The effect of antimicrobial photodynamic therapy against virulence genes expression in colistin-resistance Acinetobacter baumannii

Ebrahim Boluki 1, Maryam Moradi 2, Pardis Soleimanzadeh Azar 3, Reza Fekrazad 4, Maryam Pourhajibagher 5*, Abbas Bahador 1**

1: Department of Microbiology, School of Medicine, Tehran University of Medical Sciences, Tehran, Iran.
2: Department of Cell & Molecular Biology, Faculty of Biological Sciences, Kharazmi University, Tehran, Iran.
3: General dentist, College Station, Texas.
4: Department of Periodontology, Dental Faculty - Laser research center in medical Sciences, AJA University of Medical Sciences, Tehran, Iran.
5: Dental Research Center, Dentistry Research Institute, Tehran University of Medical Sciences, Tehran, Iran.

Background and Aims: The emergence of drug-resistant infections is a global problem. Acinetobacter baumannii has attracted much attention over the last few years because of resistance to a wide range of antibiotics. Applying new non-antibiotic methods can save lives of many people around the world. Antimicrobial photodynamic therapy (aPDT) technique can be used as a new method for controlling the infections. In this study we investigated the effect of aPDT on the expression of pathogenic genes in colistin-resistance A. baumannii isolated from a burn patient.

Materials and methods: The suspension of colistin-resistance A. baumannii was incubated with 0.01 mg/ml of toluidine blue O (TBO) in the dark; then the light emitting diode device with a wavelength of 630 ± 10 nm and output intensity of 2000-4000 mW /cm² was irradiated to the suspension at room temperature. Subsequently, after the aPDT, genes expression of ompA and pilZ was investigated by using real-time polymerase chain reaction technique.

Results: Among the genes studied, the transcript of the ompA gene after aPDT was increased significantly in comparison with control groups (P < 0.05). Whereas, there was no remarkable different in pilZ gene expression (P > 0.05).

Conclusions: It can be concluded from the results that the ompA as an outer membrane of A. baumannii is degraded after exposing aPDT and it will probably be done the penetration of antibiotics into cells of this bacterium easily.

Key words: Acinetobacter baumannii • antimicrobial photodynamic therapy • toluidine blue O • LED • colistin

Introduction

Recently, Acinetobacter baumannii has been proposed as one of the most important opportunistic pathogens in burn patients. The appearance of multiple drug-resistant (MDR) strains of A. baumannii, force physicians to use polymyxin (e.g. colistin) in the treatment of infections caused by this microorganism. Therefore, that results in increasing treatment costs and additional therapies.

In the 1970s, this bacterium was highly sensitive to various antibiotics, but currently is the main cause of drug-resistant nosocomial infections. This bacterium accounts for 2 up to 10% of total hospital infections caused by Gram-negative bacteria. Appearance of MDR, XDR (extensively drug-resistant) and PDR (Pandrug-resistant) strains and increased hospital infections due to this strain
of bacteria have attracted more attention in recent years. PDR strains are also resistant to polymyxin and tigecycline. A. baumannii does not produce special toxins or cytolyisns. Few virulence factors have been identified up to now. In fact, A. baumannii is able to accumulate diverse resistance mechanisms, innate resistance to antibiotics leading to survive in stressful environmental conditions (such as food shortages and high density of bacteria). These characteristics have introduced A. baumannii as a highly resistant opportunistic pathogen in the hospital.

OmpA (Outer membrane protein A), one of the outer membrane proteins of A. baumannii, works as a critical virulence factor. This factor is involved in the development of biofilm as well as bacteria and human cell interactions, facilitating invasion of human epithelial cells and induce apoptosis in human laryngeal epithelial cells. This bacterium can survive in the bloodstream by the OmpA. Although, A. baumannii is nonmotile, but it can quickly spread on surfaces with twitching motility phenomena. pilZ gene is one of the main genes playing significant role in control and synthesis type IV pili. Also, this gene has been identified as one of the main biofilm producers.

It is crucial to find proper treatment for A. baumannii for several reasons such as significant prevalence of A. baumannii, easy contamination, widespread infections and resistance to most of current antibiotics (even polymyxin). Nowadays, a lot of international research efforts are underway for discovering new antimicrobial agents or an alternative antimicrobial method. Antimicrobial photodynamic therapy (aPDT) is one of the recent antimicrobial methods to deal with such infections. aPDT selectively targets cells growing quickly or hyperproliferating, hence, because of their rapid growth microorganisms can be one of the objectives for aPDT. aPDT might indicates an excellent alternative or additional therapy for the treatment of local infections which represent resistance to antibiotic drugs, because it is recognized as a safe treatment strategy that is both minimally invasive and nontoxic.

aPDT is a technique containing a photosensitizer combined with light of a specific wavelength which produces cytotoxic elements such as singlet oxygen and free radicals. Consequently, these products cause some damages in essential cell components or modify metabolic activities irreversibly leading to bacterial cell death.

Although variety of studies show aPDT inhibitory effect on bacterial growth, little information is available about aPDT cellular and molecular effects on bacteria remaining after exposure to low radiation doses. Genomic studies might lead to better understanding of molecular changes on bacteria exposed to aPDT.

In this study we investigated molecular effects of aPDT on expression of genes ompA and pilZ in A. baumannii to get more information about molecular function of aPDT in physiological and metabolically process in bacteria.

Material Method

Bacterial strains and culture conditions

Colistin-resistant A. baumannii strain isolated from burn-wound infection was used. The phenotype of A. baumannii is defined as XDR consistent with the International Expert Proposal for Interim Standards Guidelines. This strain is resistant to colistin, and the minimal inhibitory concentration (MIC) value of colistin was > 32 μg/ml.

First A. baumannii was cultured on the Brain Heart Infusion (BHI) broth (CONDA, Spain) culture media under aerobic condition at 37°C for 24 h. One ml bacterial suspension was added into 10 ml fresh BHI broth then specimens were incubated with shaking incubator at 100 rpm under aerobic condition at 37°C until the bacteria have reached a log phase; were adjusted to a concentration of 1.0 × 10⁸ colony forming units (CFU)/ml, as verified by both spectrophotometry (optical density [OD] 600: 0.01 – 0.02) and colony counting.

Photonsensitizer and light source

Toluidine blue O (TBO) was used as a photosensitizer (PS). TBO powder (Merck, Germany) was solved in sterile normal saline to prepare the concentration of 0.01 mg/ml of TBO. The TBO solution was sterilized by a 0.22-micron syringe filter and stored at 4°C in the dark before use; Also in this study light emitting diode (LED) with a wavelength of 630 ± 10 nm and output intensity of 2000-4000 mW/cm² was used as a light source to activate the PS. The distance between the light source and each well surface was fixed at 1 mm.

Study design for evaluation of target genes expression

Expression of ompA and pilZ genes in studied strain was evaluated after aPDT. In the PS group (photosensitizer), microbial suspension was mixed only with the PS in the darkness, and in L group (LED irradiation), microbial suspension was only irradiated with LED light irradiation. In C group (control) microbial suspension was not exposed to any irradiation and PS material. After 10 min of incubation, gene expression was evaluated in PS, L and C groups. Microbial suspension in group L+ PS was exposed to PS and LED and then gene expression was assessed. All experimental were repeated for three times. Groups which were evaluated for expression of target genes are classified in Table 1.
Exposure of the colistin-resistant *A. baumannii* strain with aPDT

To prepare microbial suspension for groups C and L, 2 ml of bacterial suspension was centrifuged for 40 s at 14,000 × g, cell pellet in 400 μl of sterilized normal saline was suspended. In the case of PS and L + PS groups, as in previous groups, 2 ml of the microbial suspension was centrifuged for 40 s at 14,000 × g cell pellet was suspended in 200 μl of sterilized normal saline. Subsequently, for binding of PS to bacteria (cells), samples with 200 μl TBO at 0.01 mg/ml concentration were shaked on the Thermo Mixer Shaker (manufactured by Eppendorf Company, Germany) for 10 min (pre-irradiation time) in darkness and at room temperature.

After incubation in the dark, the wells were irradiated by a LED device with the wavelength of 630 ± 10 nm for 60 s. Radiation was performed under the sterile conditions in the laminar flow in a dark room. The distance between the tip of the LED to the sample was 4.5 mm and the depth of the wells was 10 mm. To prevent the spreading of light to adjacent samples, around wells were filled with TBO dye. The wells contained colistin-resistant *A. baumannii* suspension that was not exposed to any radiation and no light sensitive material (PS) was as a control.

**RNA extraction from colistin-resistant *A. baumannii* treated with aPDT**

Immediately after aPDT, the microbial suspension was transferred to the RNase free microtube and the extraction process was done according to the manufacturer’s instructions easy-spin (DNA free) Total RNA Extraction Kit (iNtRON Biotechnology Company, Korea). The total RNA samples obtained were treated with DNase I (Thermo Scientific GmbH, Germany) after assessment with NanoDrop spectrophotometer (Thermo Fisher Scientific, US). The concentration of total RNA was determined using a ultraviolet absorbance at 260 nm (A260) and 280 nm (A280) with NanoDrop spectrophotometer. The concentration of total RNA must be within an A260 / A280 ratio of 1.80 – 2.00 to ensure the integrity of the mRNA. The integrity of the RNA was determined by agarose gel 1% electrophoresis. The extracted RNA was used as a template to synthesize the primary cDNA chain by reverse transcription with a First Strand cDNA Synthesis Kit (Thermo Fisher Scientific, US) following the manufacturer’s instructions. The cDNA was stored at -20°C.

**Real-time PCR**

The resulting cDNAs were used for the quantification of virulence genes mRNA levels by real-time qRT-PCR with Line-Gene K Real-Time PCR Detection System (Hangzhou, China).

All primers utilized in this study were designed by using the Primer3Plus web tool (http://www.bioinformatics.nl/cgi-bin/primer3plus/primer3plus.cgi/). The PCR primer sequences used for quantification of the candidate reference is given in Table 2. The same primer pair was used when determining the mRNA level and DNA copy number of a certain gene. Gene-specific amplification was confirmed by a single peak in melt-curve analysis. The PCR products were subjected to electrophoresis in 2% (w/v) agarose gels to confirm the specificity of ampli-

<table>
<thead>
<tr>
<th>Group</th>
<th>Description</th>
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<tbody>
<tr>
<td>Group C</td>
<td>Microbial suspension was not exposed to any irradiation and PS material.</td>
</tr>
<tr>
<td>Group L</td>
<td>Microbial suspension was only irradiated with LED light irradiation.</td>
</tr>
<tr>
<td>Group PS</td>
<td>Microbial suspension was mixed with the PS in the darkness.</td>
</tr>
<tr>
<td>Group L+ PS</td>
<td>Microbial suspension in group L+ PS was exposed to TBO and LED irradiation.</td>
</tr>
</tbody>
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**Table 1:** Groups evaluated for expression of *ompA* and *pilZ* genes.

<table>
<thead>
<tr>
<th>Gene</th>
<th>Forward primer sequence (5' - 3')</th>
<th>Reverse primer sequence (3' - 5')</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>ompA</em></td>
<td>AGCATAAAGAAGCTACACCTGC</td>
<td>AAAGTCGCCAAGAAACCCTTGAT</td>
</tr>
<tr>
<td><em>pilZ</em></td>
<td>AGCAAAAGATGAAGTGGGTTCA</td>
<td>ACCCTTGGCGTTTAAATCCACAT</td>
</tr>
<tr>
<td>16S<em>rRNA</em></td>
<td>AAAGTTGGTATTGCGAAACGG</td>
<td>ACCCTTAAACCGGCTTTGTGCT</td>
</tr>
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**Table 2:** The sequences of primers used in this study.
fication and the absence of primer dimer formation. Amplification was followed by melting-curve analysis to check the specificity of the PCR product.

The reaction volume of 20 µl contained 10 µl of SYBR Premix Ex Taq II (Takara, Japan) under the following conditions: 95°C for 5 min, amplification for 35 cycles with denaturation at 95°C for 15 s, annealing for 10 s at the temperature reported and extension at 72°C for 10 s. The specificity of the primers was evaluated using melt curves.

The relative amounts of each target gene (ompA, pilZ) were quantified (qPCR) relative to 16S rRNA (as an internal standard). The fold changes of the target genes’ expression levels were calculated by the $2^{-ΔΔC_{t}}$ method using the Relative Expression Software Tool (REST) 2009 software (QIAGEN) 17). The mRNA expression levels were shown as n-fold differences relative to the calibrator. Changes in expression levels of the target genes were interpreted as significant if the variation was > 2-fold.

**Statistical analysis**

ANOVA and Tukey’s test were employed for statistical analysis. T-test were used to assess the difference between the relative quantities of the gene expressions in A. baumannii strains under the treatments. All experiments were performed in triplicate, and results were reported as mean ± standard deviation (SD). P-values less than 0.05 in all experiments were considered statistically significant.

**Result**

As you see in Figure 1, of the two genes studied in the L+ PS group, the transcription of the ompA gene increased considerably compared to the C, L and PS groups ($P < 0.05$). On contrast, there was not any significant change shown in the expression of the data obtained from the results of the analysis of the pilZ gene in the L+ PS group. As in all studied groups, the level of expression of this gene was similar to control group ($P > 0.05$; Fig. 1). According to the results, the level of ompA gene expression was significantly different versus pilZ in L+ PS group ($P < 0.05$), so that it was able to increase the expression of ompA gene by 8.7-fold more than pilZ.

**Discussion**

A. baumannii is known as one of the most important common agent of health care-associated infections which leads to intractable infections such as burn infections, respiratory infections (pneumonia), infections caused by the ventilator (Ventilator-Associated Pneumonia), wound and soft tissue infections, urinary tract infection, meningitis and bacteremia particularly in patients admitted to the intensive care unit (ICU) and the burn ward of a hospital 18). Its ability of resistance to antibiotics is very strong resulting in emergence of MDR strains 19). Worldwide distribution of strains resisted to various classes of antibiotics is worrying and leads to higher medical expenses 7). Recent-

![Figure 1: Effect of aPDT, TBO concentration and LED irradiation time on the virulence genes expression by colistin-resistant A. baumannii strain. Values are mean ± standard error of the mean of cDNA concentrations for each gene transcript from three replicate experiments. *, $P < 0.05$; significantly different from the control (strains grown without treatment).](image-url)
ly, the compatibility of some clinical isolates of A. baumannii to all antibacterial drugs has led to appearance of polymyxin and tigecycline resisted PDR strains. Many physicians are concerned about incurability of different bacterial infections. This concern in turn leads to greater efforts to find alternative methods to treat bacterial infections. In the recent decades, a lot of international research efforts are going to discover new antimicrobial agent. Nowadays, it is emphasized on elimination of possible MDR and how to take precautions against the establishment of MDR. New non-antibiotic approaches should be promoted in prevention and protection against infectious diseases and necessarily it should be considered as a significant priority in its research methods. In order to using aPDT techniques alone or in combination with antibiotics may be the basis for a new approach to reduce the rate of treatment failure in antibiotic treatment. Although a variety of studies shows inhibitory effect of aPDT on bacterial growth, little relevance is available about the effectiveness of these techniques on residual bacteria after low-dose radiation exposure in molecular and cellular levels.

Transcriptomic studies may lead to a better understanding of the molecular changes on the bacteria after aPDT. These tests lead to more efficient use of new techniques in medicine for treating antibiotic resistant infections.

Different light sources including coherent (lasers) and incoherent light sources have been used for irradiation in aPDT. The non-coherent light sources described in clinical studies on aPDT include the LED lamps, halogen lamps, and, more recently, intense pulsed light. Clinical application of LED light source seems to be simpler and cheaper, and field of illumination is larger. The spectra of the LED emission are relatively narrow and can correspond well with photosensitizers maximum absorption that results in time of irradiation can be shortened. In contrast to other incoherent light sources, LED is free of wavelengths that are not needed to achieve therapeutic properties. The other key fact of using LED sources is that they produce less heat than high-pressure lamps, are relatively inexpensive and highly durable in the long time.

Gram-negative bacteria contain outer membrane vesicles [Omv] facilitating transfer of virulence factors into the host. OmpA is an outer membrane protein which is released by Omv. OmpA coding gene is one of the effective genes in formation of A. baumannii biofilm causing bacteria bind to the surface of epithelial cells.

This research shows that in comparison with group C, ompA gene expression has increased as in the first few min after radiation; it was reached to highest level. According to the fact that invasion to the constituents of cellular coverage is known as an effect of the aPDT method and considering our prior study about lpsB gene expression (It plays a role in lipopolysaccharide biosynthesis) which showed a significant increase after gene expression; it is consumed that free radicals produced by aPDT cause severe damage on A. baumannii outer membrane hence to avoid cellular lysis bacteria try to maintain the integrity of their outer membrane. Thus in order to repair the damages on outer membrane and maintenance of outer membrane integrity bacteria increases expression level of ompA gene.

Also, according to Dosselli et al. research on Staphylococcus aureus showed that some proteins are fragmented following aPDT and many other after combination with oxygen conforms aggregates that will probably lead to the loss of cell function. Thus, in this study OmpA protein is probably damaged by oxygen radicals so in order to survive and organisms can repair damaged areas in outer membrane.

OmpA protein has a role in transition of antibiotics along the outer membrane and any changes in protein and decreased expression of ompA gene result in antibiotic (resistance to beta lactams) resistance. Therefore, according to damaged OmpA caused by aPDT, using combined therapy to remove infections caused by these bacteria would probably fail because damaged OmpA protein disturbs antibiotic entrance to A. baumannii. However according to Boluki et al. study, it is concluded that OmpA damage wouldn’t have effect on penetration of antibiotic agent to A. baumannii due to increased sensitization of bacteria to antibiotic which shows increased penetrance of antibiotic to bacteria.

OmpA is a virulence factor preventing A. baumannii death caused by immune system activation and complement attack. According to Kim et al. study, OmpA inhibits function of the phagocyte cells of the host; therefore, it can be concluded that for the treatment of infections caused by aPDT immune system of body can possibly act more efficient because OmpA is damaged by aPDT.

While, many studies have associated the production of reactive oxygen species (ROS) with aPDT, the association between the significant rise of local ROS level and the exposure to LED light source and photosensitizers has not been assessed. In fact, ROS are formed as a toxic byproduct of the aPDT and have important roles in significant damage to microbial cell structures and as cell signaling. ROS are also implicated in positive effects on induction of SOS response. The aPDT treatment expression of ompA was 8.7 folds higher than basal (Fig. 1); while, LED light source and photosensitizers treatment revealed no significant change in the expression of ompA expression. This suggests that stronger expression of ompA in aPDT treatment may be associated with ROS production. ompA, is an essential gene for the biosynthesis and integrity of outer membrane proteins. Increased transcription levels of ompA in the A. baumannii strains are consistent with the role of outer membrane in pro-

aPDT in genes expression of colistin-resistance A. baumannii
tecting *A. baumannii* from adverse condition. It is sup-
posed that the critical role of ompA in bacterial outer mem-
brane integrity maintenance, highlighted above, was 
corrupted due to interaction with ROSs produced fol-
lowed by PDT treatment. Therefore, the SOS response in 
bacteria will lead to the expression of ompA to prevent 
the loss of outer membrane integrity and death under 
these circumstances.

Also, according to studies conducted indicated that 
OmpA protein plays a significant role in attachment 
of the bacteria to *Candida albicans* filament and human 
epithelial cells. It is known that in burn wound *A. 
baumannii* adheres to *C. albicans* filaments by the 
OmpA protein leading to development of robust biofilms 
on burn wound. Likely, OmpA damage under effect of 
aPDT leads to decreased adherence of bacteria to eukary-
otic cells which prevents localization of bacteria at the 
site of infection. Consequently, infection will be treated 
more effectively.

Although *A. baumannii* does not increase motility 
rate in definite, it is able to spread rapidly over surfaces 
so that this motility is facilitated by type IV pili. PilZ has a 
main role in controlling type IV pili and producing bio-
film. It was identified that expression of pilZ gene has 
changed significantly following aPDT. According to 
our previous and current studies it has shown that the 
most of gene expression is assigned to proteins such as 
LpsB and OmpA involving in maintaining outer mem-
brane stability so it has been concluded that bacteria sur-
vives by using the most energy for repair of outer mem-
brane so expression of pilZ gene is not required while 
exposing to oxygen free radicals.

Since a variety of biomolecules (lipids, amino acids, 
nucleotides, proteins) and cellular structures react with 
oxxygen radicals, resistance to aPDT technique seems 
unlikely. Regarding limited access public to aPDT, and 
also requires an expert to perform aPDT, this treatment is 
limited to centers with specialized care. The most com-
monly used locally is the other limitation of this method 
and its use is difficult in systemic infections; that, of 
course, is not problematic about burn infections. Finally, 
when this technique is prescribed as a treatment, factors 
such as cost, and ease of management should be consid-

Conclusions

In summary, the main findings of this study revealed that 
aPDT as a supplement, likely decreases the failure rate in 
anti-biotic therapy and can be effective in difficult situa-
tion such as antibiotic resistance.

References

1. Hwang YY, Ramalingam K, Bienek DR, Lee V, You T, Alva-
erez R (2013): Antimicrobial activity of nanoemulsion in com-
bination with cetylpyridinium chloride in multidrug-resistant 
*Acinetobacter baumannii*. *Antimicrobial agents and chemo-
otherapy*, 57(8):3568-3575.

2. Pourhajibagher M, Hashemi FB, Pourakbari B, Aziemzadeh 
M, Bahador A (2016): Antimicrobial Resistance of *Acineto-
bacter baumannii* to Imipenem in Iran: A Systematic Re-

3. Feizabadi M, Fathollahzadeh B, Taverikalani M, Rasodinejad 
M,Sadeghifard N, Aligholi M, et al. Antimicrobial suscepti-
bility patterns and distribution of blaOXA genes among 
Acinetobacter spp. Isolated from patients at Tehran hospi-

4. Pourhajibagher M, Mokhtarzadeh M, Esmaeili D, Bahador A 
(2016): Assessment of biofilm formation among *Acineto-
bacter baumannii* strains isolated from burned patients. *Der 

(2011): *Acinetobacter baumannii* secretes cytotoxic outer 
membrane protein A via outer membrane vesicles. *PloS one*, 
6(2):e17027.

bacter baumannii* invades epithelial cells and outer mem-
brane protein A mediates interactions with epithelial cells. 

7. McConnell MJ, Actis L, Pachón J (2013): Acinetobacter bau-
mannii: human infections, factors contributing to pathogen-
esis and animal models. *FEMS microbiology reviews*, 
37(2):130-155.

The Acinetobacter baumannii oxymoron: commensal hospita-
tal dweller turned pan-drug-resistant menace. *Frontiers in 

of the Bacterial Heat Shock Response to Photodynamic 
Therapy-mediated Oxidative Stress. *Photochemistry and 
photobiology*, 87(5):707-713.

10. Raab OC (1906): On the effect of fluorescent substances on 

11. Yan R, Dai T, Avci P, Jorge AES, de Melo WC, Vecchio D, et 
al (2013): Light based anti-infectives: ultraviolet C irradia-
tion, photodynamic therapy, blue light, and beyond. *Current 

12. Perez F, Hujer AM, Hujer KM, Decker BK, Rather PN, Bonom-
o RA (2007): Global challenge of multidrug-resistant 
*Acinetobacter baumannii*. *Antimicrobial agents and chemo-
therapy*, 51(10):3471-3484.

photodynamic therapy on *Streptococcus mutans* biofilms 
using erythrosine and dental halogen curing unit. *Internation-

14. Melo MASd, Rolim JPMa, Zanin ICJ, Barros EB, da–Costa EF, 
photodynamic therapy-treated *Streptococcus mutans*: an 
atomic forces microscopy study. *Photomedicine and laser 

15. Magiorakos AP, Srinivasan A, Carey R, Carmeli Y, Falagas M, 
drug-resistant and pandrug-resistant bacteria: an internation-


17. Pfaffl MW, Horgan GW, Dempfle L (2002): Relative expression software tool (REST®) for group-wise comparison and statistical analysis of relative expression results in real-time PCR. *Nucleic acids research*, 30(9):e36.


