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**ORIGINAL ARTICLES**

# Evaluation of photodynamic therapy effect along with colistin on pandrug-resistant *Acinetobacter baumannii*

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**Background and Aims:** Pandrug-resistant *Acinetobacter baumannii* (PDRAB) are including colistin resistant starins (CoRAB) which cause infections potentially untreatable infections. Recently, incidence of these strains are increasing worldwide. Therefore, new approaches, methods and strategies are urgently needed for treatment and eradication of infections due to PDRAB. So the aim of this study was to evaluate the efficacy of photodynamic therapy (PDT) in combination treatment with colistin against PDRAB.
**Materials and Methods:** PDRAB which was isolated from burn patients was used as a test strain. PDT carried out in which toluidine blue O (TBO) and light-emitting diode (LED) were used as photosensitizer and radiation source, respectively. Then, the effect of PDT plus colistin was evaluated on CoRAB and the colony-forming units of each tested groups calculated. Finally, confirmation of antibacterial activity of combination therapy was carried out using scanning electron microscope.
**Results:** PDT declined bacterial count in comparing with control group by 83.7% of killing percentage, in other words, less than one log reduction. While PDT in combination with colistin showed high synergetic effect against *A. baumannii* in all concentrations of colistin tested by 100% of killing percentage with 9-log reduction.
**Conclusions:** According to our results, PDT alone couldn't eliminate all of the treated bacterial cells. But when combined with colistin, it killed all of the treated bacterial cells in all tested concentrations. Also PDT decreased the minimal inhibitory concentration of colistin against PDRAB by more than 11 fold.

**Keywords:** *Acinetobacter baumannii* · colistin · photodynamic therapy · toluidine blue O · wound infection · pandrug resistance

## Introduction

Burn and wound infections due to *Acinetobacter baumannii* are the major threat to global health. The treatment is remarkably difficult, not only developing extensive antimicrobial resistance but it can also form biofilms that resistant to host defense and antimicrobial treatment. Such causative factors, biofilm formation and resistance to antibiotics can lead to non-healing wounds [1]. Also these infections can develope and cause systemic infections such as pneumonia, meningitis, and bloodstream infections [1-3]. Therefore, burn infection due to *A. baumannii* has a high mortality rate [4, 5].

Multidrug-resistant (MDR) of *A. baumannii* has increased over the years and has become a major problem worldwide [6]. *A. baumannii* is defined as MDR when the organism is resistant to one or more than one agent in three or more antimicrobial cate-

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gories that would otherwise serve as treatments for *A. baumannii* infections [7]. Because of development of resistance to most available antibiotics including beta-lactams, carbapenems, fluoroquinolones, and aminoglycosides among *A. baumannii* strains and lacks of development of new antimicrobial agents against this pathogen medical community have prompted to reuse of colistin for treatment of *A. baumannii* infections in many health care centers around the world. So, it is recommended to use colistin for treating XDR *Acinetobacter* infections [8, 9]. The XDR-*A. baumannii* is non-susceptible to one or more than one agent in all but two or less than two antimicrobial categories.

The rise in colistin use for treatment of infections due to *A. baumannii* causes emergence of colistin resistance strains worldwide. Unfortunately, colistin resistant strains of *A. baumannii* several times have been reported [10, 11]. *A. baumannii* is explained as pandrug-resistant (PDR) when it is non-susceptible to all antimicrobial agents [7]. In this regard, PDR-*A. baumannii* (PDRAB) are including colistin resistant *A. baumannii* (CoRAB) strains whose infections are potentially untreatable. Recently, incidence of these strain are increasing worldwide [12]. The highest resistance rates were seen in Asia, followed by Europe and America, and also the regions in which colistin resistance rates are continually increasing [12]. In conclusion resistance to most antibiotics for treateatment of *A. baumannii* infections, this makes the treatment more difficult and cost ineffective. Therefore, new approaches, methods and strategies are urgently needed for treatment and eradication of severe infections due to highly drug resistant bacteria such as *A. baumannii* [13].

One of these strategies is photodynamic therapy (PDT) as a novel antimicrobial approach. This method commonly used for cancer and ophthalmological treatments. Today PDT has been used clinically for the treatment of skin infections caused by a variety of pathogens [14] such as treatment of ulcer and wound infection due to *Pseudomonas aeruginosa* [15] and acne bacteria-induced inflammation [16].

PDT works with generation of reactive oxygen species (ROS) through the use of a combination of oxygen, visible or near infrared light, and a photosensitizer (PS), a nontoxic dye that is photo reactive [17].

The advantages of this method are that interact with bacterial membrane, and it does not make selection of resistant strains after repeated cycles of treatment [17]. Also, this method can target bacterial cells rather than host tissue with appropriate chemical design of the PS [17].

PSs are almost photo bactericidal dyes that form metachromatic complexes with lipopolysaccharides (LPS) in Gram negative bacteria. Interactions between the PSs and LPS might contribute in the mechanism of photo killing of Gram negative bacteria [18].

Also the target of colistin is LPS of Gram negative bacteria. Resistance to the colistin in *A. baumannii* has two molecular mechanisms. These include: mutations in the *PmrAB* which lead to alterations of the lipid A component of LPS, latter mutations in the *lpxA*, *lpxC* and *lpxD* genes resulting complete loss of LPS production [19]. Commonly the cationic PSs are used against Gram negative bacteria which interact with LPS of bacterial cells. In colistin resistant strains with complete loss of the LPS or modified LPS structures, anionic polymers on the outer membrane can be a major target site for interaction with cationic PSs. Notably outer membrane proteins (OMPs) presented in outer membrane more than LPS. Therefore, significantly high amount of protein such as porins in the outer membrane of Gram negative bacteria [18] can be targeted by PDT. Therefore, our hypothesis was treatment of colistin resistant strains with PDT can increase sensitivity towards antibiotics (such as colistin). So the aim of this study was evaluation of PDT in combination with colistin against CoRAB.

## Materials and methods

### Photosensitizer and light source

TBO (Merck, Frankfurter, and Germany) was dissolved in dH2O to obtain a final concentration of 0.4 mg/mL. Then this solution was sterilized by 0.22-micron syringe filter and subsequently kept in the dark at 4 °C [20]. The light-emitting diode (LED) (FotoSan 630 nm LAD, CMS dental, Denmark), at a wavelength of 635 nm with output power of 220 mW was used as a light source.

### Bacterial strain

In this study a PDRAB isolated from burn patient was used [21]. This strain is resistant to amikacin, ampicillin-sulbactam, cefepime, ceftazidime, ciprofloxacin, colistin, gentamicin, imipenem, levofloxacin, meropenem, minocycline, piperacillin, piperacillintazobactam, rifampicin, tetracycline, tobramicin, and trimethoprim-sulfamethoxazole. The minimal inhibitory concentration (MIC) value of colistin was > 32 µg/mL. For further analysis *A. baumannii* strain was cultured in tripticase soy broth (TSB) (Himedia, India) and was stored in -70 °C, and then freshly sub-cultured on brain heart infusion (BHI) (Himedia, India) agar prior to assay.

In our experiment, a study sample is divided into

four groups including: 1. *A. baumannii* which grown on Cation-Adjusted Mueller Hinton broth (CAMHB; Himedia, India,), 2. *A. baumannii* which grown on CAMHB containing colistin (Sigma-Aldrich, Germany) (0.03 to 32 µg/mL), 3. *A. baumannii* which treated with PDT, then grown on CAMHB, 4. *A. baumannii* which treated with PDT, then grown on CAMHB containing colistin (0.03 to 32 µg/mL) **(Table 1)** .

For PDT experiments, one colony of CoRAB isolate was used for inoculation in 5 mL of CAMHB. The culture was incubated for 6 h at 37 °C with aeration at 200 rpm. Following incubation, then the number of cells in culture was adjusted to $1.5\times10^8$ CFU/mL as verified by spectrophotometry (optical density [OD] 600: 0.08–0.11).

## PDT experiments

Aliquots of 100 µL of bacterial suspensions ($2.0\times10^6$ CFU/mL) were placed in a 96-well microtiter plate (TPP, Trasadingen, Switzerland), then incubated with 100 µL TBO at a final concentration of 0.1 mg/mL in the dark and at room temperature for 5 min and exposed to LED for 3min [20].

For determination of MIC value of colistin (Sigma-Aldrich) against treated group (PDT with/ without colistin and vice versa) **(Table 1)** of *A. baumannii*, broth microdilution testing was carried out according to Clinical and Laboratory Standards Institute (CLSI) procedures using CAMHB (0.03 to 32 µg/mL) [22].

In this regard, five groups were used in present study including: 1. The wells in column 1 which contain CAMHB and inoculated by CoRAB; 2. The wells in column 2 which contain CAMHB and colistin (0.03 to 32 µg/mL) then inoculated by CoRAB as a control group; 3. The wells in column 3 which contain CAMHB and inoculated by PDT treated CoRAB; 4. The wells in column 4 which contain CAMHB and colistin (0.03 to 32 µg/mL), then inoculated by PDT treated CoRAB as a combination therapy; 5. The wells in column 5 which contain CAMHB and colistin (0.03 to 32 µg/mL) without bacteria as negative control. The colony-forming units (CFUs)/mL of test wells was calculated using Miles and Misra Method [23].

**Table 1:** Tested group for each assay

| Groups | Description |
|---|---|
| **1** | MHB[a] + *A. baumannii*[b] |
| **2** | MHB + Col[c] + *A. baumannii* |
| **3** | MHB + *A. baumannii* treated with PDT[d] |
| **4** | MHB + Col + *A. baumannii* treated with PDT |

[a] Mueller hinton broth
[b] *Acinetobacter baumannii*
[c] Colistin
[d] Photodynamic therapy

## Killing percentage and log reduction analysis

Eq. [1] was used to calculate the killing efficacy as kill percentage (%). Also, the log population reduction of the test organism was obtained using Eq [2]. Regarding the standard method, at least a one log reduction of bacterial load is required to consider an antibacterial property. The wells in column 2 which contain colistin serial dilution and inoculated by CoRAB was used as control group [24]. All experiments were repeated three times.

$$\text{Kill (\%)} = \frac{(\text{cell count of control} - \text{cell count of sample})}{\text{Cell count of control}} \times 100 \quad (1)$$

$$\text{Log population reduction} = \text{Log cell count of control} - \text{Log survivor count on sample} \quad (2)$$

## Scanning electron microscope (SEM) analysis

For confirmation of efficacy of combination therapy SEM was carried out as described previously [25]. Briefly, the specimen was coated with gold-palladium by a sputter coater (Bal-Tec SCD 005, Netherland) and examined with a SEM (5800LV, JEOL, Japan).

## Statistical analysis

All data analyzed by SPSS v.22. One-way ANOVA was used for significant differences between groups. A $P$ value < 0.05 was accepted as statistically significant.

# Result

Our analysis showed that colistin couldn't decrease significantly the CoRAB CFU/mL in comparing with media which without colistin and inoculated by CoRAB (without colistin: $8\times10^8$ CFU/mL and colistin treated *A. baumannii*: $6.0\times10^8$ CFU/mL) (pv≈0.5). Killing percentage of colistin against *A. baumannii* was 25% but in the last concentration (32 µg/mL). PDT decreased bacterial CFU/mL in compering to group 1 ($13\times10^7$ CFU/mL), and decreased CFU/mL more than colistin alone (group 2), but significant differences were not seen (pv≈0.14 and pv≈0.28, respectively). Killing percentage of PDT was 83.7% whereas the log population reduc-

tion was less than one log reduction **(Fig. 1, 2)**. But PDT in combination with colistin showed high synergistic effect against *A. baumannii* in all tested concentrations of colistin (killing percentage 100%). Moreover PDT decreased the MIC value of colistin by more than 11 fold. In this regard, MIC value decreased from > 32 to < 0.03 µg/mL, which it's the colistin susceptible pattern according to CLSI guideline [22].

SEM analysis showed that any bacterial growth were not seen in PDT plus colistin treated group in all tested concentration, while the colistin treated group produced thin layer of biofilm **( Fig. 3)**.

## Discussion

In present study, we evaluated the effect of PDT in

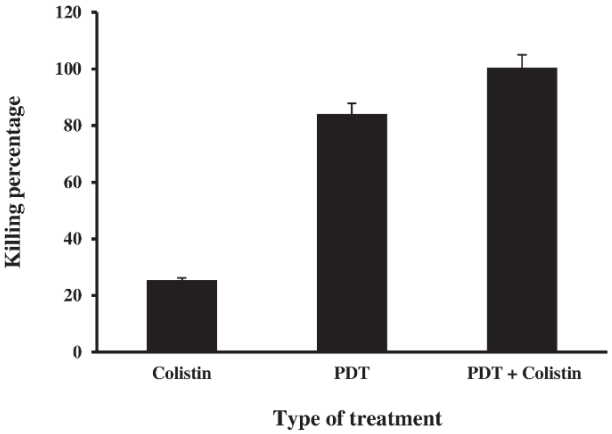


**Figure 1:** Killing percentage of different treatments against *A. baumannii*. (colistin: treatment of colistin aginst *A. baumannii*, PDT: photodynamic therapy against *A. baumannii*, PDT+ colistin: combination therapy of PDT and colistin against *A. baumannii*).

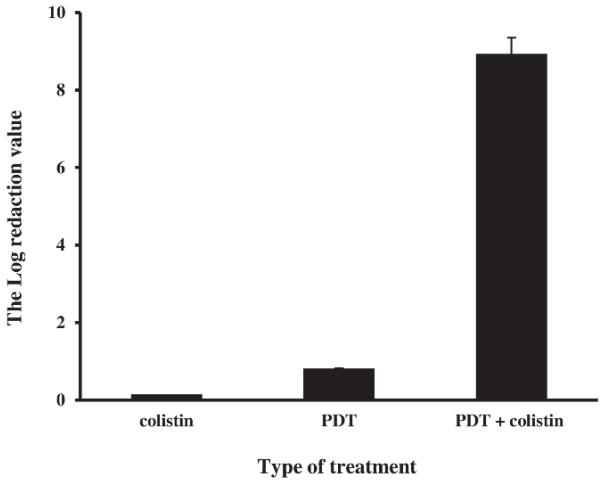


**Figure 2:** Population reduction of each assay against *A. baumannii*. (colistin: treatment of colistin aginst *A. baumannii*, PDT: photodynamic therapy against *A. baumannii*, PDT+ colistin: combination therapy of PDT and colistin against *A. baumannii*).

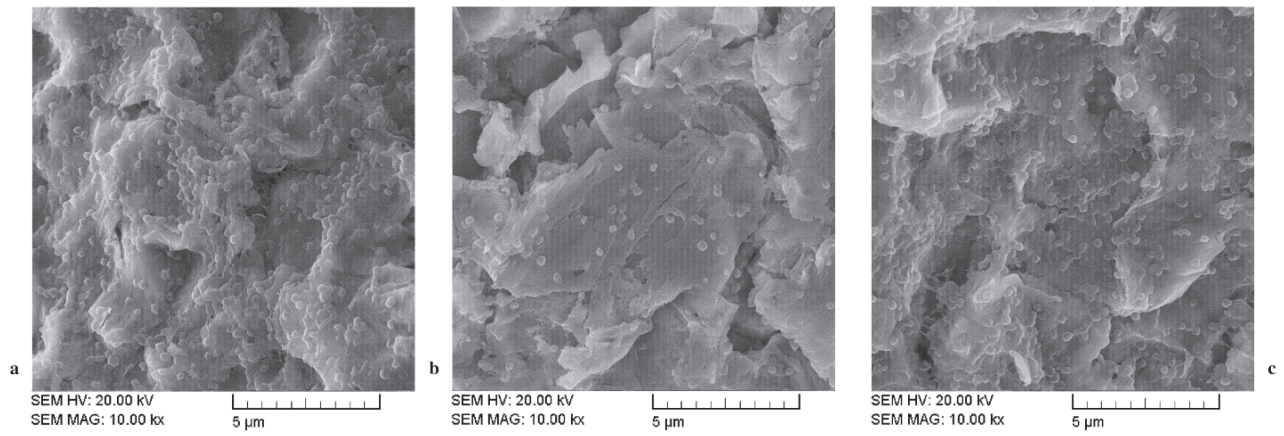


**Figure 3:** Scanning electron microscope images of *A. baumannii*. a) Control biofilm growth without treatment, b) PDT+ colistin: combination therapy of PDT and colistin against *A. baumannii*, c) colistin: treatment of colistin aginst *A. baumannii*.

combination with antibiotic therapy (colistin) for treatment of PDRAB which isolated from burn patients. Today, colistin is used as the last line of drug for treatment of *A. baumannii* infections. Nevertheless, infections caused by strains with resistance to this antibiotic have increased worldwide [7-10].

According to our results, PDT alone couldn't eliminate all of the treated *A. baumannii*. But when combined with colistin, it killed all of the treated bacterial cells in all of colistin concentrations CoRAB. The mechanisms that how PDT has synergy effects with colistin yet unknown.

Pourhajibagher et al. has been reported that PDT effects similar to EDTA, which cause alternation of permeability of the OMPs of *A. baumannii*, these proteins are responsible for nutrient, drugs and ions selective permeability and also responsible for resistance against variety of drugs [25]. It has been demonstrated that cationic PSs interact with LPS own of its negative charge. Also it has been showed that in CoRAB, LPS not expressed [19]. According to Usacheva et al. revealed that, OMPs presented in outer membrane more than LPS. In this situation anionic polymer on the outer membrane besides LPS such porins are also capable to interact with the cationic PSs [18]. Also PSs can enter the bacterial cytosol by other mechanisms including the self-promoted uptake pathway and protein transport machineries which present in the bacterial cells envelope. In this regard the 'porin' class of protein transporters facilitate the uptake of low molecular weight (600– 700 Da) hydrophilic compounds like PSs [26]. Therefore it is probable that inactivation of OMPs and porins due to PDT, may lead to passing antibiotics across the bacterial membrane, resulting reduction in the MIC value of these materials.

As described above, colistin causes bacterial cells death directly through membrane lysis [27]. Sampson et al. showed that, killing effect of colistin was increased in presence of ROS even in colistin resistant strains [27]. Also they showed that colistin is able to both induce hydroxyl radicals and kill *A. baumannii* through hydroxyl radical production [27]. On the other hand, PDT works with ROS production [17]. Also another effect of PDT on bacterial cells is mediating membrane disruption and increasing permeability [17]. In this situation colistin molecules can direct in to bacterial cells easily and interact and inactive its targets through oxidative cell death pathway [27]. Also Hood et al. showed when *A. baumannii* treated with colistin and efflux pump inhibitor, caused bacterial death more than colistin alone [28]. Therefore, in addition to LPS, efflux pump also is another mechanism for resistance to colistin. It has been reported that PDT cause disruption of efflux pump integrity and prevents effluxing of certain antimicrobials, such as minocycline, tetracycline, and tigecycline which lead to bacterial cells death.

Finally, when *A. baumannii* is treated with PDT, LPS and efflux pumps destabilize and colistin can penetrate in to the bacterial cells. Importantly, the type of PS is a major factor in PDT. For example, cationic dyes are effective against Gram negative bacteria than anionic dyes. In this regard TBO has a greater interaction with LPS than other dyes. Because TBO unlike other dyes forms higher aggregates on the surface of LPS. Among cationic dyes TBO not only have a greater affinity for LPS than other cationic dyes, but it also causes more bacterial photo damage [18]. Also it has been demonstrated that TBO-mediated photo killing through the OMPs which probably are involved in photo killing [18]. Photodynamic inactivation of Gram negative bacteria was overcome either by using cationic PS, or by combining the PS with positively charged antibiotics such colistin [26]. In this regard cationic PS can interact with LPS, anionic proteins, porins and efflux pumps and destabilize the bacterial membrane [18, 26] which lead to PS and colistin entering into the cells and makes cell death.

Recently, effects and mechanisms of action of PDT in combination with antibiotics are described [17]. For example, Cahan et al. showed that treating of Gram negative and Gram positive bacteria with PS-antibiotic conjugates has a good bactericidal activity [29].

Almeida et al. indicated that PDT in combination with antibiotics can kill MDR bacteria in hospital wastewaters [30]. Barra et al. showed that PDT in combination with gentamycin is highly effective against pathogenic bacteria, also in low dose of gentamycin is effective strategy against biofilms [31]. These data support our hypothesis which says PDT disrupts membrane integrity that allowes entering antibiotics into bacterial cells.

Also the researches that studied PDT effect in combination with antibiotics to fight bacterial biofilms published recently [32, 33]. In present study SEM analysis showed that PDT prevents biofilm formation of *A. baumannii*. Kashef et al. conducted the study that sub lethal PDT and some antibiotics cause resistance to erythromycin, amoxicillin-clavulanate and amikacin. But our study result was contrary to this study [34]. Recently, it has been reported that PDT dose has no influence on enzymatic resistant mechanisms, because it interact with selective OMPs more than enzymatic activities [26].

## Conclusion

In conclusion, our analysis showed that combination of PDT and colistin is highly effective against PDRAB. Therefore, it seems that this strategy can be used against drug resistant bacteria in localized infections. But further studies should conduct for determination of its efficacy in clinics.

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