Effect of gold nanoparticles on the expression of efflux pump mexA and mexB genes of Pseudomonas aeruginosa strains by Quantitative real-time PCR

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Abstract

Antibiotic-resistant Pseudomonas aeruginosa infections are usually difficult to treat, and there are limited antibiotics for treating them. Increased antibiotic resistance of this bacterium, especially in a multidrug form, has caused many problems for treatment. Nowadays, metal nanoparticles are considered as appropriate alternatives to antibiotics. The objective of the present study was to investigate the effect of gold nanoparticles on the expression of MexB and MexA genes in Pseudomonas aeruginosa isolates. Pseudomonas aeruginosa isolate was identified using biochemical tests and an API kit. The antibiotic sensitivity test for different antibiotics was performed with the Kirby-Bauer test according to the CLSI standard. The presence of MexB and MexA genes was assessed by PCR. The effect of gold nanoparticles was investigated by microdilution to evaluate the minimum inhibitory concentration, and the expression of MexB and MexA treated genes was done with silver nanoparticles by the Real-Time PCR method. 40 Pseudomonas aeruginosa isolates were detected and identified. These isolates showed significant resistance to various antibiotics. All strains were carriers of MexB and MexA genes, and finally, in the expression of MexB and MexA genes, a significant decrease in the expression of these genes was observed in the samples treated with gold nanoparticles compared to non-treated samples. One of the mechanisms of antibacterial activity of gold nanoparticles is through reducing the expression of mexA and mexB genes and thus reducing the number of active efflux pumps at the cell surface.

Keywords

Pseudomonas aeruginosa, gold nanoparticles, MexA, MexB, Real Time PCR

Introduction

Pseudomonas aeruginosa is a gram-negative opportunistic pathogen that is a major cause of death in immunocompromised patients due to the creation of life-threatening infections (Sadikot et al. 2005; Gellatly et al. 2013). Pseudomonas aeruginosa is one of the main causes of septicemia, urinary tract infection, and endocarditis, as well as skin, eye, and ear infections in hospitalized burn-injured patients (Ghazi et al. 2012; Jafari et al. 2013). Pseudomonas aeruginosa is resistant to antimicrobials through various mechanisms; for instance, chromosomal mechanisms of antibiotic resistance, induced
by efflux pump systems, have been reported in the membrane of this microorganism (Munna and Arias 2016). Pseudomonas aeruginosa has the potential to express 12 types of multidrug leakage pumps called Mex-Type Multidrug Resistance Efflux Pump; the five of them named MexAB-oprM, MexXY-oprM, MexEF-oprN, MexCD-oprT, and MexJK-oprM are the most significant factors of resistance to antibiotics. MexAB-oprM is the most important system for removing antimicrobial compounds from the cell and resolving inherent drug resistance in Pseudomonas aeruginosa (Goli et al. 2018). Moreover, unlike other pumps that are of the acquired type and less important clinically, MexAB-oprM is the only leakage pump that is inherently expressed in wild strains (Nehme and Poole 2007). This situation prompts the development of alternative therapeutic strategies for bacteria, and if appropriate inhibitors are used, it can disrupt the function of these pumps to inhibit multidrug resistance. Due to their antimicrobial potential, some nanoparticles, such as gold nanoparticles, can be effective as an alternative method of antibiotics to fight bacterial infections. Gold has antibacterial, viral, and fungal properties (Kalishwaralal et al. 2010). Gold nanoparticles can also simultaneously affect vital parts of the microbial cell such as protein, energy, and DNA production (Rai et al. 2009). The purpose of the present study is to determine the antibacterial effect of gold nanoparticles on the expression of important genes of the MexAB-oprM efflux pump system called mexA and mexB in Pseudomonas aeruginosa isolates.

Materials and methods

The present study was conducted on Pseudomonas aeruginosa strains in patients suspected of having Pseudomonas aeruginosa infections and referred to hospitals and health centers in the south of Fars province, Iran, within 12 months from September 2018 to September 2019. The clinical samples were burn, throat, nose, and 6 from the referred patients.

Identification and detection

Identification and detection of Pseudomonas were made biochemically using an API kit. In the biochemical method, gram staining and biochemical detection tests including oxidase, evaluation of fermentation on TSI, oxidation of glucose in the OF medium, evaluation of motility on SIM agar, oxidase, evaluation of fermentation on TSI, oxidation of glucose in the OF medium, evaluation of motility on SIM agar, identification and detection of Pseudomonas aeruginosa isolates. The present study was conducted on Pseudomonas aeruginosa isolates.

### Antibiotic sensitivity

In order to evaluate the antibiotic sensitivity of Pseudomonas aeruginosa strains, the disc diffusion method was used by employing Mueller-Hinton agar culture medium as a growth medium for bacteria and antibiotic discs Ciprofloxacin, Imipenem, Amikacin, Aztreonam, Ceftazidine, Cefotaxime, Ertapenem, Tobramycin, Gentamicin, Pipercillin, Colistin, Polymixin B, Rifampin, Tetracycline, Ticarcillin, Tobramycin, Trimethoprim, Tigecycline and Meropenem according to CLSI standard.

### PCR

After culturing the identified isolates of Pseudomonas aeruginosa on MacConkey agar culture medium, the genomes of all isolates were extracted using a DNA extraction kit (Bioneer Co. Korea). The polymerase chain reaction was performed with a final volume of 25 μL. This reaction involves 12.5 μL of mastermix (Amplicon, Denmark), 1.5 μL of forward primer, and 1.5 μL of reverse primer (offered by Bioneer Co. Korea). 2.5 μL of DNA template, and 7 μL of distilled water. The information related to primer sequence and conditions used for replication of gyrB, rhlR, exoT, lasR, PelA, and toxA genes are presented in Table 1 (3–5). The electrophoresis of PCR products was performed by 1% agarose gel, and the bands were then photographed by the Gel Doc imager. Moreover, a 50 bp marker of GenedireX Co., a joint production of Taiwan and the United States, was employed to detect PCR products.

### Polymerase chain reaction for identification of mexA and mexB

After culturing the identified isolates of Pseudomonas aeruginosa on MacConkey agar culture medium, the DNAs of all isolates were extracted using a DNA extraction kit (Bioneer Co. Korea). The polymerase chain reaction was performed with a final volume of 25 μL. This reaction included 12.5 μL of Master mix (Amplicon, Denmark), 1.5 μL of forward primer, and 1.5 μL of reverse primer (offered

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Table 1. Frequency of gyrB, rhlR, exoT, lasR, PelA, and toxA resistance genes in different antibiotics.
by Bioneer Co. Korea), 2.5 μL of template DNA, and 7 μl of distilled water. The information related to primer sequence and conditions used to replicate the mexA and mexB genes are indicated in Table 1 (3–7). Electrophoresis of PCR products by 1% agarose gel. The bands were then photographed by the Gel Doc imager. Moreover, a 1 kb marker from GenedireX Co., a joint production of Taiwan and the United States, was employed to detect PCR products.

The characteristics of the gold nanoparticles

The gold nanoparticles with a size of 40 nanometers, purity of 99.95%, the concentration of 100 ppm, and with an international number (CAS: 744-57-5) ordered from the Nano Sadra Company (Mashhad – Iran) were used in this study (Fig. 1).

RNA extraction and cDNA synthesis

Extraction of RNA isolates treated with gold nanoparticles and non-treated in the logarithmic growth phase was performed using RNA extraction kit (Qiagen, USA) according to the instructions, and finally the DNase enzyme was employed to remove the remaining DNA, and then the concentration of RNA was determined by a nanodrop. The amount of 1 μg of RNA from the sample was used for synthesizing cDNA using a QuantTect Reverse Transcription kit (Qiagen, USA). The quantitative reverse transcription PCR (qRT-PCR) using SYBR green-contained mastermix (Applied Biosystem, UK) was applied to evaluate the mexA and mexB genes of efflux pump. The materials used in 20 μL of mastermix were 2 μL of cDNA, 10 pM of forward and reverse primers, and 10 μL of SYBR green-contained mastermix performed on the Korean Bioneer device. The temperatures used in the qPCR were 90 °C for 10 minutes, 95 °C for 15 seconds, and 60 °C for 1 minute performed at 40 cycles. Moreover, the G74 gene was regarded as an internal control. Finally, the relative expression of mexA and mexB genes was calculated by the ΔΔCт method.

Statistical analysis

Statistical calculation of the present research was performed using SPSS software, and the Real-Time PCR data were analyzed by the one-way ANOVA analysis. P > 0.05 was considered statistically meaningful.

Results

Clinical monitoring

Most strains of Pseudomonas aeruginosa were isolated from wound samples, and the least strains were isolated from nasal samples (Fig. 2). The studied hospitals were
Ostad Motahhari and Peymaniyeh hospitals of Jahrom city in Iran, Imam Reza hospital of Lar city in Iran, Valiasr hospital of Lamerd city in Iran, and Omidvar hospital of Evaz city in Iran (Fig. 2). Forty non-duplicate strains of *Pseudomonas aeruginosa* were detected and identified from the studied hospitals using biochemical tests and API kits. Among 40 samples, 13 (32.5%), 10 (25%), 9 (22.5%), and 8 (20%) samples were collected from Ostad Motahhari and Peymaniyeh hospitals in Jahrom city, Imam Reza hospital in Lar city, Valiasr hospitals in Lamerd city, and Omidvar hospital in Evaz city, respectively (Fig. 3).

**Antibiotic susceptibility testing**

In the studied samples, the lowest levels of resistance were related to colestine (0%), polymyxin B (0%), meropenem (2.5%), imipenem (7.5%), amikacin (7.5%), and ciprofloxacin (7.5%), respectively. The highest resistance was observed against trimethoprim (100%), tigecycline (100%), tetracycline (100%), and rifampin (100%) (Fig. 4), respectively. According to the results, colestine and polymyxin B antibiotics are the best options for treating *Pseudomonas aeruginosa* infections.

**Results obtained from gel electrophoresis of gyrB, rhlR, exoT, lasR, Pela, and toxA genes**

The results indicated that the studied strains of *Pseudomonas aeruginosa* were carriers of most of these genes. The prevalence of exoT, rhlR, lasR, gyrB, pelA, and toxA genes in isolates were 32 (80%), 40 (100%), 40 (100%), 40 (100%), 37 (92.5%), and 40 (100%), respectively (Figs 5–11).

Since the antibiotic resistance in *Pseudomonas aeruginosa* has increased today than in the past, the presence of the above genes was investigated, which are effective factors in antibiotic resistance. According to the results, the prevalence of these genes was high in clinical isolates of *Pseudomonas aeruginosa*, indicating their role in antibiotic resistance in the evaluated isolates (Table 1).

**PCR test results for mexA and mexB genes**

The presence of MexA and MexB genes in 40 *Pseudomonas aeruginosa* isolates was evaluated before investigating the gene expression; the results demonstrated that the pump
Figure 5. Results of *rhlR* gene replication in clinical isolates of *Pseudomonas aeruginosa*. Well 1: marker size 50 bp; Well 2–26: positive samples of size 133 bp; Well 27: negative control; Well 28: positive control of *Pseudomonas aeruginosa* PTCC 17589.

Figure 6. Results of *exoT* gene replication in clinical isolates of *Pseudomonas aeruginosa*. Well 1: marker size 50 bp; Well 2–36: positive samples of size 471 bp; Well 37: negative control; Well 38: positive control of *Pseudomonas aeruginosa* PTCC 17589.

Figure 7. Results of *lasR* gene replication in clinical isolates of *Pseudomonas aeruginosa*. Well 1: marker size 50 bp; Well 2–26: positive samples of size 130 bp; Well 27: negative control; Well 28: positive control of *Pseudomonas aeruginosa* PTCC 17589.

Figure 8. Results of *Pela* gene replication in clinical isolates of *Pseudomonas aeruginosa*. Well 1: marker size 50 bp; Well 2–23: positive samples of size 148 bp; Well 24: negative control; Well 25: positive control of *Pseudomonas aeruginosa* PTCC 17589.
Efflux genes (MexA and MexB) were present in all studied isolates (Figs 12 and 13).

Expression of mexA and mexB genes before and after contact with efflux pump inhibitors through real-time PCR

In order to compare the effect of gold nanoparticles on the expression of MexA and MexB genes after RNA extraction and cDNA synthesis, the changes of gene expression in isolates of two cell groups of “gold nanoparticles-treated” and “non-treated” was quantitatively investigated using melting curve analysis and amplification plot (Figs 14–17). The expression rates of MexA and MexB genes in gold nanoparticle-treated isolates were meaningfully reduced compared to non-treated isolates, shown in Figs 18 and 19, respectively. The vertical and horizontal axes of the standard curve represent CT and quantity that indicates the number of copies of the gene per dilution. It is observed in the standard curves of MexA and MexB that the CT evaluation factors are indirectly related to concentration and increases as concentration is decreased.

According to the results, the inhibitory effect of gold nanoparticles on MexA gene is greater compared to MexB gene.

Discussion

Since no new antibiotics are available presently to replace the existing antibiotics for Gram-negative pathogens, and there is no extensively available vaccine against these infections, just one way to mitigate the effects of infections is to control their spread that can only be achieved in the case of fully understanding of causes, dynamics, and complexity of the prevalence of these organisms. The objective of the present studies was to contribute to this knowledge by studying Pseudomonas aeruginosa, which is the cause of nosocomial infections in burn-injured patients.

Much research has been carried out on the resistance of pseudomonas, the results of which are different in terms of time and place. In the present study, antibiotic resistance of 40 isolated strains from Omidvar hospital of Evin city, Valiasr hospital of Lamerd city, Imam Reza hospital of Lar city,
and Ostad Motahhari and Peymaniyeh hospitals of Jahrom city were evaluated; the resistance rates was as follows: Coles- tin 0%, ciprofloxacin 3 (7.5%), imipenem 3 (7.5%), amik- acin 3 (7.5%), aztreonam 9 (22.5%), cefepime 6 (15%), cef- tazidime 40 (100%), ertapenem 25 (62.5%), fosfomycin 21 (52.5%), gentamicin 6 (15%), piperacillin 5 (12.5%), poly- myxin B 0%, Rifampin 40 (100%), tobramycin 5 (12.5%), trimethoprim 40 (100%), tigecycline 40 (100%), and meropenem 1 (5/2%).

In a study conducted by Shahid et al on *Pseudomonas aeruginosa* strains at the burn care units, the lowest and highest resistances were against the tetracycline (0%) and gentamicin (90%), respectively (Shahid et al. 2003). According to the present study, the results indicated an increase in drug resistance for the reason of overuse over a period of time. According to the results of the present research as well as the literature, the colestine and polymyxin B are the best therapeutic options against *Pseudomonas aeruginosa* infections.

*Pseudomonas aeruginosa* is a stubborn microorganism in terms of resistance to various antibiotics and possesses three main mechanisms of limited adsorption resistance...
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Conclusion

The MexAB-oprM system is one of the main factors in the resistance of Pseudomonas aeruginosa. The results of the present investigation indicated that gold nanoparticles reduce the number of active efflux pumps on the cell surface by reducing the expression of mexA and mexB genes so that a decrease in the level of these efflux pumps reduces the excretion of antibiotics from the cell and subsequently causes lower concentrations of antibiotics to be able to kill cells.

References


