Adaptive mutation through exposure to organic and inorganic biocides lead to the antibiotics resistance

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Abstract

In this study we show the effect of some of biocides on *Acinetobacter baumannii* and reflection of this effect on resistance of bacteria to both biocides and antibiotics at the same time. We have Used inorganic biocide which represent Sodium hypochlorite compound (NaOCl). Exposure of bacteria to this compound in sub-lethal concentrations are 400 µg\ml (0.04%) and 500 µg\ml (0.05%). Also bacteria are exposed to organic biocide which represent Povidone-iodine (PVP-I) exposure of bacteria to concentrations 2000 µg\ml (0.2%) and 3000 µg\ml (0.3%), after that we observe the appearance of bacterial strains resistant to biocides by observation of increase in Minimal Inhibitory Concentration (MIC) values for biocides to inhibit the bacteria after exposure it to biocides in the concentration which are mentioned above. We observed from this study that clear differences in bacterial susceptibility to antibiotics before and after exposure by observation increase in the level of bacterial resistance to antibiotics after exposure to biocides. We conclude from this study we must know the appropriate concentrations for these biocides (Sodium hypochlorite and Povidone iodine) because if we use these biocides in sub-lethal concentration lead to appearance of bacterial strains resistant to both biocides and antibiotics and that lead to failure in the treatment.

التغيرات التكيفية خلال التعرّضِ إلى القواتل الحيوية العضوية واللاعضوية تؤدّي إلى مقاومةِ المضادات الحيوية

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الخلاصة

تم دراسة مدى تأثير بعض القواتل الحيوية (المتلفات الحيوية) Biocides على بكتريا *Acinetobacter baumannii* وانعكاس ذلك التأثير على مقاومة البكتريا للقواتل الحيوية والمضادات الحيوية في نفس الوقت إذ تم استخدام قاتل حيوي لا عضوي inorganic biocide وتمثل بمركب هايبوكلورايت الصوديوم Sodium hypochlorite (NaOCl) إذ تم تعريض البكتريا لهذا المركب بتراكيز دون التراكيز القاتلة sublethal concentrations وهي (400µg\ml) أي ما يعادل (0.04%) وتركيز (500µg\ml) أي ما يعادل (0.05%). كذلك عرضت البكتريا لقاتل حيوي عضوي organic biocide وتمثل في مركب Povidone-iodine حيث عرضت البكتريا لتراكيز (2000 µg\ml) أي ما يعادل (0.2%) وتركيز (3000 µg\ml) أي ما يعادل (0.3%). لوحظ ظهور سلالات بكتيرية مقاومة للقواتل الحيوية بدلالة ارتفاع قيم التركيز المثبط الأدنى (MIC) للقواتل الحيوية لتثبيط البكتريا بعد تعرضها للقواتل الحيوية بالتراكيز المذكورة أعلاه. كما لوحظ من خلال البحث ان هناك فروقات معنوية واضحة في حساسية البكتريا للمضادات الحيوية قبل وبعد التعريض إذ لوحظ ارتفاع في مستوى مقاومة البكتريا للمضادات الحيوية بعد التعريض للمتلفات الحيوية. نَستنتجُ مِنْ هذه الدراسةِ انه يَجِبُ أَنْ

نَعْرِفَ التراكيز الملائمةَ لهذه القواتل الحيوية Sodium hypochlorite و Povidone -iodine التي تقتل الجراثيم لأن استعمال هذه القواتل الحيوية في التركيزِ دون أو أقل من التركيز القاتلِ يُؤدّي إلى ظهورِ سلالات جرثوميةِ مقاومةِ إلى كل من القواتل الحيوية والمضادات الحيوية واللذان يُؤدّيانِ إلى الفشلِ في المعالجةِ.

Introduction

Biocides (Antiseptics and disinfectants) are used broadly in hospitals and health care places for different topical sterilization because biocides have dramatic effects on the structure and function of the different compounds that make up microbial life, which include (carbohydrates, proteins, lipids, and nucleic acids), which represent essential structures that enter in formation of cell walls, cell membranes, and viral envelopes, and other structures for microorganisms, therefore biocides are an essential part for infection control and help in the prevention of nosocomial infections (1). Many studies signal that troubles which are associated with the evolution and spread of antibiotic resistance in the clinic is increased (2,3,4).Now a number of scientists have worry from the use of biocides and preservatives which use in different applications and in local and industrial places may be a contributory factor to evolution and selection of antibiotic-resistant strains (5,6) because that biocide and antibiotic act on the same target site might lead to selection of mutants changed in such targets by emergence of cross-resistance. The bacteria which used in this study is:

Acinetobacter baumannii

Acinetobacter is a Gram-negative bacterium, aerobic, often capsulate, non-motile that is readily found throughout the environment including drinking and surface waters, soil, sewage and various types of foods. *Acinetobacter* is also commonly found as a harmless coloniser on the skin of healthy people and usually poses very few risks (7). *A.baumannii* can be taken from several human sources, including skin, pharynx, sputum, urine, vaginal secretions, and stool. therefore, it forms opportunistic infections, and it cause a wide spectrum of infections, including pneumonia, meningitis, bacteremia, soft tissue infections, surgical site infections, peritonitis, endocarditis, catheter-related infections, and urinary tract infections (8). There have been many reports of *A. baumannii* different infections with strains resistant to the major classes of antibiotics: beta lactams and inhibitors, aminoglycosides, fluoroquinolones, cholamphenicol, tetracycline, and rifampin (9). The aim of this study was not only to understand about the relationship between exposure to biocides and reduced sensitivity to antibiotics and biocides, but to attempt for clarification the practical implications for the use of biocides in the domestic environment.

Materials and methods

- **Bacterial strains:** 6 isolates from *Acinetobacter baumannii* were collected from wounds. *Acinetobacter baumannii*. were identified by Gram's stain, and use of an identification kit (the API 20NE system.; bioMérieux, Marcy L'Etoile, France). *Acinetobacter baumannii* were cultured on nutrient agar and blood agar and identification of bacteria depend on Clinical Manual microbiology (10).
- **Antimicrobial susceptibility testing to antibiotics:** Bacteria were assayed for susceptibility to many antibiotics before and after exposure to biocides this antibiotics include: amoxicillin\clavulanic acid (AMC), cefixime (CFM), Aztereonam (ATM), Cefodizine (CDZ), Impenem (IMP), Tetracycline (TE), Chloromphenicol (C), Gentamycin (GN), Kanamycin (K), Tobramycin (TOB), Ciprofloxacin (CIP), Ceftazidime (CAZ), Cefotaxime (CTX), Ticarcillin (TIC), Refampicin (RA), Ticarcillin\ clavulanic acid (TIM).

- **Antimicrobial susceptibility testing of Biocides by Disk Diffusion method**

- Use Sodium hypochlorite (The chemical structure (NaOCl)) in concentration (5%) as working solution and use Povidone –iodine (Molecular Formula: (C6H9I2NO)n· Ix) in concentration (10%) as working solution. from this working solution prepare serial dilutions of each biocide by take amount of biocide and diluted it by using distilled water to get a final concentration (11).
- Prepare sterile filter disks by using a hole punch to make small circular disks from filter paper in diameter (6 mm). put the disks in aluminum foil and sterilize in oven.
- Take amount of solutions which contain on biocides prepared at different concentration by using micropippete and add this solution on the disks from filter paper.
- Take cotton swab and dipped it in the bacterial suspension and spread the bacterial suspension on Mueller-Hinton agar plate by using cotton swab.
- Put the filter paper disks on Mueller-Hinton agar plates.
- Incubate all of the plates overnight (24 hours) at 37ºC.
- Measure the diameter of the zone of inhibition around each disk. Use a ruler to measure the diameter of the clear area in millimeters.

- **Measurement of Minimum Inhibition Concentration (MIC) of Biocides**

- Use Mueller-Hinton agar which contain on (Sodium hypochlorite, Povidone-iodine) in different concentrations.
- Spread the bacterial suspension by cotton swab on the medium.
- Incubate the plate at 37ºC for 24 hours.

- **Selection of mutants resistant to biocides:** We can select mutant colonies resistant to biocides depend on Randall method (12).

- Mutants resistant to biocides were obtained from stationary-phase Mueller-Hinton broth cultures of *Acinetobacter spp.* at 37˚C
- Use Mueller-Hinton agar contain on sodium hypochlorite (400 µg\ml (0.04%), 500 µg\ml (0.05%)), Povidone iodine (3000 µg\ml (0.3%),2000 µg\ml (0.2%)) .
- Dilute the bacterial suspension 1:10 to get on inocolum (10^6 cfu\ml) by using normal saline.
- Use cotton swab for spreading the bacterial suspension on the medium which prepared to select the mutation by input the swab in bacterial suspension tube and spread it on the medium's surface.
- Incubate the cultured dishes at 37ºC for (1 – 6) days and enumerate the colonies which appeared every day.

Results and Discussion

In this study we selected *Acinetobacter baumannii* to study effect of biocides on the bacteria because the adaptation and mutation which take place on it rapidly and because the studies about this bacteria are few and limited.

Table (1) showed sensitivity of *Acinetobacter baumannii* toward antibiotics these No. is clear diameter of zone inhibition in unit (mm)

No. of strain	K	CN	TOB	CIP	IMP	ATM	CFM	CTX	TIM	CDZ	CAZ	AMC	TIC	RA	TE	C
1	24	20	23	32	19	17	12	14	6	16	17	6	9	11	15	19
2	26	22	25	30	32	27	16	18	10	18	22	19	7	10	18	25
3	15	10	13	19	21	18	12	8	11	9	14	6	6	6	7	16
4	13	8	11	15	15	12	7	6	9	8	6	6	6	7	11	10
5	23	22	23	25	30	22	6	6	12	14	21	11	10	9	17	20
6	25	24	27	35	30	30	15	20	13	21	25	14	12	15	29	33

In this table we observe increase in the resistance of *Acinetobacter baumannii* to most of antibiotics because it has different of mechanisms of resistance lead to antibiotic resistance. In this study we used biocides because the lack of information about many of biocides and because biocides have been studied less than antibiotics in terms of efficacy, mechanism of action, and mechanisms of bacterial resistance. On the whole, biocides have a broader spectrum of activity than antibiotics because biocides may have multiple targets, susceptibility of each target is different and dependant on the concentration of the biocide. While antibiotics tend to have specific intracellular targets. We study effect of inorganic biocide (Sodium hypochlorite) on bacteria by minimum inhibitory concentration (MIC) method (Table 2) and disc diffusion method (Table 3). Sodium hypochlorite solutions are widely used for hard-surface disinfection (household bleach) (13,14).

Table (2) showed the minimum inhibitory concentration (MIC) values for *Acinetobacter baumannii* toward Sodium hypochlorite before and after exposure to Sodium hypochlorite in concentration 400µg\ml (0.04%) and in 500µg\ml (0.05%)

Con.	*100 (0.01%)	*200 (0.02%)	*300 (0.03%)	*400 (0.04%)	*500 (0.05%)	*600 (0.06%)	*700 (0.07%)	*800 (0.08%)	*900 (0.09%)	*1000 (0.1%)	*1100 (0.11%)	*1200 (0.12%)	*1300 (0.13%)	*1400 (0.14%)	*1500 (0.15%)
Before exposure	+	+	+	-	-	-	-	-	-	-	-	-	-	-	-
After exposure (0.04%)	+	+	+	+	-	-	-	-	-	-	-	-	-	-	-
After exposure (0.05%)	+	+	+	+	+	-	-	-	-	-	-	-	-	-	-

(+) Growth ,(-) No growth (inhibition).
(*) µg\ml

Table (3) sensitivity of *Acinetobacter baumannii* toward Sodium hypochlorite in disc diffusion method these No. is clear diameter of zone inhibition (mm)

*100 (0.01%)	*200 (0.02%)	*300 (0.03%)	*400 (0.04%)	*500 (0.05%)	*600 (0.06%)	*700 (0.07%)	*800 (0.08%)	*900 (0.09%)	*1000 (0.1%)	*1100 (0.11%)	*1200 (0.12%)	*1300 (0.13%)	*1400 (0.14%)	*1500 (0.15%)
6	6	6	7	12	13	13	13	14	14	14	15	15	15	16

(*)µg\ml

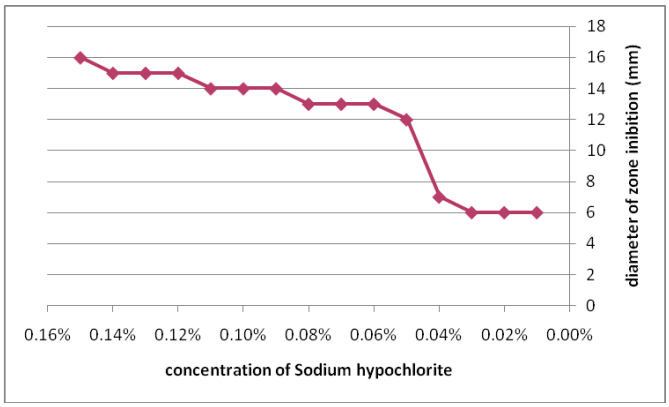


Fig. (1) showed the relation between the concentration of Sodium hypochlorite (NaOCl) and diameter of zone inhibition

When prepare many dilutions from Sodium hypochlorite by adding distilled water In water, sodium hypochlorite ionizes to produce (Na) and the hypochlorite ion, OCl^-, which establishes an equilibrium with hypochlorous acid, HOCl . Pécora et al. showed that sodium hypochlorite exhibits a dynamic balance as is shown by the reaction(15):

$$NaOCl + H_2O \leftrightarrow NaOH + HOCl \leftrightarrow Na^+ + OH^- + H^+ + OCl^-$$

The antimicrobial effectiveness of sodium hypochlorite, based on PH value. The cell wall of pathogenic microorganisms is negatively charged by nature. when be pH of the sodium hypochlorite solution is between 4 and 7, the chlorine is existed predominantly as Hypochlorous acid (HOCl), HOCL can penetrate cell walls and protective layers of microorganisms followed by inhibition of main enzymatic reaction within the cell and act on protein denaturation and effectively kills pathogens as a result HOCL is split into hydrochloric acid (HCl) and Oxygen atom (O). Oxygen is a very powerful disinfectant(13). Hypochlorous acid (HOCl) and hypochlorite ions (OCl^-) act on amino acid degradation and hydrolysis(14). While when be pH of Sodium hypochlorite solution above than 9, Hypochlorite Ion (OCl^-) predominates. Hypochlorite contain on chlorine. Chlorine (strong oxidant) has antimicrobial action inhibiting bacterial enzymes. This enzyme inactivation can be observed in the reaction of chlorine with amino groups (NH2-) which found in amino acids to form chloramines that interfere in cell metabolism, and an irreversible oxidation of sulphydryl groups (SH) of essential bacterial enzymes which contain on (cystein), and phospholipid degradation which found in the bacteria (13). Organic biocide which used in this study is povidone iodine. Povidone iodine Similar to chlorine, the antimicrobial action of iodine is rapid, even at low concentrations (16).

Table (4) sensitivity of *Acinetobacter baumannii* toward Povidone iodine in disc diffusion method these No. is clear diameter of zone inhibition (mm)

0.1%	0.2%	0.3%	0.4%	0.5%	0.6%	0.7%	0.8%	0.9%	1%	1.1%	1.2%	1.3%	1.4%	1.5%
6	7	7	7	7	7	7	7	8	9	9	10	11	11	12

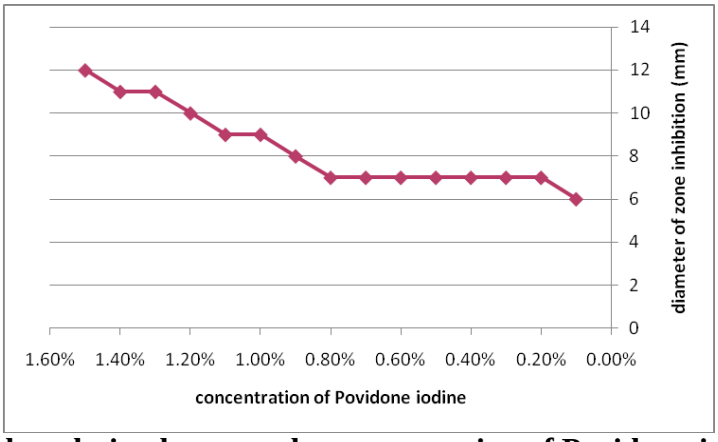
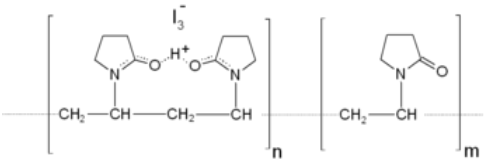


Fig. (2) show the relation between the concentration of Povidone-iodine (PVP-I) and diameter of zone inhibition

Table (5) showed the sensitivity of *Acinetobacter baumannii* toward Povidone iodine in MIC method

Con.	0.1 %	0.2 %	0.3 %	0.4 %	0.5 %	0.6 %	0.7 %	0.8 %	0.9 %	1%	1.1 %	1.2 %	1.3 %	1.4 %	1.5 %
Before exposure	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-
After exposure (0.2%)	+	+	-	-	-	-	-	-	-	-	-	-	-	-	-
After exposure (0.3%)	+	+	+	-	-	-	-	-	-	-	-	-	-	-	-

(+) Growth ,(-) No growth (inhibition).
Povidone iodine is a polyvinylpyrrolidone with iodine complex (PVP-I)
Povidone-iodine



Although less reactive than chlorine, iodine is rapidly germicidal. It has a broad spectrum of activity against bacteria, mycobacteria, fungi, protozoa and viruses and spores. Free Iodine rapidly pass through the cell walls and penetrates into microorganisms (16), and attacks groups of proteins. Iodine is thought to affect protein structure by oxidizing thiol S-H groups are found in cysteine and methionine by the rapid formation of disulfides (R-SS-R), and reacting with the phenolic groups which found in tyrosine and reacting with N-H groups in amino acids (such as arginine, histidine and lysine) to block hydrogen bonding (3). Povidone iodine acts by destroying microbial DNA It reacts with bases of nucleotides (such as adenine, cytosine and guanine) to prevent hydrogen bonding, and it attack and react with unsaturated carbon bonds (C=C) in fatty acids which enter in membrane structure and lead to change in membrane structure (17, 18).

Table (6) showed the sensitivity of *Acinetobacter baumannii* toward the antibiotics before And after exposure to Sodium hypochlorite in concentration 400µg\ml(0.04%) and 500µg\ml(0.05%) these No. is clear diameter of zone inhibition (mm)

antibiotic	K	CN	TOB	CIP	IMP	ATM	CFM	CTX	TIM	CDZ	CAZ	AMC	TIC	RA	TE	C
Before	25	24	27	35	30	30	15	20	13	21	25	14	12	15	29	33
After (0.04%)	18	19	22	31	26	15	10	12	6	18	23	12	6	14	23	22
After (0.05 %)	16	16	19	30	21	10	8	9	6	7	23	7	6	15	20	16

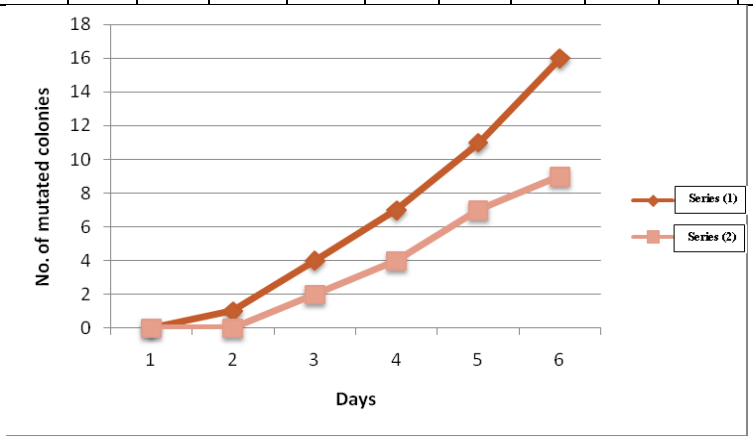


Fig. (3) mutation of *Acinetobacter baumannii* after exposure to Sodium hypochlorite NaOCl. Series (1) represent to No. of mutated colonies when use Sodium hypochlorite in concentration (0.04%) while Series (2) represent to No. of mutated colonies when use Sodium hypochlorite in concentration (0.05%)

Adaptation of organisms to hypochlorite can be determined by comparison of the Minimum Inhibitory Concentration (MIC) such as showed in table (2) observe increase in MIC after exposure to sodium hypochlorite. Bacteria are able to produce stress response proteins when subjected to sub-inhibitory levels of stress. The different cases can act transcription and translation of stress response proteins, which carry increased resistance to a multitude of stressor.

Table (7) showed the sensitivity of *Acinetobacter baumannii* toward the antibiotics before And after exposure to Povidone-iodine in concentration 2000µg\ml(0.2%) and 3000µg\ml(0.3%) these No. is clear diameter of zone inhibition (mm)

antibiotic	K	CN	TOB	CIP	IMP	ATM	CFM	CTX	TIM	CDZ	CAZ	AMC	TIC	RA	TE	C
Before	25	24	27	35	30	30	15	20	13	21	25	14	12	15	29	33
After (0.2%)	24	16	25	29	30	9	11	17	11	16	20	7	11	13	21	23
After (0.3%)	20	13	24	25	25	6	10	6	6	10	17	11	6	10	18	22

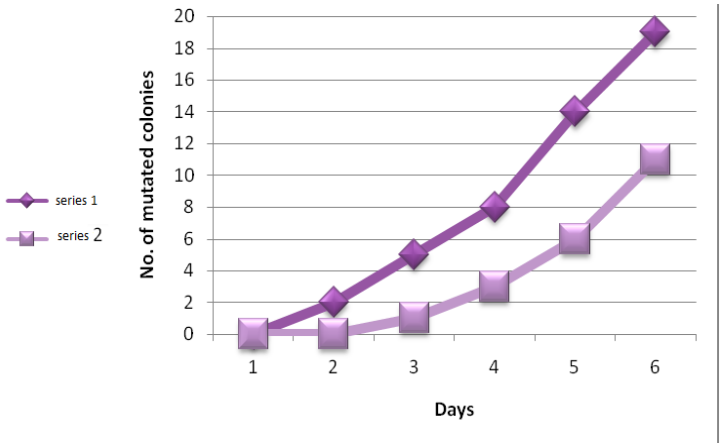


Fig. (4) mutation of *Acinetobacter baumannii* after exposure to Povidone iodine. Series (1) represent to No. of mutated colonies when use povidone iodine in concentration (0.2%) while Series (2) represent to No. of mutated colonies when use povidone iodine in concentration (0.3%)

The cell phenotype expressed can vary significantly which depend on the environmental conditions which is grow under it the bacteria. Adaptation of micro-organisms (that reduces susceptibility to biocides in response to environmental changes) is also considered as intrinsic resistance (19). The resistance of *Acinetobacter baumannii* to both biocides such as clear in (Table (2) and Table (5)) and antibiotics as observed in (Table (6) and table (7) which clear increase in *Acinetobacter baumannii* resistance to antibiotics after exposure to biocides) this resistance may derive partly from changes in outer cell layers that prevent access to their site of action, but other changes are also involved. also the bacteria resistance take place by formation of a biofilm (20). Reduced susceptibility of bacteria in biofilms to antimicrobials can sometimes be extreme and is probably caused by a variety of factors including nutrient depletion within the biofilm, reduced access of the biocide to cells in the biofilm, chemical interaction between the biocide and the biofilm, and the production of degradation enzymes and neutralising chemicals (21). Other mechanism for *Acinetobacter baumannii* resistance to both biocides and antibiotics by efflux pumps. Efflux pump is an important and the main mechanism for the emergence of multidrug-resistant pathogens. which relies on the over-expression of active efflux pumps that extrude multiple antimicrobial agents outside the bacterial cell wall(22). There are three multidrug efflux pumps have been identified in *A. baumannii* so far, are: AdeABC, AbeM and AdeIJK, which display broad substrate specificity including antibacterial agents, biocides and dyes (23, 24). The AdeABC system was the first efflux pump which was described in *A. baumannii* and it is regulated encoded by AdeS and AdeR. (25). AdeABC system mainly confers resistance to aminoglycosides, ß-lactams, chloramphenicol, erythromycin and tetracycline. Over-expression of efflux pump (AdeABC system) led to increase in MIC values to both Sodium hypochlorite as showed in (Table 2) and Povidone iodine as showed in (Table 5). When the

antimicrobial drugs attack disease which is caused by bacteria, they also affect non-pathogenic bacteria which are found in the body, They exterminate these bacteria and make room for more resistant bacterial growth. Acquired resistance to biocides may arise by cellular mutation or by the acquisition of genetic elements in the form of plasmids or transposons (26). Disinfectants which present in the hospital wastewater may act as selective pressure for the retention of the bacterial plasmids that often contain the genes for resistance to numerous antibiotics (27). Differ results of this study with the results of another study which showed that there is not a correlation between utilization of common antibacterial cleaning agents and the formation of antibiotic-resistant bacteria in the environment (28).

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