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Original Research

Assessment of biofilm formation among clinical isolates of *Acinetobacter baumannii* in burn wounds in the west of Iran

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Abstract: Burn wound infection by *A. baumannii* is one of the predominant cause of mortality worldwide. The present investigation aimed at determination of antimicrobial resistance profile and expression of the biofilm-related genes in *A. baumannii* isolated from hospitalized patients with burn wound infection in Kermanshah hospitals. Sixty four isolates of *A. baumannii* were recovered from burn wound of hospitalized patients at hospitals in Kermanshah. The antimicrobial susceptibility testing (AST) was performed. Biofilm formation was measured and antibiotic resistance was compared between before and after of biofilm formation. The polymerase chain reaction (PCR) and Real-Time PCR were performed to detect of *abaI* and *pgaD* genes. The biofilm producer isolates and the most resistant isolates were exposed to ozone gas .More than 70% strains were resistance to Erythromycin, Ofloxacin, Ceftazidime, Ceftriaxone, and Ticarcillin-clavulanic acid and 50% isolates were resistant to Imipenem. Thirty one (48.4%) isolates were biofilm producer. The *pgaD* and *abaI* genes were positive in 29 (45.3%) and 9 (14%) isolates, respectively. Real time PCR demonstrated that the copy numbers of the *pgaD* and *abaI* genes after biofilm formation were increased. After exposure to ozone, biofilm formation reduced in all very strong biofilm producing isolates. Our results showed that after biofilm formation, an increased resistance was observed in most isolates. Also rising expression of *abaI* gene was associated with biofilm formation and an increase of antibiotic resistance. In the current study, both biofilm formation and antibiotic resistance were reduced after O3 exposure.

Key words: *Acinetobacter baumannii*, Antibiotic resistance, Biofilm, Minimum Inhibitory Concentration (MIC), Ozone therapy.

Introduction

The cost of wound care in worldwide is so much and until now wound infection especially burn wound infection is a major challenge in hospitals. Burn wound infection is one of the predominant cause of morality in worldwide especially in developing country (1). Although in Iran a significant progress about wound care is observed but yet wound infection is a major problem. *Acinetobacter baumannii* is one of the most notorious pathogens in burn wards.

A. baumannii is an opportunistic pathogen cause of nosocomial infections especially in immunocompromised patients (2). The antibiotic resistance development and emerging multidrug resistance (MDR) among *A. baumannii* strains isolated from hospitalized patients are routine (3). Acquire new genes related to resistance, biofilm formation, and overexpression of efflux pumps are important elements in the spread of MDR *A. baumannii*. These MDR isolates are related to diverse infections includes respiratory, bloodstream, urinary tract, skin, wound, and soft tissue infections (4).

In addition to antibiotic resistance, biofilm formation plays a determinants role in infectious capacity, pathogenesis and persistence of *A. baumannii* in the hospital environment (5). Biofilms permit the bacteria resist to disinfectant while also helping the participating cells to exchange resistance genes and resistance mechanisms, further simplifying the persistence of the pathogen in the clinical environment (6).

Because antibiotic resistance, biofilm formation and limit the efficacy of topical application of antibiotic on wound infection, different studies investigated the new options and alternatives for control of wound infection caused by *A. baumannii*. For example, blue light (415 nm) showed a strong antimicrobial activity against MDR isolates of *A. baumannii* (7). Also, demonstrated that pulsed electric field (PEF) has a suitable capability to reduce the load of MDR isolates of *A. baumannii* (1).

Some studies have investigated the effect of ozone on the healing of wound infection (8). The ozone as naturally occurring gas, an unstable allotrope of oxygen, reacts rapidly with most hydrocarbons to effectively destroy biofilms, microorganisms, and organic residue material within these films (9). The effect of ozone on bactericidal activity in numerous studies was confirmed (10, 11). Sharma *et al*, demonstrated that several bacterial pathogens such as *Clostridium difficile*, methicillin-resistant *Staphylococcus aureus* (MRSA), and *A. baumannii* were inactivated after exposure to ozone (12).

The aims of this study were to determine of i) antibiotic resistance profile and biofilm formation, ii) eva-

luation of relative expression of biofilm-related genes iii) and ozone potency for the eradication of MDR isolates of *A. baumannii* recovered from burn wards of Kermanshah hospitals.

Materials and Methods

Isolation of *A. baumannii*

In May 2016 to Jun 2017, 64 isolates of *A. baumannii* were recovered from burn wound of hospitalized patients at Imam Reza and Imam Khomeini hospitals, Kermanshah, Iran. The collected samples were transferred to hospitals laboratory in thioglyculate broth. The strains were identified as *A. baumannii* using biochemical test and API 20NE kit (bio-Merieux, France). The *A. baumannii* ATCC 19606 used as positive control.

Antimicrobial susceptibility testing

Antimicrobial susceptibility testing (AST) was performed using both disk diffusion and micro-broth dilution methods according to CLSI guideline protocols (13). The AST was performed using disk diffusion method for Ceftriaxone (30 µg), Ampicillin-Sulbactam (20 µg), Erythromycin (15 µg), Ofloxacin (5 µg), Cefepime (30 µg), Amikacin (30 µg), Ceftazidime (30 µg), Ticarcillin-clavulanic acid (85 µg), Tigecycline (15 µg), Piperacillin-tazobactam (110 µg), Colistin (10 µg), Azithromycin (15 µg), Imipenem (10 µg), and Tobramycin (10 µg). Also, micro-broth dilution was applied to the determination of minimum inhibitory concentration (MIC) for Imipenem. Biofilm formation was induced in isolated strain using a concentration of 0.5 McFarland and antibiotic resistance was compared between before and after of biofilm formation.

Analyzing of biofilm formation using the microtiter plate method

For investigation of biofilm formation, strains were cultured in tryptic soy broth (TSB) (including 0/25%) and were incubated at 37°C for 24 hours. Then 250 µl of each medium containing 10^7 CFU/ml of bacteria were inoculated in each well of the microtiter plate and was added at 37°C for 24 hours. Also, the medium without bacteria was considered as control negative (AC). Subsequently, the microtiter plate was decanted and washed with phosphate buffered saline (PBS) 5 times. After washing, 100µl crystal violet was added in each well and incubated for 10 minutes at room temperature. Then the microtiter plate was washed and air dried in an inverted position at room temperature. Subsequently, 160 µl acid acetic was added to wells and microtiter plate was read with the enzyme-linked immunosorbent assay (ELISA) reader (Synergy; HTX Multi-Mode Microplate Reader, USA) at wavelength 600-650 nm. The results were compared with control negative and the result was interpreted as below (14):

1. No biofilm producer: A≤ Ac
2. Weak biofilm producer: Ac<A≤ 2×Ac
3. Moderate biofilm producer: 2×Ac<A ≤4×Ac
4. Strong biofilm producer: 4×Ac<A.

(A: Sample, Ac: Control wells)

Polymerase Chain Reaction (PCR)

Genomic DNA was extracted using boiling method. The PCR was performed to detection of *16sRNA*, *abaI* and *pgaD* genes as described previously (15-19). Using the primers shown in table 1. The PCR program was initiated by 94°C for 5 minutes and followed by 35 cycles of 94°C for1 minute, 54°C for1 minute, 72°C for 1 minute, finally followed by 72°C for 10 minutes. The amplified products were visualized on gel agarose electrophoresis (2%) stained using DNA safe stain (SinaClon, Iran).

Real-Time PCR

The RNAs were extracted using RB100 mini kit (Geneaid, Taiwan) and concentration was evaluated by Nanodrop (Synergy; HTX Multi-Mode Microplate Reader, USA). Subsequently, the cDNA was prepared by cDNA Prime ScriptTM RT reagent Kit (Takara, Japan).

Quantification of *abaI* and *pgaD* transcripts was performed by SYBR Green method (Fermentas, Germany) with 250 ng cDNA and 0.2 µM each forward and reverse primers, 10 µl SYBR Premix Ex and 0.4 µl ROX reference (50 X) in a final volume of 20 ml on the RT devices (Applied Biosystems, USA). The PCR program was initiated by 95°C for 30 s, followed by 38 cycles of 95°C for 5 s, 54°C for 34 Cycles. The calculator can be found at: www.scienceprimer.com/ and copy number calculator for Real Time PCR:

$$\text{Number of copies} = \frac{x\,ng * 6.0221 * 10^{23}\ (molecules/mol)}{(N * 660\ g/mole) * 1 * 10^{9}\ ng/g}$$

Where: X= amount of amplicon (ng)
N= length of dsDNA amplicon
660 g/mol= average mass of 1 bp dsDNA

The effect of ozone gas

The biofilm producer isolates and the most resistant isolates were exposed to ozone gas that according to a previously described protocol with some modifications (8). For this purpose first, prepared 0.5 McFarland suspension from biofilm producer isolates and the most resistant isolates and then 100µL of this suspension was inoculated in TSB containing ELISA plate and the plates were exposed to ozone gas (80µg/ml) for 40 minutes. This was done twice for any of strains, one for evaluation of biofilm production and one for antimicrobial susceptibility testing.

Table 1. Primer sequences used in this study.

Primer	Sequences*(5′ to 3′)*	Product size
pgaD	F: TTG ATC AGC CTG AAT ATG TGA R: CAC ACA TAG TCA TAA ATG AGG	145 bp
abaI	F: AAT GCC TAT TCC CTG CTC AC R: AAT GCT TGC AGA ATT GC	132 bp
16sRNA	F: CAG CTC GTG TCG TGA GAT GT R: CGT AAG GGC CAT GAT GAC TT	150 bp

Results

Sex and age distribution

From 64 patients 40 (62.5%) were men and 24 (37.5%) patients were women. The mean age was 39.5 years old (SD=±26.2 ranged from 1 to 84 years). Of 64 identified *A. baumannii*, 36 and 28 strains were isolated from Imam Reza and Imam Khomeini hospitals, respectively.

Antibiotic susceptibility testing

The results of AST were illustrated in Chart 1&2. The most resistance frequency was observed in Erythromycin (100%), Ofloxacin (89%) and Ceftazidime (89%). Also, Colistin (43.7%), Imipenem (50%) and Tigecycline (77.1%) were the most active antibiotic agents against *A. baumannii*. more than 70% strains were resistance to Erythromycin, Ofloxacin, Ceftazidime, Ceftriaxone, and Ticarcillin-clavulanic acid. In contrast to disk diffusion results, results of micro-broth dilution demonstrated that 64 (50%) isolates were resistant to Imipenem (Figure 1).

Investigating the formation of biofilm

Of 64 isolates, 31 (48.4%) isolates were biofilm producer. Comparison of antibiotic resistance between before and after biofilm formation. resistance to Colistin, Tigecycline, and Azithromycin were not increased. Also, 4 (6.2%) of 31 isolates produced a very strong biofilm (Figure 1 & 2).

PCR

Genus and species of all isolates were confirmed using PCR targeted *16sRNA*. The *pgaD* and *abaI* genes were positive in 29 (45.3%) and 9 (14%) isolates, respectively. Furthermore, 6 (9.37%) isolates were positive for both *pgaD* and *abaI* genes.

Real-Time-PCR

The positive *pgaD* and *abaI* isolates subjected to Real-Time PCR experiment. Results demonstrated the copy numbers of the *pgaD* gene before biofilm formation was ranged from 7.29 to 11.29 (mean=9.11) and after biofilm formation was ranged from 7.84 to 10.79 (mean=9.44). Also, the copy numbers of *abaI* gene before biofilm formation was ranged from 9.16 to 11.02 (mean=9.63) and after biofilm formation was ranged from 10 to 11.73 (mean=10.63).

Ozone effect

Four isolates that were resistant to all antibiotics (except Colistin), after exposure to of ozone, were susceptible to most antibiotics (5 to 9 antibiotics) (table 2). Biofilm formation was also decreased in all of very strong biofilm producing isolates after exposure to ozone.

Discussion

Skin is the first barrier in human defence against infective agents. Skin irritation, burns and wound led to the development of skin infections (20). Prevalence of wound infection is 2.5 to 25% in different countries. The cost of wound care was more than 20 billion dollars per years. Numerous agents play role in wound infection; *A. baumannii* is one of the most important causative agents of burn wound infection. Management and treatment of wound infection caused by *A. baumannii* are still remaining challenging (1). Due to exorbitance use of antibiotics in the recent decades, MDR *A. baumannii* isolates have been reported worldwide (21). Resistance to Cephalosporin and Tigecycline obtained in the current study were close to other studies (22-26). Resistance to Ofloxacin was less than other studies performed in Iran (24, 26, 27). The most important reason

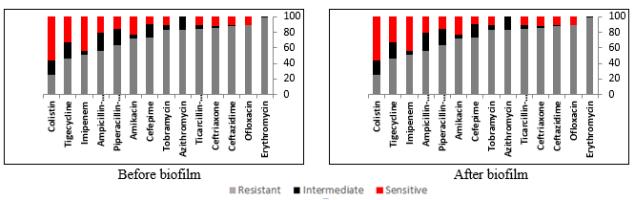

Figure 1. Frequency (percent) of resistance to various antibiotics before and after biofilm.

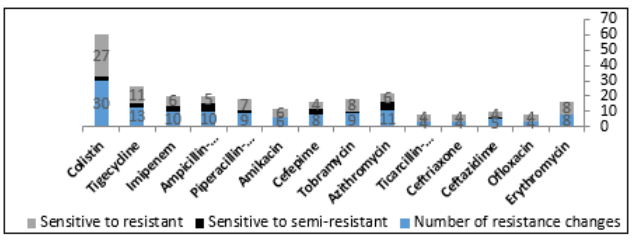

Figure 2. Changing of resistance frequency among isolated strains after biofilm formation.

Table 2. The effect of ozone gas on the antibiogram pattern of the most resistant isolates of this study.

Ozon gas	Code of isolates	AST													
		CRO	SAM	E	OFX	CPM	AK	CAZ	TIM	TGC	PTZ	CO	ATH	IMI	TN
Pre-effect ozone gas	1	R	R	R	R	R	R	R	R	R	R	S	R	R	R
	2	R	R	R	R	R	R	R	R	R	R	S	R	R	R
	3	R	R	R	R	R	R	R	R	R	R	S	R	R	R
	4	R	R	R	R	R	R	R	R	R	R	S	R	R	R
Pro-effect ozone gas	1	R	S	R	R	R	R	R	S	S	S	S	I	S	R
	2	R	S	R	R	S	S	R	S	S	S	S	R	S	S
	3	R	S	R	R	R	S	R	S	S	S	S	R	S	R
	4	R	S	R	R	R	R	S	S	S	S	S	R	S	R

*CRO=Ceftriaxone, SAM=Ampicillin-Sulbactam, E=Erythromycin, OFX=Ofloxacin, CPM= Cefepime, AK=Amikacin, CAZ=Ceftazidime, TIM=Ticarcillin-clavulanic acid, TGC=Tigecycline, PTZ=Piperacillin-tazobactam, CO=Colistin, ATH=Azithromycin, IMI =Imipenem, TN=Tobramycin. *R=Resistant, S=Sensitive, I=Intermediate.

for this difference is associated with geographical areas and predominantly used drug regime. In past years Carbapenems (especially Imipenem) used as the first and suitable choice for treatment of infections caused by *A. baumannii* worldwide. But recently, in this study in accordance with other studies resistant isolates to Imipenem was detected. In some studies, resistance to Imipenem was more than 90% (28). After the increase of resistance to Imipenem that reported by several scientists, Colistin and Tigecycline were described as new drug choice for treatment of *A. baumannii* infections. Resistance to Colistin and Tigecycline in this study and other study was reported (29). The high level of resistance to these antibiotics is related to overuse of them in hospitals.

Several factors such as long period of hospitalization, long use of catheters, ventilator, and implants are involved in colonization, persistence, and biofilm formation of *A. baumannii* in hospitals environment (30). Our results showed that after biofilm formation, an increased resistance was observed in most isolates, also an increased resistance was observed in two important drugs Tigecycline and Colistin (20% and 45%, respectively). Thus, biofilm producer isolates are challenging pathogens in treatment and management of *A. baumannii* infections. The relation between biofilm and change in antibiotic resistance is associated with several factors including limited access of antibiotic to pathogens, the transfer of related genes to antibiotic resistance among isolates, and low level of bacterial metabolism in the biofilm (30). After biofilm formation, different expression between *abaI* and *pgaD* genes was detected and 100% increased expression and 64% reduced expression for *abaI* and *pgaD* genes were observed, respectively. However, the previous study demonstrated that *pgaD* gene has a high-level expression in *A. baumannii* strains recovered from burn wound infections (31). The *pgaD* gene is a part of *pgaABCD* operon (involved in quorum sensing (QS)) and encodes a protein with the unclear role (32). Other study demonstrated that in the biofilm formation process, increased expression of *abaI* gene has a capacity for regulation of the *pgaD* gene (33). Anbazhagan *et al* demonstrated that AbaI protein has autoinducer role in *QS* and *abaI* mutant of *A. baumannii* unable to biofilm development (34). On other hands, in the previous study, a relation between *QS* and antibiotic resistance was described (34, 35). Our results confirm this relation, and rising expression of *abaI* gene was associated with biofilm formation and an increase of antibiotic resistance. This revealed that indirectly, *abaI* gene plays an important role in the development of antibiotic resistance and emerging MDR isolates.

In the current study, both biofilm formation and antibiotic resistance were reduced after O3 exposure. In accordance with current study ozone activity against pathogens in the different studies demonstrated. For example, Kowalski et al. showed that *Escherichia coli* and *Staphylococcus aureus* were eradicated in 300 ppm ozone for 15 s. Also, *S. aureus* was eradicated in 1500 ppm for 15 s (36). Furthermore, Summerfelt et al. showed that most pathogens available in the aqueous environment were eradicated by ozone exposure (37). Also, ozone activity against bacterial spores (*Clostridium* and *Bacillus*) was demonstrated (38-40). Briefly, ozone has a powerful impact on the cell wall, nucleic and amino acids of bacteria and led to cell death. Application of ozone at hospital environment is favourable because the ozone has numerous advantages including, suitable activity against different pathogens (board spectrum), cost-effective, high penetration to the various environment, low half-life (about 20 minutes), and fast neutralization. The most important disadvantage of ozone applying is its adverse effects on human health, especially on the lung. This adverse effect is controlled by preventing the inhalation of this gas by patients and health workers. On other hands, ozone has irritating effects on skin when used for a long time (8, 12). However, in the previous studies ozone used for the treatment of diabetic foot and wound infection and suitable results were observed (41, 42). Thus, use of this gas in burn ward is efficient propose for control and management of infections.

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Interest conflict
Non of authors have a conflict of interest.

Author's contribution
Dr Amirhoushang Alvandi: Design and management of research implementation, Dr Neda Akbari, Dr Parvaeh Jafari and Dr Ramin Abiri: Cooperation in data analyzing and manuscript writing, Reza Hatami Moghadam: Performing of research, data analyzing and manuscript writing.

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