ORIGINAL ARTICLE

Antisense *mqsR*-PNA as a putative target to the eradication of *Pseudomonas aeruginosa* persisters

L. B. Al Husseini[1], **A. Maleki**[2] **and M. F. Al Marjani**[1]

1) *Department of Biology, College of Science, Mustansiriyah University, Baghdad, Iraq and* 2) *Clinical Microbiology Research Centre, Ilam University of Medical Sciences, Ilam, Iran*

Abstract

Chemotherapy is frequently unsuccessful in fully eradicating bacterial biofilm infections. Persisters are a main cause for the failure of antibiotic therapies and are assumed to significantly impact the increased multidrug tolerance and unsuccessful elimination of chronic biofilm infections. *Pseudomonas aeruginosa* infections are frequently linked to high rates of drug-tolerant persisters, triggering a major challenge to human health. It is crucial to classify persisters to develop novel useful therapeutic strategies to fight infectious diseases. In this study, the *mqsR* gene was selected as a novel antimicrobial target, and silencing was with antisense peptide nucleic acid (PNA) assay to eradicate the *P. aeruginosa* persisters. First, they were analysed by experimental procedures. Functionality was assessed by stress conditions. We found that the expression of *mqsR* (as the toxin) compared with *mqsA* (as antitoxin) was increased under stress conditions. We demonstrated that when *mqsR* was targeted and treated with different concentrations of *mqsR*-PNA after 24 hours; the formation of *P. aeruginosa* persisters was eradicated. Antisense *mqsR*-PNA in concentrations of 35 μM or more could eradicate persister cell formation in *P. aeruginosa*. It was suggested that other toxin–antitoxin loci in *P. aeruginosa* are examined by antisense PNA to detect their functionality. However, considering the importance of persisters in human infections, *ex vivo*, *in vivo*, preclinical and clinical settings should be highlighted.

Keywords: Eradication, peptide nucleic acid assay, persister cells, *Pseudomonas aeruginosa*, TA system
Original Submission: 3 May 2020; **Revised Submission:** 10 February 2021; **Accepted:** 12 March 2021
Article published online: 19 March 2021

Corresponding author: M.F. Al Marjani, Mustansiriyah University, Palestine Street, P.O.Box: 10052, Baghdad, Iraq.
E-mails: marjani20012001@gmail.com, dr.marjani@uomustansiriyah.edu.iq

Introduction

Pseudomonas aeruginosa is a human opportunistic, life-threatening pathogen, especially in individuals who lack normal defences such as those with serious illness or who are immunocompromised; it rarely causes disease in healthy individuals. It is the most prevalent nosocomial pathogen causing morbidity and mortality in hospitalized patients. The important of this pathogen rests in the presence of multidrug-resistant strains [1–3].

Toxin–antitoxin (TAs) systems are abundant genetic modules presented on plasmids or chromosomes of bacteria as an operon. TAs are typically comprised of closely linked genes for toxin and its cognate antitoxin. A wide variety of cellular activities belonging to TA systems have been clarified in the last decade and most of them are involved in crucial cellular processed. The first TA system shown to be involved in biofilm formation was *mqsRA*, a typical type II TA, in which the toxicity of the protein MqsR is neutralized by its conjugate antitoxin MqsA. Several studies have demonstrated that toxin MqsR is significantly stimulated by biofilm formation and enhanced cell motility and has a major role in persister formation in *P. aeroginosa* [4–6].

Persisters are dormant, slow-growing or growth-arrested phenotypic variants of normal cells in bacterial populations and are transiently antibiotic-tolerant. These cells form a subpopulation of non-dividing cells that exhibit multidrug tolerance and survive treatment by all known antimicrobial agents. They

https://doi.org/10.1016/j.nmni.2021.100868

TABLE 1. The sequences of the primers that were used in the PCR and Real Time PCR

Gene	Primer sequence (5′→3′)	Denaturation temperature (°C)	Annealing temperature (°C)	Extension temperature (°C)	Cycle
mqsR	F: ACGCACACCACATACACGTT R: GCCTGGGTCTGTAAACATCCT	95	58.7	72	34
mqsA	F: AATGTCCGGTTTGCCACCAG R: GCATTCACCGAAGCCCGAAA	95	58.7	72	34
gyrB	F: CTGCTTCACCAACAACATCC R: GGTGGCGATCTTGAACTTCT	95	58.7	72	34

withstand stress by entering a dormant state in which cellular processes are inactive [7–16].

Persister cells are formed by Gram-negative and Gram-positive bacteria related to biofilms, such as *P. aeruginosa*, *Escherichia coli* and *Staphylococcus aureus* [17–21]. Hence, it is important to categorize them in order to develop alternative and novel therapeutic strategies to fight infectious diseases. Antisense treatment approaches are a biotechnological form of antibacterial agents that use chemical analogues of short single-stranded nucleic acid sequences modified to form stable oligomers. Peptide nucleic acid (PNA) is characterized as a novel approach in antisense treatment. The properties of PNA make it appropriate for antisense treatment in bacterial cells [22,23]. In this study, antisense treatment is applied for artificial blocking of TA systems as an antibacterial strategy. The aim of the current study was the eradication of *P. aeruginosa* persister cells by antisense *mqsR*-PNA.

Materials and methods

Bacterial isolates and identification

In this study, a total of 80 unduplicated *P. aeruginosa* isolates were collected from different clinical samples (burn, wound swab, urine, sterile body fluids, ear and catheters) and identified by routine biochemical and microbiological tests between May 2017 and March 2018 from Baghdad hospitals, Iraq. Stock cultures were preserved in TSB (HiMedia, Mumbai, India) containing 15% glycerol (HiMedia) at −80°C.

Bacterial persister cells assay

To identify the persistence of *P. aeruginosa* isolates, we used the previously described protocol of Canas-Duarte *et al.* [24]. In brief, overnight cultures were diluted to a turbidity of 0.5 McFarland, then 1 mL of 0.5 McFarland was used to induce persister formation. Then the bacterial cultures were exposed to 200 µL of lysis solution and lysozyme. Finally, cell survival was determined by overnight drop culture.

DNA extraction and evaluation of the *mqsR*, *mqsA* genes

Total DNA was extracted using the genomic DNA extraction kit (WizPrep gDNA mini Kit; WizBio Solutions, Seongnam, South Korea) according to the manufacturer's protocol. DNA purity, quality and quantity were measured using a NanoDrop instrument (Titertek Berthold, Pforzheim, Germany). Total extracted DNAs of isolates were immediately stored at −20°C for further analysis. PCR were carried out using specific primers for amplifying *mqsRA* genes as described previously [25]. Subsequently, PCR products were observed using electrophoresis with 1% agarose gel stained by DNA safe on a Gel Doc XR+ system (Bio-Rad, Hercules, CA, USA). The sequences of the primers and conditions are listed in Table 1. PCR products were analysed on 1% (weight/volume) agarose gels by electrophoresis (Merck, Darmstadt, Germany). PCR products were visualized under UV and photographed.

Growth of *P. aeruginosa* in normal and stress conditions

Pseudomonas aeruginosa persister cells were cultured in Mueller–Hinton agar and incubated at 37°C overnight. One colony was resuspended in Mueller–Hinton broth medium for normal conditions, while to create stress conditions other bacteria were cultured in Mueller–Hinton broth media in the presence of ciprofloxacin (256 µg/mL).

Evaluation of *mqsR* and *mqsA* gene expression in normal and stress conditions

First, RNA was extracted under normal and stress conditions with a Total RNA Prep Kit (BioFACT™, Daejeon, South Korea). Briefly, the isolates were cultured on nutrient agar (Merck) for 24 hours and incubated at 37°C. Then, the isolates were inoculated into Luria–Bertani broth medium (Merck) and incubated for 24 hours at 37°C, and in the presence of ciprofloxacin (256 µg/mL) as normal and stress conditions, respectively. RNA was extracted under normal and stress conditions using Total RNA Prep Kit (BioFACT™) according to the manufacturer's instructions.

In the next step, the cDNA was synthesized using BioFact™ RT Series (BioFACT™). The specific primers for the TA genes and *gyrB* (housekeeping gene) were synthesized by Alph DNA (Montreal, Quebec H3C 0J7, Canada).

To measure the activities of *mqsR* and *mqsA* genes in normal and stress conditions, real-time PCR was performed. The *gyrB* gene was used as a housekeeping gene. The Real-Time PCR System (Bio-Rad) was used with the following conditions: 95°C for 3 minutes as an initial step for DNA polymerase activation; 95° C for 15 seconds, 58.5°C for 30 seconds as the annealing step and 68°C for 30 seconds as the extension step (40 cycles for each). The lack of primer dimers was proved by the melting curve, as well as by running the PCR products in a 1% agarose gel. The primer sequences for *mqsR* and *mqsA* genes are listed in Table 1.

PNA design and PNA array

To design antisense PNAs complementary to *mqsR* mRNAs, the −20 to +20 region of the start codon was targeted. The PNA synthesized by Panagene Company (Daejeon, South Korea) was as follows: *mqsR*: (KFF) 3K-AEEA- ATGGAAAA GTAC-k. To improve the cell entry (KFF) 3K (K is lysine and F is phenylalanine) was covalently conjugated to PNA, with the linker AEEA (aminoethoxyethoxyacetic acid).

Antisense *mqsR*-PNA treatment of *P. aeruginosa* persisters

An MIC assay was performed for each PNA and isolates according to the protocol of broth microdilution methods [26,27]. To determine the MIC for *mqsR*-PNA in persister *P. aeruginosa* we used the turbidity assay by McFarland scale. Briefly, a 0.5 McFarland standard of persister *P. aeruginosa* containing the *mqsR* TA gene was prepared. Then, 200 µL of 0.5 McFarland of persister suspension was added to Costar microplates (Sigma-Aldrich Chemie GmbH, Taufkirchen, Germany), and various concentrations of PNAs 1−35 µM and fivefold of MIC of ciprofloxacin were added for evaluation. The Costar microplate was incubated at 37°C, then the plate was read with a spectrophotometer at OD_{620} every 2 hours. The effects of PNA at different concentrations (1−35 µmol) against *P. aeruginosa* were examined. The negative control was Mueller−Hinton broth, and Mueller−Hinton broth containing bacteria without PNA was used as the positive control.

Evaluation of genes after PNA treatment

The quantitative RT-PCR was performed to evaluate the expression level of *mqsR* and *mqsA* genes after treatment of the culture with PNA. This method was used to detect the ability of PNA in controlling expression of *mqsR* in persistence of *P. aeruginosa*. The method of quantitative RT-PCR has been described above.

Statistical analysis

To determine the fold change of the turbidity assay, the optical densities of samples were obtained as follows: OD_{real} = OD samples − OD negative control; second, the OD was changed to CFU/mL as McFarland scale. Then, folds of both quantitative PCR and turbidity assay were obtained as follows: Fold change = concentration of positive control/concentration of samples. The negative control was PNA plus medium without bacteria.

Results

Bacterial persisters and TAs loci in *P. aeruginosa*

Using the Canas-Duarte *et al.* protocol [24], six *P. aeruginosa* persister isolates were found. The presence of *mqsR* and *mqsA* genes was investigated, and results showed that all 80 isolates (100%) were positive for both TA genes.

The expression of TA loci on normal and stress conditions

In normal conditions, both genes were expressed but we found that the expression of *mqsR* (as the toxin) compared with *mqsA* (as antitoxin) was increased under stress conditions. Hence, *mqsR* could play a role in persistence formation.

Eradication of persister cells by antisense *mqsR*-PNA therapy

For a gene to be targeted for drug discovery, it must play a key role in the survival of the organism. Accordingly, these genes play an essential role in biofilm formation and stringent responses. However, because of the important role of the *mqsRA* TA system in pathogenic bacteria, the expression of *mqsR* and *mqsA* genes, as attractive targets, under stress and normal conditions, was investigated. Surprisingly, our findings showed that the expression of the *mqsA* gene under stress conditions was stopped, whereas expression of the *mqsR* gene showed higher expression. Interestingly, the expression of these two genes in normal conditions was almost equal.

Our results clearly confirmed that when *mqsR* mRNA was inhibited, the formation of persisters was eradicated (Figs. 1−3). After treatment with different concentrations of *mqsR*-PNA, the findings indicated that the expression of *mqsR* was decreased and there was an increase of antitoxin (*mqsA*), which confirmed the eradicated persistence activity of *mqsR*-PNA (Fig. 1). Fig. 2 also depicts the eradication of persister *P. aeruginosa* after treatment of *mqsR*-PNA at 35 µM concentration after 24 hours. Fig. 3 also shows that a concentration of 35 µM from *mqsR*-PNA led to complete removal of all forms of bacteria, both normal and persister cells.

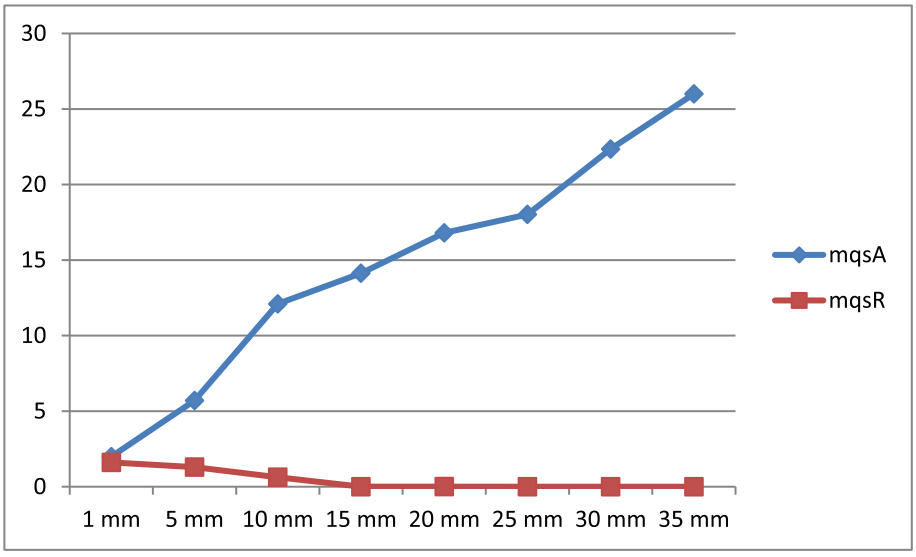

FIG. 1. Results of RT-qPCR for *mqsR*-PNA in different concentrations (1–35 μmol). *mqsR* silencing with peptide-PNAs in *Pseudomonas aeruginosa*. *mqsR* was slowly inhibited by *mqsR*-PNA in different concentrations. The *y*-axis is the rate of expression in fold change. The *x*-axis is the different concentrations of *mqsR*-PNA. * mm: μmol.

Discussion

Chronic infections present a therapeutic challenge and removal of *P. aeruginosa* persisters is difficult. On the other hand, *P. aeruginosa* persisters also show resistance to several antibiotics and are responsible for many infections [1].

This persistence is a major medical challenge, as it leads to the overuse of antibiotics and therefore to increased antimicrobial resistance [5,6]. As we know the stresses that bacteria encounter during the infection of a host are triggers for the formation of persisters [5,6].

Various biological functions are proposed for TA systems and experimental evidence has shown that these systems have a

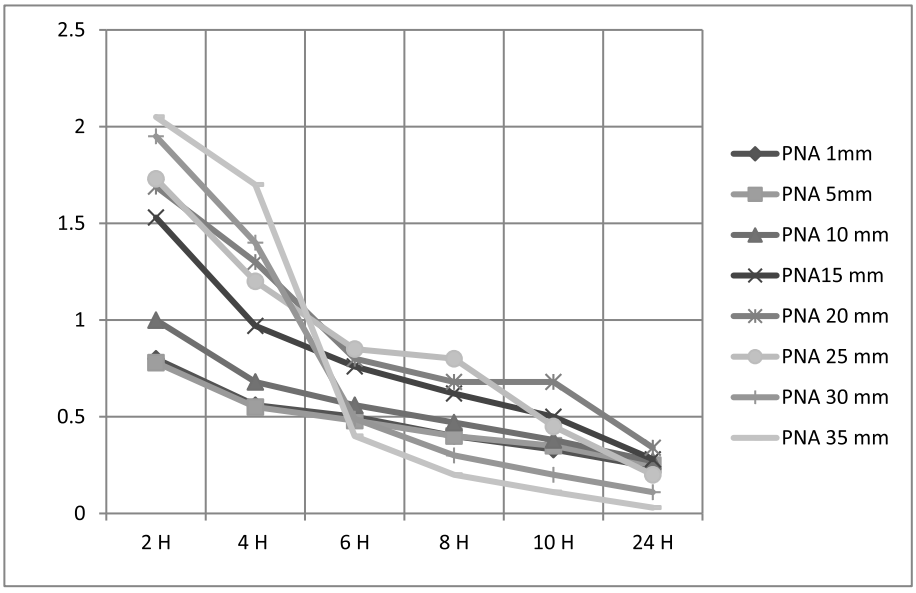

FIG. 2. Alterations OD of persister *P. aeruginosa* after treating with different concentrations of *mqsR*-PNA after 24 hours. The *mqsR*-PNA led to growth inhibition of persister *P. aeruginosa* at 35 μM concentration after 24 hours. Y-axis: OD 620nm; X-axis: time (hour).

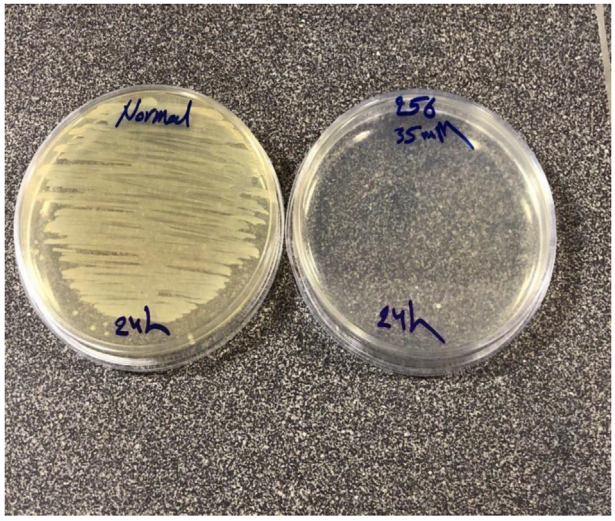

FIG. 3. A concentration of 35 μM from *mqsR*-PNA completely removes bacteria and precursor cells. Left plate shows normal conditions without the use of any *mqsR*-PNA and the right plate is after treatment with 35 μM *mqsR*-PNA.

wide range of functions, including defence against bacteriophages, formation of persister cells and programmed cell death. Generally, given the nature of TA systems, the toxin is neutralized permanently by the antitoxin [28].

Many studies have been conducted with PNA, and they have confirmed that because of the good stability of PNA, its non-toxicity even at high concentrations and its high specificity for the target mRNA, it is an exciting procedure for researchers to pursue. We targeted the TA system of *P. aeruginosa* persisters with antisense PNA and have collected valuable data. Several previous reports have demonstrated the effective role of the PNA method against pathogens [29–31].

On the one hand, as shown in the results, the *mqsRA* TA system showed low *mqsA* antitoxin expression and higher *mqsR* expression under stress conditions. Therefore, the functionality was confirmed, when the antitoxin was exposed by PNA, the toxicity killed the bacteria, and its function was confirmed. Hence, our findings represent a new candidate for antimicrobial targeting in *P. aeruginosa* persisters.

Considering the importance of *P. aeruginosa* and its potential for infection, the focus is on finding genes that are involved in pathogenesis and are essential for the survival of the organism, which could open a new window on the microbiology of the organism.

It is important that PNA can kill microorganisms in a short time and at very low concentrations, such as micromolar, compared with antibiotics. Therefore, it is recommended that other virulence genes in *P. aeruginosa* are analysed by antisense PNA to detect their functionality and identify their role. Also, it is clearly necessary for *mqsR*-PNA to be extensively assayed for its safety in subsequent research in cell culture, animal studies, preclinical studies or clinical trials.

Conflict of interest

There are no conflicts of interest.

Authors' contributions

LBAH and AM, contributed to the conception and design of the work. MFAM contributed in drafting the work and revising it critically for intellectual content. AM and MFAM contributed to revising the article and gave final approval of the version to be published.

Acknowledgements

We are grateful for the useful comments and suggestions from anonymous referees.

References

[1] Sultana ST, Call DR, Beyenal H. Eradication of *Pseudomonas aeruginosa* biofilms and persister cells using an electrochemical scaffold and enhanced antibiotic susceptibility. NPJ Biofilms Microbiomes 2016;2:1–8.
[2] Alhusseini LB, Maleki A, Kouhsari E, Ghafourian S, Mahmoudi M, Al Marjani MF. Evaluation of type II toxin-antitoxin systems, antibiotic resistance, and biofilm production in clinical MDR *Pseudomonas aeruginosa* isolates in Iraq. Gene Rep 2019;17:100546.
[3] Sedighi M, Moghoofei M, Kouhsari E, Pournajaf A, Emadi B, Tohidfar M, et al. In silico analysis and molecular modeling of RNA polymerase, sigma S (RpoS) protein in *Pseudomonas aeruginosa* PAO1. Rep Biochem Mol Biol 2015;4:32.
[4] Neidig A, Yeung AT, Rosay T, Tettmann B, Strempel N, Rueger M, et al. TypA is involved in virulence, antimicrobial resistance and biofilm formation in *Pseudomonas aeruginosa*. BMC Microbiol 2013;13:77.
[5] Gerdes K, Maisonneuve E. Bacterial persistence and toxin–antitoxin loci. Annu Rev Microbiol 2012;66:103–23.
[6] Szabó MÁ, Varga GZ, Hohmann J, Schelz Z, Szegedi E, Amaral L, et al. Inhibition of quorum-sensing signals by essential oils. Phytother Res 2010;24:782–6.
[7] Moradkasani S, Kouhsari E, Jazi F, Kalani B, Pakzad I. Evaluation of putative toxin–antitoxins systems in clinical *Brucella melitensis* in Iran. Infect Disord Drug Targets 2021;21(1):38–42.
[8] Christensen SK, Gerdes K. RelE toxins from bacteria and Archaea cleave mRNAs on translating ribosomes, which are rescued by tmRNA. Mol Microbiol 2003;48:1389–400.
[9] Christensen SK, Pedersen K, Hansen FG, Gerdes K. Toxin–antitoxin loci as stress-response-elements: ChpAK/MazF and ChpBK cleave

translated RNAs and are counteracted by tmRNA. J Mol Biol 2003;332:809–19.
[10] Yoshida H, Maki Y, Kato H, Fujisawa H, Izutsu K, Wada C, et al. The ribosome modulation factor (RMF) binding site on the 100S ribosome of *Escherichia coli*. J Biochem 2002;132:983–9.
[11] Dawson CC, Intapa C, Jabra-Rizk MA. "Persisters": survival at the cellular level. PLoS Pathogens 2011;7(7).
[12] Hu Y, Kwan BW, Osbourne DO, Benedik MJ, Wood TK. Toxin YafQ increases persister cell formation by reducing indole signalling. Environ Microbiol 2015;17:1275–85.
[13] Jaiswal S, Paul P, Padhi C, Ray S, Ryan D, Dash S, et al. The Hha-TomB toxin–antitoxin system shows conditional toxicity and promotes persister cell formation by inhibiting apoptosis-like death in *S. Typhimurium*. Sci Rep 2016;6:38204.
[14] Kuroda A, Nomura K, Ohtomo R, Kato J, Ikeda T, Takiguchi N, et al. Role of inorganic polyphosphate in promoting ribosomal protein degradation by the Lon protease in *E. coli*. Science 2001;293(5530):705–8.
[15] Wiuff C, Zappala R, Regoes R, Garner K, Baquero F, Levin B. Phenotypic tolerance: antibiotic enrichment of noninherited resistance in bacterial populations. Antimicrob Agents Chemother 2005;49:1483–94.
[16] Lewis K. Persister cells. Annu Rev Microbiol 2010;64:357–72.
[17] Al-Dhaheri RS, Douglas LJ. Absence of amphotericin B-tolerant persister cells in biofilms of some *Candida* species. Antimicrob Agents Chemother 2008;52:1884–7.
[18] Jabra-Rizk MA, Falkler WA, Meiller TF. Fungal biofilms and drug resistance. Emerg Infect Dis 2004;10:14.
[19] Keren I, Shah D, Spoering A, Kaldalu N, Lewis K. Specialized persister cells and the mechanism of multidrug tolerance in *Escherichia coli*. J Bacteriol 2004;186:8172–80.
[20] Kumamoto CA. *Candida* biofilms. Curr Opin Microbiol 2002;5:608–11.
[21] LaFleur MD, Kumamoto CA, Lewis K. *Candida albicans* biofilms produce antifungal-tolerant persister cells. Antimicrob Agents Chemother 2006;50:3839–46.
[22] Braasch DA, Corey DR. Novel antisense and peptide nucleic acid strategies for controlling gene expression. Biochemistry 2002;41:4503–10.
[23] Sahu N, Shilakari G, Nayak A, Kohli DV. Peptide nucleic acids: a novel approach. Curr Chem Biol 2008;2:110–21.
[24] Canas-Duarte SJ, Restrepo S, Pedraza JM. Novel protocol for persister cells isolation. PLoS One 2014;9(2).
[25] Hemati S, Azizi-Jalilian F, Pakzad I, Taherikalani M, Maleki A, Karimi S, et al. The correlation between the presence of quorum sensing, toxin-antitoxin system genes and MIC values with ability of biofilm formation in clinical isolates of *Pseudomonas aeruginosa*. Iran J Microbiol 2014;6:133.
[26] Coyle MB. Manual of antimicrobial susceptibility testing. BCIT Imaging Services; 2005.
[27] Stephen J, Ronald J, Yvette S, José H, Ivonne D, Robert L, et al. Manual of antimicrobial susceptibility testing. Washington. Am Soc Microbiol 2005:39–40.
[28] Page R, Peti W. Toxin-antitoxin systems in bacterial growth arrest and persistence. Nat Chem Biol 2016;12:208.
[29] Good L, Awasthi SK, Dryselius R, Larsson O, Nielsen PE. Bactericidal antisense effects of peptide–PNA conjugates. Nat Biotechnol 2001;19:360–4.
[30] Maleki A, Ghafourian S, Pakzad I, Badakhsh B, Sadeghifard N. mazE antitoxin of toxin antitoxin system and fbpA as reliable targets to eradication of *Neisseria meningitidis*. Curr Pharmaceut Des 2018;24:1204–10.
[31] Valadbeigi H, Sadeghifard N, Salehi MB. Assessment of biofilm formation in *Pseudomonas aeruginosa* by antisense mazE-PNA. Microb Pathog 2017;104:28–31.