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ORIGINAL ARTICLE

The Study of Cross-Resistance Between Silver and Antibiotics in Isolated Bacterial Strains From A Burns Unit

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ABSTRACT

Silver is an effective, broad-spectrum antimicrobial agent commonly incorporated into topical creams (such as silver sulfadiazine) and wound infection. The genes associated with silver resistance provided the basis of the current investigation to determine the prevalence of silver resistance genes in clinical wound isolates. Isolated strains were biochemically identified by conventional tests. Isolates were tested for antimicrobial and silver nitrate susceptibility by standard methods. The presence of silver (*sil* genes) and aminoglocoside resistant gene (*aac(6′)-IIa*) was proved by PCR. From 106 bacterial strain isolated from burn patients, respectively 70 strains (69%) and 30 strains (31%) were belonged to *Pseudomonas aeruginosa* and *Staphylococcus aureus*. The highest and lowest minimal inhibitory concentration for *Ps.aeruginosa* strains were to gentamicin and imipenem respectively. The range of MIC for silver nitrate was 0.01-35µg/ml. The range of MIC isolated *S.aureus* strains was obtained about 25-400 µg/ml for penicilin. Cross-resistance were observed in 14% of isolated *Ps.aeruginosa* strains. The strains with *silP* and *silE* genes (14%) have *aac(6′)-IIa* gene. 18.5% , 17% and 4% of strains had *silP* gene, *silE* and *silS* respectively. Silver resistance genes were not seen in any of *S.aureus* isolated strains. Multi-resistant species, such as methicillin-resistant *Staphylococcus aureus* and *Pseudomonas aeruginosa* could create major problems in treatment of hospital infections. Our findings show that cross-resistance between antibiotics and biosides such as silver nitrate were existed in isolated bacterial strains. This review was determined that silver nitrate resistance was associated with antibiotic resistance in several strains. The phenomenon of cross- resistance 54% clinical strains isolated from burns can be observed and the indiscriminate use biocides silver-containing compounds resulting in increased antibiotic-resistant strains and the treatment of burn infections serious problems with making.

Key words: silver, cross-resistance, *sil* genes.

Introduction

Most of the time *Ps.aeruginosa* infection is a general problem in burn patients and the reason of death in these cases are mostly because of this bacteria. Based on published reports related to clinical infections in Iran *Ps.aeruginosa* infections is the most important reason especially in urine burn and infections. The most serious and important thing in these cases is high resistance of *Ps.aeruginosa* strains to microbial treatment [13]. Systemic gentamicin and topical silver preparations have been used in this unit since the mid-1960s [4]. Silver nitrate compounds are used widely as effective antimicrobial agents to combat pathogens (bacteria, viruses and eukaryotic microorganisms) in the clinic and for public health hygiene [32]. Ag^+ resistance is most likely to be found in environments where greatest Ag^+ usage of silver-containing products might be expected, such as in the dental setting where amalgams are known to contain 35% silver, burns units in hospitals or the use of silver-coated catheters [22]. Despite the medical benefits of using ionic silver to manage infections, concern has been raised regarding the potential for development of bacterial resistance and an association with cross-resistance to antibiotics has been implied. Cross-resistance is of concern because plasmids encoding silver-resistant genes also may encode for antibiotic resistance [36]. The use of silver preparations for

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topical antimicrobial therapy in the Burns Unit of hospitals has prompted routine screening of bacterial isolates for resistance to this metal. During some early tests two strains of *Enterobacter cloacae* were isolated which displayed high but unstable resistance to silver nitrate [23]. In recent years, a number of scientists have expressed concern that the use of antimicrobial chemicals (biocides, preservatives) in general practice and in domestic and industrial settings may be a contributory factor to the development and selection of antibiotic-resistant strains [8]. This has been particularly the case with regard to the recent trend towards inclusion of antibacterial agents within a multitude of otherwise traditional consumer products [11].

Resistance to an antimicrobial agent can occur either by intrinsic or acquired mechanisms. Acquired resistance can arise by either mutation or acquisition of various types of genetic material such as plasmids. Biocides (such as silver) and antibiotics have differing modes of action. Biocides tend to target multiple sites on or within bacterial cells and hence have broad- spectrum activity. Antibiotic tend to target specific sites on or within bacterial cell and have a narrower spectrum activity. Antiseptic or biocide resistance can be acquired via mutation in normal cellular genes, plasmids or transposons [7]. Plasmid-mediated biocide resistance has been documented as occurring in *Staphylococcus aureus*, coagulase-negative *Staphylococci*, members of the *Entrobactreiceae* and *Pseudomonas* spp [34,19,16,15].

Plasmid mediated resistance has been extensively studied in the facultative anerobes against heavy metals. Resistance to silver compounds as determined by bacterial plasmids and genes has been defined by molecular genetics. Silver resistance conferred by the plasmid pMGH100 involves nine genes in three transcription units. A sensor/responder (SilRS) two-component transcriptional regulatory system governs synthesis of a periplasmic Ag (I)-binding protein (SilE) and two efflux pumps (a P-type ATPase (SilP) plus a three-protein chemiosmotic RND Ag (I)/H^+ exchange system (SilCBA)). The same genes were identified on five of 19 additional IncH incompatibility class plasmids but thus far not on other plasmids [32].

Plasmid pMG101 is a 182 kb, transferable plasmid[26] encoding resistance to silver (nine Open Reading frames [ORFs] in three transcriptional units), mercury, tellurite, ampicillin, chloramphenicol, tetracycline, streptomycin, and sulphonamide. It confers resistance in bacteria at silver concentrations six or more times the concentration of what a sensitive *Escherichia coli* can tolerate [36]. Changing Enzyme for Aminoglycosides are usual factors of resistance in *Ps.aeruginosa* strains to aminoglycosides [24]. There are three main groups of Enzymes in bacteria cytoplasm: Aminoglycoside acetyltransferas (ACC), Aminoglycoside phosphoryltransferas (APH) and Aminoglycoside nucleotidetransferas (ANT). These Enzymes depends on changing drug or resistance divide to different subgroups [31]. II(6́) AAC is the most usual Aminoglycoside transferas in *Ps.aeruginosa* and caused the resistance to gentamicin and tobramycin [24]. The goals of this study were to investigate the presence and prevalence of silver resistance and silver-resistant genes in clinical burn wound isolates and to examine the cross- resistance of isolated strains to antibiotics and silver.

Material and Methods

The micro-organisms used in this study were routinely isolated from the burn wounds of patients attending the burn unit in Motahari Hospital in Tehran city. All wounds were swabbed using calcium alginate swabs. About 106 collected samples were cultured and identified by specific medium (such as cetrimide agar, manitol salt agar and mcconkey agar Difco Ltd) and biochemical tests using standard protocols [12]. All bacteria were screened for resistance to silver.MIC values and categorical breakpoints (traditionally, susceptible, intermediate and resistant) are now defined by various professional organizations such as the European Committee on Antimicrobial Susceptibility Testing the CLSI (formerly the NCCLS). All isolated *Pseudomonas aeruginosa* and references strains were tested for sensitivity to gentamicin (10µg/l), amikacin (30µg/l), ciprofloxacin (5µg/l), tubramicin (10µg/l), Ticarcillin (30µg/l), ceftazidim (30µg/l), piperacilin/nasobactam (100+10 µg/l) and imipenem (10µg/l), and *S.aureus* isolates were tested for sensitivity to methicilin (10 µg/l), penicillin (10µg/l), erythromycin (15µg/l), Ciprofloxacin (5µg/l), gentamicin (10 µg/l), vancomicin (30 µg/l) and Rifampicin (5µg/l) by a macro dilution broth test (National Committee for Clinical Laboratory Standards). Resistance to heavy nitrate silver was determined by an agar dilution method with modifications as described by Rilay by using nutrient agar as Mueller hinton can show the exaggerated MIC in presence of Mg and Ca as reported in previous studies in case of *Pseudomonas aeruginosa* [9,5]. Plates containing 20 ml nutrient agar prepared in deionized water and 4 graded final concentrations of the ions are prepared on the day of the experiments, by adjusting the final pH of the medium to 7.4 and plates were dried at 37ºC for 20 minutes. The approximate the concentrations were 10mmol, 20 mmol, 30 mmol, 40 mmol and 50 mmol for silver ions. The inoculums size was adjusted to 0.5 McFarland turbidity. A strain of *Pseudomonas aeruginosa* ATTC 27853and *Staphylococcus aureus* ATCC29213 known to be susceptible to these concentrations of the silver ions and the agar plate without the ions were used as control. The plates

were incubated in 37ºC for 24 h. The presence of the growth indicated that the strain is resistant and absence of the growth indicated the susceptibility of strains. The tests were carried out duplicates to verify the results.

Polymerase chain reaction (PCR):

The isolated and control strains were incubated at 37ºC for overnight in Luria Bertani media (Sigma Aldrich, Mo) that selected for 100 µg/mL ampicillin to stationary phase (*A*540; 2.6). Then bacterial plasmids were extracted by Fermentas Co. kit. After adding the RNaseI with precipitated proteins solutions, centrifuged twice and sediment DNA was soluble in 100µl sterile deionized water. The optical absorbance was readed at 260 and 280nm wave lengths. For PCR amplification of the ***sil*** and *aac(6)IIa* coding region from plasmid DNA templates, four primers were designed as shown in table1.

Table 1: Designed primers for *sil* gene and *aac (6) IIa* gene.

	Oligonocleotide sequence(5'–3')	Genes
FF20	GTA CTC CCC CGG ACA TCA CTA ATT	*silE gene*
RR20	AAT AAC GGT CAG TCT GGCC	*silE gene*
FF21	CATGACATATCCTGAAGACAGAAAATGC	*silP gene*
RR21	TATCTGTTATTGCTGGTCTGCCCG	*silP gene*
RR22	GATGTCATTAGCCTGTCATGCAGCAAAC	*silS gene*
FF22	GGAGATCCCGGATGCATAGCAA	*silS gene*
XF	TTG CCC TCC CGC ACG ATG	*aac(6)IIa*
XR	GCT AGA TTT TAA TGC GGA TGT TGC	*aac(6)II a gene*

The PCR program consisted of standard steps at temperatures and durations relevant to the size of PCR products produced (13 in 20 F) Tables 2 and 3).

Table 2: PCR programs for detection of *sil* genes.

Heat	*Lid*	105°	
In	*denature*	95°	2 min
Number	*cyle*	40	
Seg	95°	1 min	
Seg	55°	1 min	
Seg	72°	3 min	
Final	*Exten*	72°	5 min
Final	*hold*	4°	

Table 3: PCR programs for detection of *aac(6)- IIa* gene.

Heat	*Lid*	105°	
In	*denature*	95°	3 min
Number	*cyle*	30	
Seg	95°	1 min	
Seg	63.4°	1 min	
Seg	72°	3 min	
Final	*Exten*	72°	10 min
Final	*hold*	4°	

Agarose gel electrophoresis. The gel wells were filled with 12 µl of PCR products from the wound isolates and controls with a 1 kb plus Ready Load DNA Marker "ladder" (Metabion Co.) providing size standards. Electrophoresis was run at 65 V for 90 minutes; the amplification products were separated on a 0·7% agarose gel and visualized under UV after staining with ethidium bromide. All experiments were carried out in duplicate.

Results:

Out of 106 isolated strains from burn patients respectively were belonged to, 70 strains (69%) *Ps.aeruginosa*, 30 strains (31%) *S.aureus*. The results of bacterial susceptibility tests were shown that 90% of *Ps.aeruginosa* strains were resistante to gentamicin, amikacin, tobramycin and ciprofloxacin (Fig.1). The highest resistante strains appeared to gentamicin and tobramycin(100%) and the lowest resistante strains appeared to emipenem(35%). After antibiogram test out of 70strains of *Ps.aeruginosa* , 25strains multi-resistant species, have been selected for The minimum inhibitory concentration (MIC) determination. The MIC to gentamicin was 2500 µg/ml. The MIC of silver nitrate for the resistant-strains to gentamicin and emipenem were 70 µg/ml and 0.01 µg/ml respectively. The values of MIC of silver nitrate of about 85% of all the strains were approximately 0.01-35 µg/ml (table1). Thus all of isolated *S.aureus* strains show resistante to Penicillin and the lowest resistant appeared to vancomycin (3%) (Fig.2).

The amounts of MIC and MBC of *S.aureus* strains for penicillin were obtained MIC=MBC=25 µg/ml-400 µg/ml. 69% of all strains had MIC of 25 µg/ml for Penicillin.

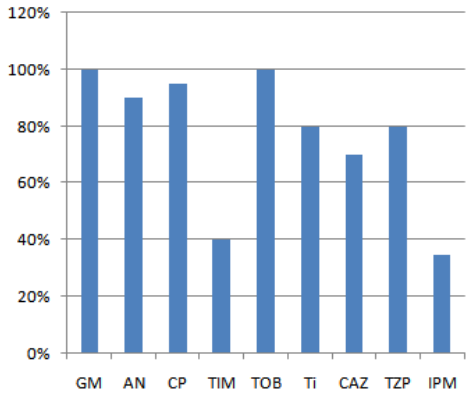

Fig. 1: Antibiotic-resistant *Ps. aeruginosa* isolated from burn wounds of patients.
GM: Gentamicin **AN:** Amikacin **CP**: Ciprofloxacin **TOB**: Tobramycin **Ti**: Ticarcilin **CAZ**: Ceftazidime **TZP**: Piperacilin / Nazobactam IPM: Emipenem

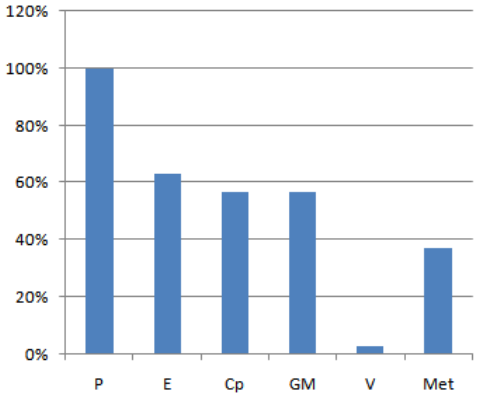

Fig. 2: Antibiotic resistance in ***S.aureus*** strains isolated from burn wounds of patients.
Horizontal column shows tested antibiotics **P**: penicillin **E**: erythromycin **Cp**: Ciprofloxacin GM: gentamicin **V**: vancomycin **Met**: meticilin Vertical column shows percentage of resistance to every specific antibiotic.

Table 2: Antibiotic and MIC of susceptible Mean annular radius Acceptable range (mm).

Antibiotic and Disc content (µg)	MIC of susceptible strains (µg/ml)	Mean annular radius ± standard deviation (mm)
Pseudomonas aeruginosa		
Amikacin	(312) ≤ 160.0	9.1± 0.9
Aztreonam	(234) ≤78.0	9.7± 0.8
Ceftazidime	(20) ≤ 40.0	9.8± 1.2
Imipenem	(250) ≤ 40.0	8.7±0.7
Piperacillin	(432) ≤ 160.0	10.2±1.2
Ticarcillin	(175) ≤ 32.0	9.5±1.1
Tobramycin	(159) ≤ 40.0	8.5± 0.6
Gentamicin	(625) ≤ 40.0	7.9± 0.8
Ciprofloxacin	(12.5) ≤ 20.0	11.4 ± 1.3
Staphylococcus aureus		
Penicillin	(400) ≤20.06	12.3±0.7
Ciprofloxacin	(244.5) ≤ 10.0	11.0± 0.9
Erythromycin	(154) ≤ 10.5	9.3± 0.9
Gentamicin	(523) ≤ 20.0	8.4 ± 0.5
Methicillin	(560) ≤ 40.0	9.2 ± 7.0
Rifampicin	(50) ≤ 5.5	10.5 ± 1.0
Vancomycin	(670) ≤ 10.2	6.1 ± 0.2

From 106 isolated *Ps.aeruginosa* and *S.aureus strains*, 25 of them which were resistant to silver nitrate and also had multi-drug resistance have been selected for PCR. The results have been shown that from of 13 strains of *Ps.aeruginosa* (18.5%), 12 strains (17%) and 3 strains (4%) had *silP*, *silE* and *silS* genes respectively. Thus 10 strains of *Ps.aeruginosa* (14%) had *aac(6)´-IIa* gene . All of strains with *silE* gene also had *silP* gene and strains which had both *silP* and *silE* genes also had *aac (6) ´-IIa gene*.

Therefore it can be concluded that cross-resistance between silver nitrate and antibiotics only found in 14% of *Ps.aeruginosa* strains. In refrence strains of *Ps.aeruginosa and S.aureus* no cross-resistance had been observed. Any of *sil* genes haven't been observed in clinical isolated strains of *S.aureus* (Fig3).

Discussion:

Today use of silver nitrate and silver sulphadiazine have got wide spread applications in the management of the burns infections [36]. There does, however, appear to be some similarity between bacterial resistance to antibiotics and antiseptics. A study by Akimitsu et al. reported methicillin-resistant *Staphylococcus aureus* mutants resistant to benzalkonium chloride that exhibited increased resistance to various beta-lactam antibiotics compared with the parent strain [1]. Vishnu Prasad found that there is no common connection between resistance to heavy metals and antibiotics in *Ps.aeruginosa* and also there is no connection between resistance and sensitivity to antibiotics and metal ions except Silver in multi-resistance strains [36]. A study by Mc Hugh on some of *Salmonella typhimurium* strains which were resistance to AgNO3,ampicillin and chloramphenicol that isolated from burn patients shows that multi-resistance pattern can transfer to some *E.coli* and S.*typhimurium* sensitive strains(in invitro). He mentioned that the danger of these strains which have achieved resistance to chosen antibiotics is the result of using AgNO3 creams on burn wounds [14]. Ugur and Ceylan reported an increase in outbreak of *S.aureus* by plasmid which is resistance to antibiotics and heavy metals like $AgNO_3$ and $Hgcl_2$ in clinical samples [35]. Many clinical researches have been done about the connection between using silver and cross-resistance to antibiotics. Some scientists think that using Ag immethodically will cause more resistance in bacteria. Although the conjunction between bacteria to transfer Ag resistance gene is not common. Studying of Ag resistance frequencies shows that it is not stable and it mostly happen in *E.coli, Entrobacter cloacae*, *Klebsiella and pneumonia* strains [12]. Plasmid determined metal resistance has been demonstrated in members of *Entrobacteriaceae*, *Pseudomonas* and *Staphylococcus* [2,20]. Although studies by Rutala *et al.* have shown that hospital strains of antibiotic-resistant bacteria do not display increased resistance to biocides, and that *S. aureus*, after exposure to biocides, does not increase the transfer of antibiotic-resistant plasmids. It is evident that bacteria tolerate biocides and antibiotics by employing the same types of cellular mechanism [29]. Levy stated that it is probable that the increasing use of biocides will eventually result in the selection of bacteria that are less susceptible. In fact, bacterial adaptation and resistance to biocides is certainly not a new phenomenon [17]. Furthermore, the contribution of biocides to the development of bacterial antibiotic resistance has yet to be fully elucidated. Additional research is required to examine the modes of action of biocides and bacterial

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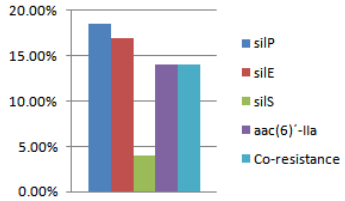

Fig. 3: The percentage of *Ps.aeruginosa* strains which have *sil* and *aac (6) ´-IIa* ***genes***.

Fig. 4: Electrophorase of PCR products (*silE* gene) are from isolated *Ps.aeruginosa* strains on 0.7% agarose gel (Lines 1-12). The sizes of products are indicated and the primers used for PCR are given below each panel.

Fig. 5: Electrophorase of PCR products (*silS* gene) are from isolated *Ps.aeruginosa* strains on 0.7% agarose gel (Lines 1-3). The sizes of products are indicated and the primers used for PCR are given below each panel.

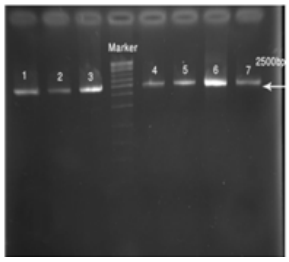

Fig. 6: Electrophorase of PCR products (*silP* gene) are from isolated *Ps.aeruginosa* strains on 0.7% agarose gel (Lines 1-3and 4-7). The sizes of products are indicated and the primers used for PCR are given below each panel.

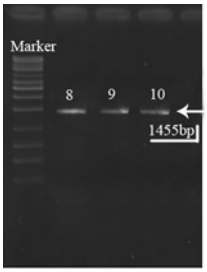

Fig. 7: Electrophorase of PCR products (*aac(6)´-IIa* gene) are from isolated *Ps.aeruginosa* strains on 0.7% agarose gel (Lines 8-10). The sizes of products are indicated and the primers used for PCR are given below each panel

biocide resistance mechanisms, as well as to better characterize potential cross-resistance with antibiotics [26,27,28]. The measure of susceptibility often used in such instances is the minimum growth-inhibitory concentration (MIC), the appropriateness of which to biocides is applied in this survey. As in the clinical setting, certain groups of microorganism are innately resistant to certain groups of biocide and can be described as tolerant [11]. To date, it is unclear whether the use of heavy metals, such as Ag^{+}, is contributing to the emergence and spread of antibiotic-resistant bacteria; however, this is unlikely [3]. A recent paper by Cole *et al*. concluded that there was a lack of antibiotic and antiseptic agent cross-resistance in target bacteria from the homes of antibacterial product users and non-users, as well as increased prevalence of potential pathogens in non-user homes. Gilbert and McBain's paper concluded that the risks associated with the overuse of biocides has been overstated, and that it is now imperative that confidence is restored in products that form an essential part of domestic and hospital hygiene. Preferably the term resistance should be avoided, and reference should only be made to the susceptibility of an organism other than when treatment outcomes are changed [10]. In this study most of samples have been isolated from burn patients in Motahari hospital in Tehran because in this center Silver-sulfadiazine cream is the main drug for burn wounds and the probability to isolatation of resistant bacteria have been more possible. Identification of *Ps.aeruginosa* and *S.aureus* strains has been performed. Based on a study on antibiotical resistant in near years the minimum resistant was related to Emipenems [21]. Our results shows the minimum resistance to Emipenems(35%) which was similar to Abduli's results. Furthermore the minimum resistance to Vancomycin (3%) and the maximum resistance to Penicillin (100%) in *S.aureus* strains were reported which was similar to the results of the Saderi et al. findings [30]. The highest MIC to Gentamicin was 2500 µg/ml which was similar with Rahmani's results [25]. We observed that *Ps.aeruginosa* strains had MIC=0.01-80 µg/ml to silver nitrate. Furthermore the percentage of resistant strains to emipenem was 35% and to ciprofloxacin was 95%. In these strains the amount of MIC was 0.01 mg/ml and 70mg/ml to AgNO3. We demonstrated that 25 *Ps.aeruginosa* strains multi-drug resistant were resistant to AgNO3. The results of this study shows that more than72% of isolated strains were from burn cases related to *Ps.aeruginosa*. Bridge assumed strains with MIC around 8mg/l and less as sensitive to Gentamicin and strains with MIC around 16-32 mg/l as resistance. He proved the role of plasmid in appearing resistance to Ag, Gentamicin and carbonicillin [3]. Designed silver resistance gene's primers (*sil* genes) and aminoglycoside resistance gene's primer (*aac(6)´-IIa)* for PCR observed that 12 *Ps.aeruginosa* strains (17%) have coding gene for adhesive protein to Ag+(SilE)with 400bp molecular weight,13 *Ps.aeruginosa* strains (13.5%) have *silP* gene with 2500bp molecular weight and 3 *Ps.aeruginosa* strains with coding gene for membrane receptor ATP kinase which include Histidine (*silS*) with 1500bp molecular weight. These results are similar with Persival et al. findings. Persival and his assistances proved that *sil* genes exist in every strains of *Enterobacteriaceae* which isolated from foot wounds in Diabetes patients [23]. In isolated *S.aureus* strains none of *sil* genes existed. In 2006 during a study Silver and his assistances found that although *silE* gene shows the minimum resistance to Silver never seen without other *sil* gene [32]. The result of this study is similar with Silver's observations. In 12 *Ps.aeruginosa* strains which include *silE* gene *silP* gene also founded.We concluded that *aac(6)´-IIa* gene exists in only 10 *Ps.aeruginosa* strains(14%) which also include *silE* and *silP* genest. Nevertheless in the same strains the amount of MIC to silver nitrate was 80µg/ml. In these strains all three *silP* ، *silE*و *aac (6) ´-IIa* genes can be found together.The primary results show that resistance to silver may appear extensively but because of the lack of research tools is not totally clear. The large extensibility of Homolog factors of Sil proteins in plasmids and in bacterial chromosome may become a danger against using Silver and its derivatives as Bactericide when using antibiotics has been increased.

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