Nanomed. J., 4(1): 37-43, Winter 2017

ORIGINAL RESEARCH PAPER

Study of antimicrobial effects of several antibiotics and iron oxide nanoparticles on biofilm producing pseudomonas aeruginosa

*Khadijeh Ramezani Ali Akbari; Ahya Abdi Ali**

Department of Microbiology, Faculty of Biological Sciences, Alzahra University, Tehran, Iran

ABSTRACT

Objective(s): Pseudomonas aeruginosa is a nosocomial pathogen resistant to most antimicrobial treatments. Furthermore, it persists in adverse environments thereby forming biofilms on various surfaces. Researchers have therefore focused on antibiofilm strategies using nanoparticles due to their unique physicochemical properties. Superparamagnetic iron oxide nanoparticles (SIONPs) have recently shown to possess antimicrobial and anti-biofilm characteristics. In this study, the effects of SIONPs and some antibiotics were tested against strong biofilmproducing *P. aeruginosa* isolates.

Materials and Methods: 60 isolates of P.aeruginosa were screened for biofilm formation on microtiter plates using 0.1%w/v crystal violet (CV) staining. Twenty isolates producing strong biofilms were selected for further study on the effects of antimicrobial agents. Microdilution method was used to assay twenty isolates susceptible to antibiotics. The effects of antibiotics and SIONPs on biofilm formation were determined by the microdilution method and 0.1% CV staining. The checkerboard dilution technique was used to determine the combined effects of SIONPs and imipenem.

Results: In twenty isolates, the rate of resistance to ciprofloxacin, levofloxacin, amikacin, azithromycin was 65, 75, 45 and 95% respectively. SIONPs at 30 µg/ml reduced biofilm biomass in 11 isolates; however it stimulated biofilm formation in 9 isolates. The effects of SIONPs in combination with imipenem in the 10 isolates were different exhibiting synergistic or antagonistic relationships.

Conclusion: *P. aeruginosa* has increasingly developed resistance to many antimicrobial agents but the resistance to nanoparticles is less frequently been reported. However, iron oxide nanoparticles could enhance biofilm production in isolate- dependent manner because they may possibly utilize this nanoparticle as an iron source, an important element in biofilm production. The exact mechanism of these effects however, remains to be elucidated.

Keywords: *Antibiotics, Biofilm, Iron oxide nanoparticles, Pseudomonas aeruginosa, Resistance*

How to cite this article

Ramezani Ali Akbari K, Abdi Ali A. Study of antimicrobial effects of several antibiotics and iron oxide nanoparticles on biofilm producing pseudomonas aeruginosa. Nanomed J. 2017; 4(1): 37-43. DOI:10.22038/nmj.2017.8051

INTRODUCTION

P. aeruginosa is a mobile, non-fermentative Gram–negative pathogen that survives in various metabolic environments. This organism colonizes immunocompromised hosts using its secretory, cell- related virulence factors. In these individuals, the pathogen can infect body tissues and persist under various antimicrobial treatments. *P. aeruginosa* is often resistant to common antimicrobial agents because of its innate, acquired resistance mechanisms and ability to transfer genetic materials to the other bacteria [1, 2]. This high level of resistance increases 1000 times in biofilm cells versus planktonic cells. Biofilm is defined as a mono or multispecies adhesive community enclosed in a matrix. This matrix creates a tight barrier against the permeation of antimicrobial agents [3]. In biofilm, *P. aeruginosa* survives on biotic and abiotic surfaces such as catheters and medical devices. The biofilm phenotype plays an important role in urinary tract and respiratory infections particularly chronic respiratory infections in cystic fibrosis (CF) patients [4]. Biofilm cells are not killed by antibiotic treatments which are effective against planktonic

*Corresponding Author Email: *abdialya@alzahra.ac.ir*
Tel: (+98) *02188044040*

Note. This manuscript was submitted on November 5, 2016; approved on December 23, 2016

cells [5]. Consequently, biofilm formation in *P. aeruginosa* complicates the cure for chronic infections. This problem emphasizes the importance of anti-biofilm techniques in modern research. There are a few anti-biofilm strategies including, inhibiting bacterial adhesion to various surfaces, interfering with the quorum-sensing system, removing attached biofilms on surfaces and degrading the biofilm matrix. In some situations, these anti-biofilm methods reduce catheter -associated infections but more techniques are required *in vivo* to improve local and lasting delivery of their antimicrobials effects [6, 7].

Nanotechnology has found a special position in medical science. The production of nanoparticles has started an evolution in various medical treatments, diagnoses and drug deliveries [8].

Superparamagnetic iron oxide nanoparticles (SIONPs) recently have been used in biomedical areas such as MRI, drug delivery and hyperthermia because of its biocompatibility and magnetic properties [9]. Many studies have shown the size-dependent antimicrobial and anti-biofilm effects of these nanoparticles and their physico-chemical properties [10]. Nanoparticles do not induce the drug resistance in pathogens. This advantage of nanoparticles is possibly due to the following reasons: 1) using various antimicrobial mechanisms that reduce the possibility of resistance emerging to the nanoparticles 2) the high surface to volume ratio, in result the pathogens do not have a chance to develop the resistance and 3) nanoparticles can target antimicrobial agents to the site of infection, thus higher doses of drug can be given at the infected site, which overcomes resistance [11]. Therefore, in present study, the effects of iron oxide nanoparticles and some antibiotics were tested against various biofilm-producing *P. aeruginosa* strains.

MATERIALS AND METHODS

Bacterial strains

To investigate the ability to form the biofilm on surfaces, 60 strains of *P.aeruginosa* were collected from respiratory excretions and burn wounds (40 isolates from Burn hospital, Tehran, Iran and 20 isolates from cardiovascular hospital, Tehran, Iran). 60 isolates were characterized then cultured in Skim milk medium (15% glycerol), stored at -70 °C. Isolates were transferred from the stock cultures into tryptic soy agar (TSA) and were aerobically incubated at 37 °C for 24 h.

Antimicrobial agents

In this study, the four antibiotics were used including ciprofloxacin, levofloxacin, amikacin and azithromycin. Stock solution were prepared in suitable solvents and diluted as described by CLSI protocol [12]. SIONPs at concentrations 44 mg / ml and size 6-13nm was kindly provided by Dr J.Raheb (from National Institute of Genetic Egineering and Biotechnology) that were synthesized by co-precipitation method. In order to prevent aggregation of SIONPs together and its oxidation, it was coated by oleic acid.

MIC determination of antibiotics against 20 strong biofilm producing isolates

To test the isolates susceptibility to antibiotics, serial dilutions 0.25-512 µg /ml of four antibiotics (ciprofloxacin, levofloxacin, amikacin, and azithromycin) prepared in the wells of microtiterplate according to the CLSI standard. Suspension of cells in Muller Hinton broth (MHB) was adjusted to 0.5 McFarland tube and diluted 1:100 in MHB medium. Then, 100 µl of diluted antibiotics was added to each row of microtiter plate followed by addition of 100 µL of each isolates to the wells. Finally, microtiter plates were incubated at 37°C for 24 h. After incubation, a minimal dilution of antibiotic inhibited visually the bacterial grow, intended as MIC antibiotic. *P. aeruginosa* ATCC 27853 was used as standard strain. All tests were carried out in triplicate .The susceptibility of 20 isolates to antibiotics were measured as described by CLSI protocol [12].

Biofilm formation and assay in microtiterplate

The biofilm formation of 60 isolates on polystyrene microtiterplate was measured by 0.1%crystal violet (CV) staining. After 24 hour incubation, the bacterial suspension was adjusted to McFarland standard 0.5 in tryptic soy broth (TSB supplemented with 0.2% glucose). 100 of each suspension was inoculated to each well of plates that subsequently were incubated at 37 °C. Then, the planktonic cells were removed and plates were rinsed 3 times using physiological saline. Later, 250 µl of 0.1% CV solution was added to wells. After 20 min at room temperature, the wells were emptied and washed in order to remove excess CV dye. In next step, the microplates were forcefully tapped on napkins to remove any excess liquid and dried in air. After 20 min, 250 µl of 30% glacial acetic acid was added to

all wells. Following 10 min, 200 of any well were transferred to a new microplate and the absorbance was measured at 550 nm using ELISA plate reader. The experiments were repeated for 3 times. *P. aeruginosa* PAO1 is considered as a positive control and OD of medium as control OD (OD(c)). The isolates were divided into 4 classes according to the OD values: No adherent OD ≤ OD (c), Weak adherent OD (c) < OD ≤ 2 × OD (c), moderately adherent 2 × OD (c) < OD ≤ 4 × OD (c), Strong adherent 4 × OD (c) <OD [13, 14].

Twenty isolates producing the strong biofilm on microtiterplate were selected to examine effects of antimicrobial agents. To find out the anti-biofilm activities, TSB medium (0.2 % glucose) utilized to dilute serially the antibiotics (0.25-512 µg /ml) and SIONPs 0.234-30 µg/ml) through micro dilution method then 100 µl of 0.5 McFarland suspension added to wells of microtiterplate. Crystal violet staining was handled again to demonstrate the inhibition of biofilm formation in the presence of antibiotics and nanoparticles. A dilution of nanoparticles and antibiotics had absorption less than 10% positive control absorption was considered as minimum biofilm inhibition concentration (MBIC). To assay the anti-biofilm effects, the antibiotics including ciprofloxacin, levofloxacin, amikacin and SIONPs were examined separately for the isolates and each experiment was repeated 3 times. Azithromycin did not used in this experiment because of its high resistance (in some isolates, MIC was >1250 µg/ml) [15].

FBIC determination

To determin MBIC of SIONPs in combination with imipenem, diluted imipenem were added to plates via checkerboard dilution technique and fractional biofilm inhibition concentration ("FBIC) were then determined. In this method, 10 strong producing biofilm isolates exposured the dilutions 3.2-3275µg /ml of imipenem and 0. 3-30 g /ml SIONPs alone and in combination together. Finally, FBIC was calculated according to the following formula:

Index A and B show the first and second of antimicrobial compounds (imipenem and iron oxide nanoparticles).

$$FBIC\ (A) = \frac{MBIC\ (A\ in\ the\ presence\ of\ B)}{MBIC\ (A\ alone)}$$

$$FBIC\ (B) = \frac{MBIC\ (B\ in\ the\ presence\ of\ A)}{MBIC\ (B\ alone)}$$

$$\sum FBIC = FBIC\ (A) + FBIC\ (B)$$

To determine the relationship between the antimicrobial agents, $\sum FBIC$ was computed and interpreted as following: "FBIC ≤0.5: synergism, 0.5 ≤ "FBIC <1: partial synergism, 1 < "FBIC ≤ 2 indifference, "FBIC >2: antagonism [22].

Scanning Electron Microscopy (SEM)

Scanning electron microscopy was used to observe effects of SIONPs on biofilm formation. For this purpose, 1.5 mL bacterial suspension and 1.5 mL SIONPs added to 12-wells microtiter plate then glass slides (1cm× 1cm) were located in the wells. After incubation for 24h, slides were washed by sterile distilled water and immersed in Glutaraldehyde 4% solution for 2 h to dehydrate. In order to fix, all slides were placed for 15 min in the serial concentrations of 40 %, 60 %, 80 %, 90 % and finally 100 % of absolute ethanol for 24h respectively.

Biofilms on the surface of the glasses were lyophilized and glass surface images by VEGA3 TESCAN were recorded [16].

Statistical analysis

Statistical analysis is conducted by SPSS software using paired samples T test (P ≤ 0.05, 0.01). Reproducibility of data was determined by the coefficient of variation (CV).

RESULTS

Among the 60 isolates, 20 isolates (33.33%) formed strong biofilm in the microtiter plates. Ten of these isolates were from the cardiovascular hospital and the other 10 isolates were from the Burn hospital (Fig. 1).

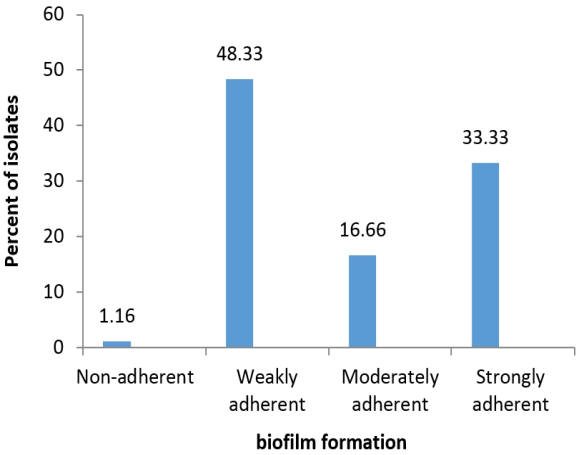


Fig.1. Percent of *P.aeruginosa* Isolates Formed Biofilm in Microtiter Plates

Table 1. The susceptibility of 20 strong biofilm producing *P.aeruginosa* to antibiotics

Isolates / Antibiotics	Resistant	Intermediate	Susceptible
Ciprofloxacin	65%	25%	10%
Levofloxacin	75%	15%	10%
Amikacin	45%	20%	35%
Azithromycin	95%	-	5%

As shown in Table 1, the rate of resistance of the 20 isolates producing strong biofilms to ciprofloxacin, levofloxacin, amikacin, azithromycin was 65, 75, 45 and 95% respectively. One isolate was susceptible to azithromycin and the MIC for azithromycin exceeded 1250 µg/ml in some isolates.

The Burn hospital isolates had higher resistance than the cardiovascular hospital isolates: 50% of the Burn hospital isolates were resistant to all the used antibiotics while 10% of the cardiovascular hospital isolates were resistant. Among the 4 antibiotics, amikacin showed the least resistance (10%) in the cardiovascular hospital isolates.

The most isolates formed the biofilms at 512 µg/ml of 3 antibiotics. Biofilm formation of some isolates was inhibited at 0.5-512 µg/ml.

In the 20 isolates studied 37%, 40 % and 60% of isolates were susceptible to ciprofloxacin, levofloxacin and amikacin respectively as shown in Table 2. The majority of isolates had higher MBIC higher than MIC for the antibiotics tested.

Iron oxide nanoparticles at 30 µg/ml reduced biofilm biomass (Fig. 2) for 11 isolates. However, it increased biofilm formation in 9 other isolates (Fig. 3). Paired samples T test showed significant differences in biofilm production between the presence and absence of SIONPs ($P \leq 0.01$).

Inhibitory effects of SIONPs in combination with imipenem differed among the 10 *P. aeruginosa* isolates that produced strong biofilms. Synergistic effects were observed for in 4 isolates ("FBIC $\leq$ 0.5) and 2 isolates showed antagonistic effects ("FBIC > 2) (Table 3).

Paired samples T test showed significant differences between SIONPs alone and in combination with imipenem ($P \leq 0.05$).

Table 2. MBIC of antibiotics among strong biofilm producing *P.aeruginosa* isolates

Isolates / Antibiotics	S15	B28	S21	S2	B51	B25	B5	S14	S16	S4	B293	B279	S17	B307
Ciprofloxacin (µg/ml)	128	16	16	8	16	32	32	R*	R	R	R	R	R	R
Levofloxacin (µg/ml)	2	16	16	0.5	16	64	32	16	R	R	R	R	R	R
Amikacin (µg/ml)	8	512	R	8	256	512	512	R	512	64	64	256	16	32

R*: resistant (>512 µg/ml concentrations)

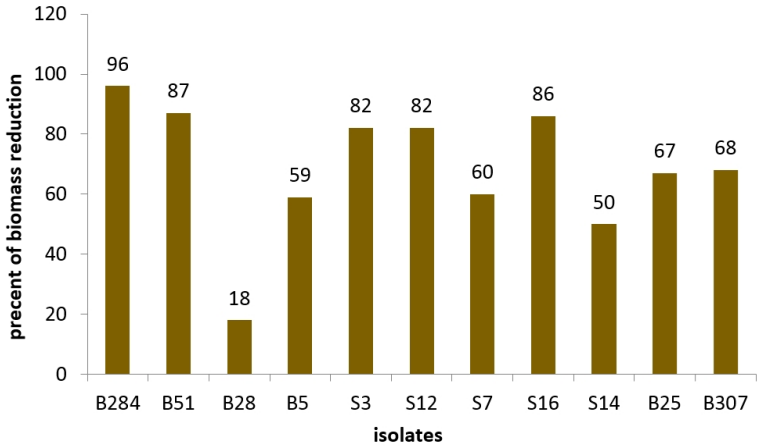


Fig.2. Reduction of biofilm biomass of 11 isolates in presence of iron oxide nanoparticles

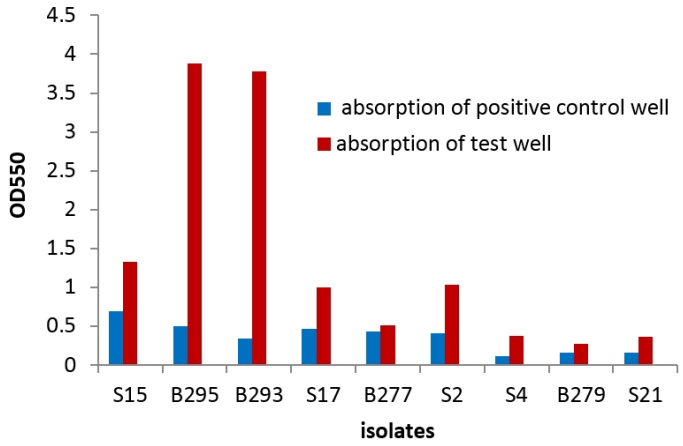
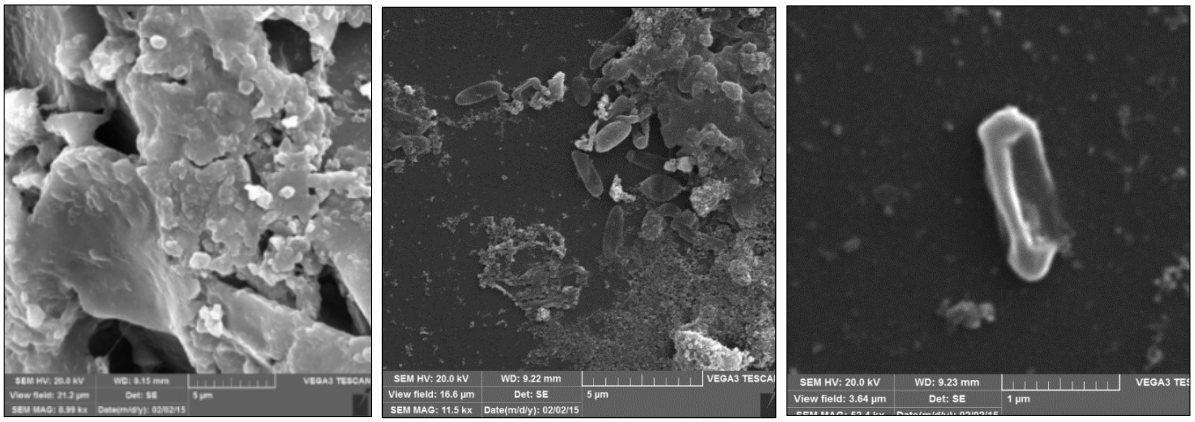


Fig.3. Increase of biofilm biomass of 9 isolates in presence of the iron oxide nanoparticles



a) b) c)

ig. 4. a) The biofilm formation in the positive control sample (magnification 8.99Kx)
b)The biofilm formation in presence of iron oxide nanoparticles(magnification 11.5Kx)
c) Morphologic modifications of bacterial cells in presence of iron oxide nanoparticles (magnification 62.4 Kx)

As shown in the scanning electron microscopy micrographs, SIONPs reduced biofilm formation on the surface of glass

(Fig. 4). Furthermore, it caused a morphologic change in the cells .This could be due to the toxic effects of active radicals released from the nanoparticles on the cell wall.

DISCUSSION

P. aeruginosa is a nosocomial pathogen involved in severe infections worldwide and is difficult to treat with the most common antibiotics (β-lactams, aminoglycosides and fluoroquinolones). Emergence of multidrug resistant *P. aeruginosa* has been increasingly reported worldwide in healthcare centers due to the inadequate or over-prescribed use of antibiotics in medical practices [2]. In this study, a high rate of resistance to 4 antibiotics (ciprofloxacin, levofloxacin, amikacin and azithromycin) was detected in 20 isolates producing strong biofilms. In the majority of isolates (70% isolates), biofilm formation was inhibited at higher concentrations of antibiotics than planktonic cells (MBIC>>MIC). The mechanisms of resistance in biofilm cells are different from planktonic cells. These include the biofilm matrix acting as a barrier against antimicrobial penetration into the biofilm, slow growth of the biofilm cells due to nutrient limitation, induction of the general stress response and the emergence of a biofilm-specific phenotype [4].

Table 3. Anti-biofilm combined relations of iron oxide nanoparticles and imipenem

Relationship	$\sum FBIC$	$FBIC\ (B^{**})$	$FBIC\ (A^{*})$	isolates
Indifference	$2 < \sum$FBIC ≤ 1	1	0.5	S_3
Antagonism	$\sum$FBIC > 2	2	2	S_{16}
Synergism	$\sum$FBIC ≤ 0.5	< 0.0153	0.062	S_{12}
Synergism	$\sum$FBIC ≤ 0.5	< 0.25	0.25	S_7
Synergism	$\sum$FBIC ≤ 0.5	≤ 0.00195	≤ 0.03	S_{14}
Partial synergism	$1 \leq \sum$FIC < 0.5	≤ 0.5	0.25	B_{25}
antagonism	$\sum$FBIC > 2	1	2	B_{307}
indifference	$2 < \sum$FBIC ≤ 1	$1 <$	1	B_5
Synergism	$\sum$FBIC ≤ 0.5	0.002	0.062	B_{51}
Partial synergism	$1 \leq \sum$FBIC< 0.5	< 0.031	0.5	B_{284}

Drug A*: Imipenem, Drug B**: Iron oxide nanoparticles.

Consequently, *P. aeruginosa* biofilm development on surfaces is considered as an additive strategy to overcome antibiotic therapy.

Nanoparticles are small enough to penetrate the biofilm matrix and have a high surface to volume ratio, which promotes effective interactions with bacteria. Taylor and Webster showed that 12 h treatment of SIONPs at 10 µg/ml disrupted *Staphylococcus epidermidis* colony assembly and prevented biofilm formation [10]. In this study, 30 µg/ml SIONPs reduced biofilm biomass in 11 isolates. However, this concentration increased biofilm formation in 9 other isolates. The opposite effects of SIONPs on biofilm formation may be attributed to differences in the isolates. Edwin Haney reported an increase in *P. aeruginosa* PAO1 biofilm biomass in the presence of SIONPs from Brown University and US Research Nanomaterial Inc. However, nanoparticles from Novacentrix had the opposite effect [17]. Another study reported the stimulatory effect of SIONPs at 5 mg/ml on biofilm formation in Gram-negative (*P. aeruginosa* and *E. coli*) and Gram – positive (*E.faecalis* and *B. subtilis*) bacteria. However, at lower concentrations of SIONPs, a slight anti-biofilm effect was observed [18]. These results show the importance of the physico-chemical features of SIONPs and its effective concentration on different bacteria. The anti-biofilm effect of SIONPs is formed by the release of active oxygen radicals (based on the fenton reaction) which consequently induces oxidative stress in bacterial cells and decreases eDNA levels in the biofilm matrix [17, 19]. On the other hand, these nanoparticles could serve as an iron source to enhance the biofilm production in some isolates and these isolates use from it for their pathogenicity and metabolism. The effects of SIONPs on *P. aeruginosa* biofilm should be studied in more details at the molecular level.

Previous studies have demonstrated the synergistic effects of silver nanoparticles with antibiotics against bacterial biofilm formation [20, 21]. Therefore, the present study focused on the combined effects of iron oxide nanoparticles in combination with imipenem on *P. aeruginosa* biofilm development.These effects were different in 10 isolates producing strong biofilms: synergistic and antagonistic effects were observed in 4 and 2 isolates, respectively. These paradoxical results could not be easily explained and need to be investigated further.

CONCLUSION

The suitable administration of antibiotics in patients would prevent resistance developing in bacterial and consequently reduce the cost of treatments and the time spent in hospitals.

The effects of SIONPs on *P. aeruginosa* biofilm are complex and isolate dependent. More studies should be conducted to further investigate the use of SIONPs as an anti-biofilm agent in medicine.

ACKNOWLEDGEMENTS

The authors wish to thank Vice Chancellor of Alzahra University for the financial support and Burn and Cadiovascular hospitals for providing the bacterial isolates.

CONFLICT OF INTEREST

The authors declare that there are no conflicts of interest regarding the publication of this manuscript.

REFERENCES

1.Morita Y, Tomida J, KawamuraY. Responses of Pseudomonas aeruginosa to antimicrobial. Front Microbiol. 2014; 4 (422): 1-8.

2.Strateva T and Yordanov D. Pseudomonas aeruginosa – a phenomenon of bacterial resistance. J Med Microbiol. 2009; 58: 1133–1148.

3.Drenkard E. Antimicrobial resistance of Pseudomonas aeruginosa biofilms. Microbes Infect. 2003; 5(13): 1213-1219.

4.Thien-Fah C Math and O'Toole G A. Mechanisms of biofilm resistance to antimicrobial agents. Trends Microbiol. 2001; 9(1): 34-39.

5.Costerton JW, Stewart PS, Greenberg EP. Bacterial Biofilms: A Common Cause of Persistent Infections. Science. 1999; 284(5418): 1318–1322.

6.Danese P N. Antibiofilm Approaches: Prevention of Catheter Colonization. J Biol Chem .20002; 9(8): 873–880.

7.Francolini1 I and Donel G. Prevention and control of biofilm-based medical-device-related Infections. FEMS Immunol Med Microbiol. 2010; 59(3): 227–238.

8.Lellouche J and Friedman A. Antibacterial and antibiofilm properties of yttrium fluoride nanoparticles. Int J Nanomedicine. 2012; 7: 5611-5624.

9.Wu W, He Q, Jiang C. Magnetic Iron Oxide Nanoparticles: Synthesis and Surface Functionalization Strategies. Nanoscale Res. Lett. 2008; 3: 397–415.

10.Taylor EN and Webster TJ. The use of superparamagnetic nanoparticles for prosthetic bioflm prevention. Int. J. Nanomedicine. 2009; 4: 145–152.

11.Pelgrift RY and Friedman AJ. Nanotechnology as a therapeutic tool to combat microbial resistance. Adv Drug Deliv Rev. 2013; 65: 1803–1815.

12.CLSI. (2012). Methods for Dilution Antimicrobial Susceptibility Tests for Bacteria That Grow Aerobically; Approved Standard—Ninth Edition. CLSI document M07-A9. Wayne, PA: Clinical and Laboratory Standards Institute.

13.Stepanovic S, Vukovic D, Hola V. Quantification of biofilm in microtiter plates: overview of testing conditions and practical recommendations for assessment of biofilm production by staphylococci. APMIS. 2007; 115(8): 891-899.

14.Sabaeifard P, Abdi-Ali A, Soudi M, Dinarvand R. Optimization of tetrazolium salt assay for Pseudomonas aeruginosa biofilm using microtiter plate method. J Microbiol Methods. 2014; 105: 134–140.

15.Stepanovic S, Vukovic D, Dakic, I, Savic, B, Svabic-Vlahovic M. A. modified microtiter-plate test for quantification of staphylococcal biofilm formation. J Microbiol Methods. 2000; 40(2): 175-179.

16.Kolari M, Mattila, Mikkola K, Salkinoja-Salonen R. Community structure of biofilms on ennobled stainless steel in Baltic Sea water. J Ind Microbiol Biot. 1998; 21(6): 261-274.

17.Edwin Haney C. Effects on iron nanparticles on Pseudomonas aeruginoa biofilms. Electronic Thesis & Dissertations Center. 2011; 1-74.

18.Proda AM, Iconaru SL, Chifiriuc CM. Magnetic Properties and Biological Activity Evaluation of Iron Oxide Nanoparticles. J Nanomater. 2013; 1-7.

19.Touati D. Iron and oxidative stress in bacteria. Arch Biochem Biophys. 2000; 373: 1–6.

20.Hendiani S, Abdi- Ali A, Mohammadi P, Kharrazi Sh. Synthesis of silver nanoparticles and its Synergistic effects in combination with imipenem and two biocides against biofilm producing Acinetobacter baumannii. Nanomed J. 2015; 2(4): 291-298.

21.Li P, Li J, Wu Ch. Synergistic antibacterial effects of â lactam antibiotic combined with silver nanoparticles. Nanotechnology. 2005; 16(9): 1912–1917.

22.Gould I, M., Wilson, D., Milne, K., Paterson, A., Golder, D., Russell, D. Interaction of imipenem with erythromycin and tetracycline assessed by microdilution checkerboard techniques. Antimicrob Agents Chemother. 1991; 35(11): 2407-2409.