Hospitals, following: Ethanol 70%, Chlorhexidine 2%, Sodium hypochlorite 5%, Chloroxylenol (Dettol) 4.8%, Sayasept- HP 2%. The MICs and MBCs of the all mentioned disinfectants were assessed by broth micro-dilution method (micro titer assay) [22]. Briefly, 100 µl nutrient broth was added to all wells. Then 100 µl of disinfectant was added to well one, after serial dilution, 100 µl of bacterial inoculum (10^6cfu/ml) was added to all wells. The dilutions included 1/2, 1/4, 1/8, 1/16, 1/32, 1/64, 1/128, 1/256, 1/512. MICs AND MBCs calculated (68). The lowest concentration of biocide that inhibits bacterial growth and does not show turbidity is reported as the minimum inhibitory concentration (MIC).

Subsequently, 100µl of four final clear diluted wells of each disinfectant, were cultured on Muller Hinton agar medium and if after 48 hours at 37 ° C 99.9% of the bacteria did not grow (i.e. no growth or growth Less than 15 colonies) That dilution is considered the minimum bactericidal concentration(MBC) (69). Rideal-Walker Phenol Coefficient Test was used to determine the efficacy of the disinfectants (70).

Efficacy of Ethylene-diamine-tetra acetic acid (EDTA) on selective biocides

For this purpose, the selected disinfectants were mixed equally with the EDTA 17% and placed at room temperature for 15 minutes. Then, for all isolates MIC and MBC with new mixture were calculated. The obtained results were compared with the previous results and its synergistic effect was investigated.

Assessment of Biofilm Formation Capacity

The biofilm-forming ability was determined using the crystal violet staining method in triplicates and at three independent replicates, as previously described, as positive control *Acinetobacter baumannii* ATCC19606 was used, and LB medium was used as a negative control (71).

Statistical analysis

SPSS/16 software was used for Statistical analysis. In all evaluations, it was considered significant in terms of statistical analysis ($P < 0.05$) or Fisher method.

Abbreviations

MDR: multidrug-resistant; XDR: Extensively drug-resistant; MIC: minimum inhibitory concentration;
MBC: minimum bactericidal concentration; WHO: World Health Organization; EDTA: Ethylene-diamine-tetra acetic acid

Declarations

Availability of data and materials

All data and material are available upon request to correspondence author.

Authors' contributions

Farhad Nikkhahi designed the study, Mehdi Bakht and Sara Rahimi performed the study and wrote the manuscript. All authors have read and approved the manuscript.

Ethics approval and consent to participate

Current study was performed by approval of the Ethics Committee of Qazvin Medical University with approval number IR.QUMS.REC.1398.156. In addition, the committee approved the utilization of human samples within this study. The clinical samples were taken as part of standard patient care and therefore no informed consent was applied from participants. Also it should be noted that biological samples are handled by the authors in the

present study. The adopted methods for handling of human samples were carried out in accordance with relevant guidelines and regulations provided in the Declaration of Helsinki. The research protocol was approved by the Research Ethics Committee at the Qazvin Medical University, Iran.

Funding

This research did not receive any specific grant from funding agencies in the public, commercial, or not-for-profit sectors

Consent for publication

Not applicable.

Acknowledgement

Not applicable.

Competing interests

The authors declare no conflict of interest

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Figures

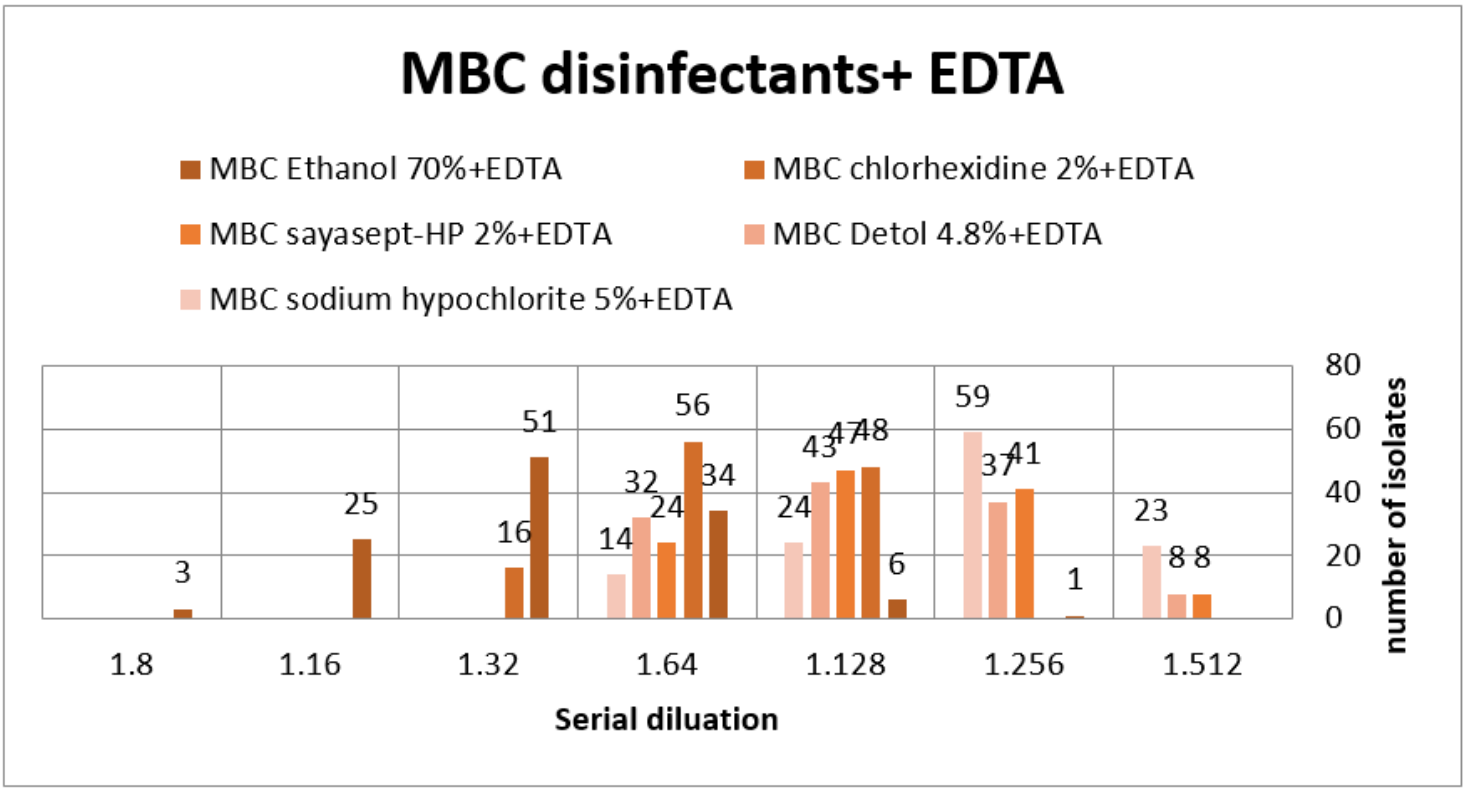

Figure 1

Comparative diagram of MBC disinfectants at different concentrations before and after mixing with EDTA

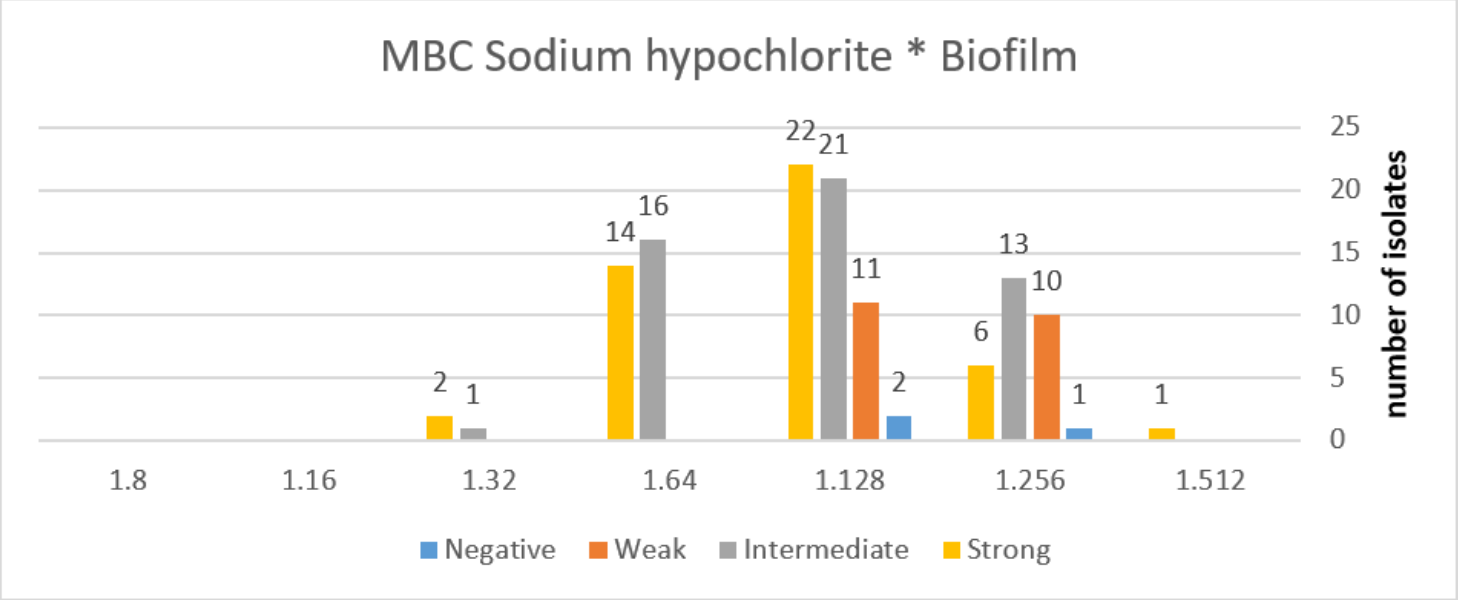

Figure 2

Comparative diagram of the results of antibiotics susceptibility test before and after exposure to sodium hypochlorite

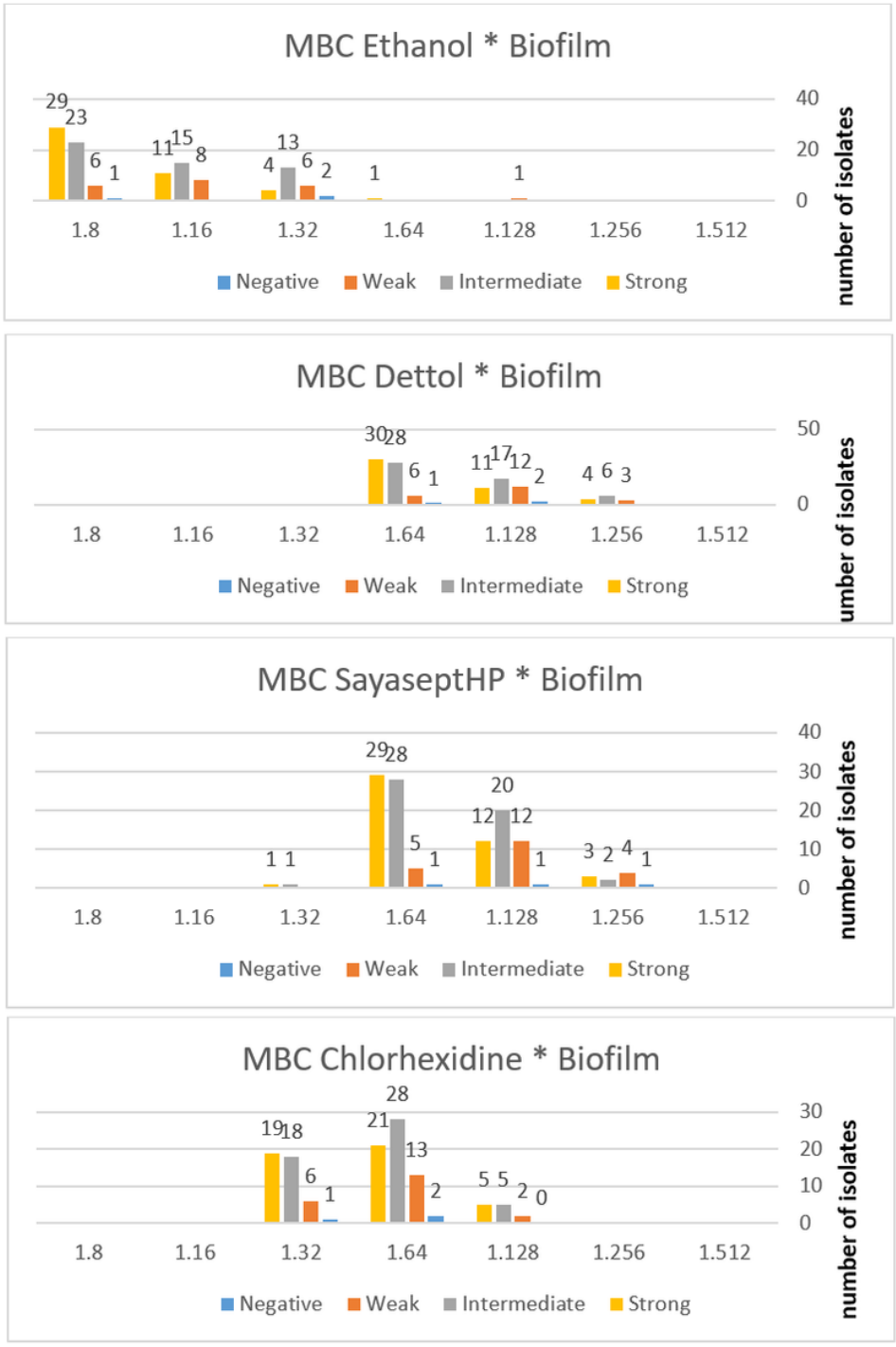

Figure 3

Comparative diagram of the results of MBC disinfectants and the power of biofilm formation