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Biofilm production and biocidal efficacy in multidrug-resistant *Pseudomonas aeruginosa* and *Acinetobacter baumannii* isolates

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SUMMARY

Objective: Nosocomial infections caused by resistant Gram-negative bacilli have become a major problem for hospitals in recent years. Antiseptics and disinfectants play an important role in the prevention of nosocomial infections and in the management of infections. Some Gram-negative bacilli also show resistance to antiseptics and disinfectants. Therefore, the selection of proper antiseptics and disinfectants is crucial to prevent nosocomial infections produced by these resistant organisms. In this study, we investigated the biofilm production, antimicrobial susceptibility, and biocidal activity of commonly used antiseptics and disinfectants in our hospital setting against *Pseudomonas aeruginosa* and *Acinetobacter baumannii* isolates.

Methods: Between January and December-2014, a total of 50 *Pseudomonas aeruginosa* and 50 *Acinetobacter baumannii* strains, which were multidrug-resistant (MDR) strains, were included in this study. Biofilm production was identified spectrophotometrically by the microplate assay. Activity of sodium hypochlorite, chlorhexidine, orthophthalaldehyde (OPA), peracetic acid (PA), and peracetic acid/hydrogen peroxide was studied with suspension tests.

Results: Commonly used disinfectant-antiseptics were found to be effective against multi-drug resistant *A. baumannii* and *P. aeruginosa* strains as follows, chlorhexidine 98%, sodium hypochlorite 90%, OPA 96%, PA and peracetic acid/hydrogen peroxide 94%. The rates of efficacy against the antibiotic-susceptible *A. baumannii* and *P. aeruginosa* were found to be 100% for chlorhexidine, OPA and PA, 98% for sodium hypochlorite, and 94% for peracetic acid/ hydrogen peroxide. Considering the relationship between the biofilm production and biocidal activity, 22% of biofilm-producing strains of *A. baumannii* were found to be resistant to any all disinfectants-antiseptics tested, while this rate was 2% in the *P. aeruginosa* strains. Disinfectant resistance rates were 2% and 6% for biofilm-negative *A. baumannii* and, *P. aeruginosa* strains, respectively. Biofilm production and disinfectant resistance were found to be significantly associated with *A. baumannii*, compared to *P. aeruginosa* ($p<0.05$).

Conclusion: Tested antiseptics-disinfectants showed 90% efficacy to Gram-negative non-fermentative bacteria isolated in the intensive care unit in our hospital. It would be reasonable to perform further efficacy tests for commonly used antiseptics and disinfectants on a regular basis.

Keywords: *Acinetobacter baumannii*; biofilms; disinfectants; *Pseudomonas aeruginosa*; resistance.

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Pseudomonas aeruginosa and *Acinetobacter baumannii* are common agents responsible for nosocomial infections among other Gram-negative bacteria. *A.baumannii* ranks the first as the causative agent of nosocomial infections in most centers.[1] This infectious agent is important not only for being the cause of outbreaks associated with a high mortality in the intensive care unit (ICU), but also emergence of multidrug-resistant strains. There has been a significant increase in the prevalence of multidrug-resistant strains in recent years. The outbreaks caused by *Acinetobacter* strains are associated with the ability of bacteria to remain viable for prolonged periods in a dry environment and emergence of antibiotic-resistant infections.[2] *P. aeruginosa* accounts for 10 to 25% of all nosocomial infections.[3] This agent, in particular, causes nosocomial infections in patients who receive long-term broad-spectrum antibiotherapy and in those who receive chemotherapy or undergo mechanical ventilation and surgical procedures.[4]

It is well-established that bacterial resistance can be acquired not only against antibiotics, but also against disinfectants and antiseptic materials used. Disinfection of the environment and materials with the selection of appropriate disinfectants, and proper use are helpful to prevent the development of many nosocomial infections.[5] In addition, biofilm protects bacteria from phagocytosis and effects of the complement, and this layer forms a physical barrier rendering bacterial resistance to the effects of antibiotics and disinfectant materials.[6]

In this study, we aimed to investigate the biofilm production, antimicrobial susceptibility, and biocidal activity of commonly used antiseptics and disinfectants in our hospital setting against *P. aeruginosa* and *A. baumannii* isolates.

Materials and methods

Between January and December 2014, 164 *Pseudomonas spp.* and 395 Acinetobacter spp. strains were isolated from patients hospitalized in the intensive care unit at Republic of Turkey MoH, Marmara University, Pendik Training and Research Hospital. Of these isolates, 50 *Pseudomonas aeruginosa* and 50 *Acinetobacter baumannii* strains were found to be multidrug-resistant (MDR) strains.

The strains were identified using the mass spectrophotometry (VITEK MS, bioMérieux, France). The antibiotic susceptibility was tested using the disc diffusion test.[7] *P. aeruginosa* strains[8] resistant to all carbapenem, aminoglycoside, and fluoroquinolone groups and *A. baumannii* strains resistant to all penicillin antibiotics plus at least three of the cephalosporin, quinolone, carbapenem, and fluoroquinolone groups were considered multidrug-resistant strains.[9] In the study, biofilm forming was tested using *A. baumannii* ATCC19606 and *P. aeruginosa* PAO-1 strains positive controls and *P. aeruginosa* PAO-JP3 strain as negative control.[10-12]

Clinical strains incubated overnight in the MacConkey agar (BioMerieux, France) and a colony of control strains were inoculated into tubes containing a 5-ml fresh Luria Bertani (LB, Sigma, USA) liquid medium at 37°C for 24 hours to calculate the biofilm production. After incubation, 1:100 dilution was performed in a fresh LB liquid medium and the dilution was transferred to three wells each containing 100 ml on a sterile, flat-bottomed 96-well polystyrene microplate (Greiner, Germany). The plates were incubated at 37°C for 24 hours, and, then the content of the wells was removed and irrigated three times with distilled water. After irrigation, each well was filled with 100 µl crystal violet solution (0.1%) and the plates remained in room temperature for 10 minutes. The plates were, then, irrigated three times with distilled water to remove excess dye solution and the wells were added 200 µl ethanol 95% to quantify the biofilm layer. Following five minutes of incubation, the absorbance was read in an optic reader (Labsystem Multiskan MS, Thermo Scientific, USA) at 550 nm. The mean absorbance of three wells was recorded for each strain and the experiments were repeated three times. The cut-off value of biofilm production was estimated using the mean absorbance values and standard deviation for non-biofilm forming *P. aeruginosa* PAO-JP3. A mean + two standard deviations were considered as the cut-off value.[11]

Sodium hypochloride (1%), OPA (Orto-Phthalaldehyde) (0.5%) (Anios), chlorhexidine (4%) (Anios), Peracetic acid (2%) (EcoLab), Peracetic acid + hydrogen peroxide (0.2% + 7.5%) (EcoLab) were used in the disinfectant activity studies. The disinfectant concentrations used in the experiments were as 1%, 4%, 0.5%, 2%, 0.2%+7.5% freshly prepared in sterile distilled water. The suspension test method reported by Michel and Zach was used to evaluate the effects of antiseptic and disinfectants on selected strains.[13,14] This method is a modified version of qualitative and quantitative suspension test recommended by the German Society for Hygiene and Microbiology. A suspension was prepared from *Pseudomonas aeruginosa* ATCC 15442, *Escherichia coli* ATCC 10536, *Staphylococcus aureus* ATCC 6538, clinical *A.baumannii* (n=50), and clinical *P. aeruginosa* (n=50) strains reproduced in the MacConkey agar at 0.5 McFarland standard in a phosphate buffer and the suspension was, then, diluted to 1/100. A 100 µl of this suspension was transferred into sterile Eppendorf tubes

containing 100 µl disinfectant solution at room temperature (20-25°C) and serial dilutions were performed after five minutes (eight times). A 10 µl of each serial dilution was transferred on tryptic soy agar (TSA) using the drip inoculation method and incubated at 37°C for 18 hours. Disinfectant was considered effective in the absence of growth in a specified contact time and concentration; disinfectant solution was considered ineffective, if there was no 99.999% decline (≥10 colony), compared to the positive control. The suspensions of each strain without a disinfectant solution were used as the positive controls.

Statistical analysis was performed using SPSS v15 software (SPSS Inc., Chicago, IL, USA). A p value of <0.05 was considered statistically significant.

Results

The experiments performed on the negative control strain (*P. aeruginosa* PAO-JP3) showed a cut-off value of 0.169 for the biofilm production. The biofilm production of *P. aeruginosa* and *A. baumannii* was evaluated on the basis of this cut-off value.

Biofilm production was positive in 42.8% of MDR *P. aeruginosa* strains and 75.6% of MDR *A. baumannii* strains. Biofilm production was also positive in 37.9% of antibiotic resistant *P. aeruginosa* strains and 71.4% of *A. baumannii* strains (Table 1). Biofilm production and disinfectant resistance were significantly higher in the *A. baumannii* group, compared to the *P. aeruginosa* group (p<0.05).

The rates of efficacy against MDR *A. baumannii* and *P. aeruginosa* strains (n=50) were found to be 98% (n=49/50) for chlorhexidine, a commonly used hand antiseptic at our hospital, 90% (n=45/50) for sodium hypochlorite, 96% (n=48/50) for OPA, 94% (n=47/50) for PA, and 96% for peracetic acid/hydrogen peroxide. The rates of efficacy against susceptible *A. baumannii* and *P. aeruginosa* strains (50%) were 100% (n=50/50) for chlorhexidine, OPA, and PA, 98% (n=49/50) for sodium hypochlorite, and 94% (n=47/50) for peracetic acid/hydrogen peroxide (Table 2).

Three out of 50 *P. aeruginosa* strains showed resistance against various disinfectants, while only one strain was found to be positive for the biofilm production. The relationship between the biofilm production and disinfectant susceptibility was not significant in *P. aeruginosa* strains. One non-biofilm forming *P. aeruginosa* strain was found to be resistant to both chlorhexidine and peracetic acid/hydrogen peroxide.

A total of 10 strains among *A. baumannii* isolates were resistant to more than one disinfectant, while only

Table 1. Biofilm production in multidrug-resistant *Pseudomonas aeruginosa* and *Acinetobacter baumannii* strains.

	***Pseudomonas aeruginosa* (n=50)**						***Acinetobacter baumannii* (n=50)**					
	MDR		**Sensitive**		**Total**		**MDR**		**Sensitive**		**Total**	
	n	**%**	**n**	**%**	**n**	**%**	**n**	**%**	**n**	**%**	**n**	**%**
Biofilm- positive	9	42.8	11	37.9	20	40	22	75.8	15	71.4	37	74
Biofilm- negative	12	57.2	18	62.1	30	60	7	24.2	6	28.6	13	26
Total	21		29		50		29		21		50	

MDR: Multidrug-resistant.

Table 2. The efficacy rates of disinfectants against *P. aeruginosa* and *A. baumannii* strains.

	Pseudomonas aeruginosa				***Acinetobacter baumannii***			
	MDR (n=21)		**Sensitive (n=29)**		**MDR (n=29)**		**Sensitive (n=21)**	
	Biofilm-positive (n=9)	**Biofilm-negative (n=12)**	**Biofilm-positive (n=11)**	**Biofilm-negative (n=18)**	**Biofilm-positive (n=22)**	**Biofilm-negative (n=7)**	**Biofilm-positive (n=15)**	**Biofilm-negative (n=6)**
Sodium hypochlorite	E	1	E	E	4	E	1	E
OPA	E	E	E	E	2	E	E	E
Chlorhexidine	E	1	E	E	E	E	E	E
Peracetic acid (PA)	E	E	E	E	3	E	E	E
PA+Hydrogen peroxide	E	1	1	E	1	E	1	1

MDR: Multidrug-resistant; OPA: Orto-Phthalaldehyde; E: Effective.

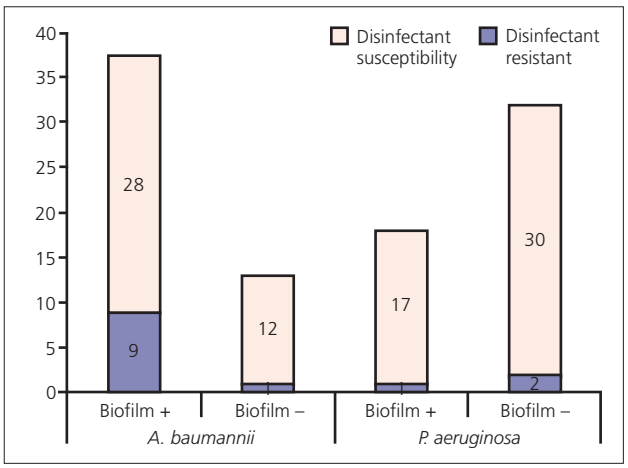

Fig. 1. Disinfectant susceptibility and biofilm production in multidrug-resistant *Pseudomonas aeruginosa* and *Acinetobacter baumannii* strains.

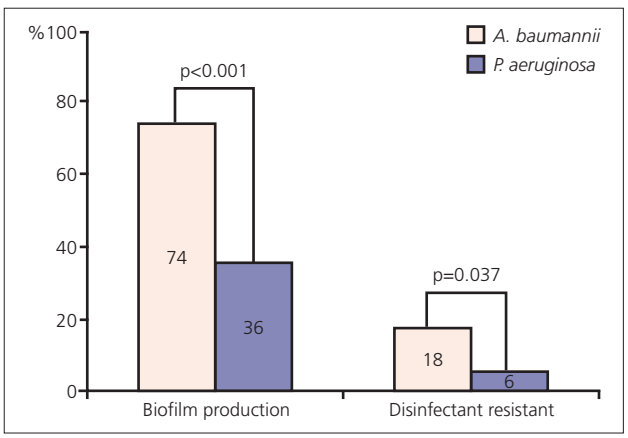

Fig. 2. Disinfectant susceptibility in *P. aeruginosa* and *A. baumannii* strains.

one strain was negative for the biofilm production. Of 10 disinfectant-resistant *A. baumannii* strains, nine were found to be positive for the biofilm production.

In the *A. baumannii* group, three strains were resistant to two disinfectants. Of these strains, two were resistant to sodium hypochlorite and peracetic acid, whereas one strain was resistant to OPA and peracetic acid.

Disinfectant susceptibility and biofilm results are presented in Figure 1.

The rate of disinfectant resistance was significantly higher in *A. baumannii* strains, compared to *P. aeruginosa* strains (Figure 2).

Discussion

The presence of bacteria in the hospital setting leads to continuous contamination of the patients and hospital staff which makes impossible to control hospital infections related to contamination. Thus, disinfection of the environment and hand washing practices are of utmost importance. *Pseudomonas aeruginosa* and *A. baumannii* are the most common agents responsible for nosocomial infections among other Gram-negative bacteria.[15] The importance of these agents has been increasing day by day due to their ability to develop antibiotic resistance, which remain viable for prolonged periods on the surfaces and the ability to cause outbreaks.[1]

Inappropriate use of antiseptic and disinfectant solutions in terms of contact time with an adequate concentration leads to the selection and emergence of microorganisms resistant to these materials in the hospital setting. In addition, bacteria enclosed with a biofilm layer are more known to be resistant to disinfectant materials, compared to free planktonic form. Several studies have shown that inactivation of microorganisms in the biofilm layer requires the use of up to 1000-fold higher concentrations.[16,17]

When the isolates in the present study were analyzed in terms of biofilm production, 40% of *P. aeruginosa* strains and 74% of *A. baumannii* strains produced a biofilm layer. The rate of biofilm production was 75.8% in multidrug-resistant *A. baumannii* strains and the rate in *P. aeruginosa* strains was 42.8%, showing an inverted trend.

When the relationship between the biofilm production and antiseptic/disinfectant materials used in the study was examined, three out of four resistant strains in the *P. aeruginosa* group were biofilm-negative, while nine out of ten resistant strains in the *A. baumannii* group were biofilm-positive, as expected (90%). The efficacy of the disinfectants in the present study was tested on the planktonic form of the bacteria. The study by Spoering and Lewis[18] showed that both planktonic cells and biofilm layer of *P. aeruginosa* exhibited similar resistance to the germicide effects of antibiotics and peracetic acid. The authors emphasized that biocidal efficacy of the bacteria depends on its metabolic activity and bacteria in steady state showed the highest degree of resistance.

Among the materials tested, OPA and peracetic acid showed the highest efficacy against *P. aeruginosa* strains. Using a method similar to that used in the present study, Ekizoglu et al.[19] reported that chlorhexidine 4% and sodium hypochlorite at a dilution rate of 1:50 (1000 ppm) were the most efficient materials against *P. aeruginosa* strains, while sodium hypochlorite at a dilution rate of 1:500 (100 ppm) did not exert efficacy adequately.

Considering *A. baumannii* strains, chlorhexidine 4% was found to be the most effective disinfectant in

this group (100%). Similarly, Ekizoglu et al.[5] found chlorhexidine 4% to be highly efficient against this agent, while sodium hypochlorite at a dilution rate of 1:50 (1000 ppm) did not show an adequate efficacy. In the present study, sodium hypochlorite showed the lowest efficacy against *A. baumannii* strains with a 90% success rate. However, sodium hypochlorite 5% at dilution rates of 1:10 and 1:100 was found to be among the most efficient disinfectant against clinical *P. aeruginosa* and *A. baumannii* strains in the study by Inan et al.[20]

In the present study, five-minute contact time was used in the efficacy tests considering the routine daily practices. Both the rate of the biofilm production and resistance to antiseptic/disinfectant materials were significantly higher in the *A. baumannii* group, compared to the *P. aeruginosa* group ($p<0.05$ and $p<0.001$, respectively). The most efficient disinfectant against all study strains was chlorhexidine (99%), followed by OPA (98%) and PA (97%). Sodium hypochlorite showed the lowest efficacy (94%) based on the clinical isolates. In another study, Gorgul et al.[21] studied sodium chlorite at a dilution rate of 1:100 and reported efficacy against clinical isolates within 15-minute contact time.

In conclusion, antiseptic/disinfectant solutions tested in the present study yielded 90% and higher efficacy rates, compared to non-fermentative Gram-negative bacteria recovered from the intensive care unit of our hospital. The importance of antisepsis and disinfection must be further emphasized considering patients hospitalized in the intensive care units. Proper application and appropriate concentration are the key drivers for success in disinfection and antisepsis. It would be reasonable for each unit to test the efficacy of antiseptic/disinfectant materials used against the isolated agents on a regular basis.

Conflict of interest: None declared.

Peer-review: Externally peer-reviewed.

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ÖZET

Çoklu ilaç dirençli *Pseudomonas aeruginosa* ve *Acinetobacter baumannii* izolatlarında biyofilm üretimi ve biyosidal etkinlik

Burcu SEBİT, Burak AKSU, Ayşegül KARAHASAN YAĞCI

Amaç: Dirençli Gram-negatif bakterilerin neden olduğu nozokomiyal infeksiyonlar, son yıllarda hastanelerin önemli problemi haline gelmiştir. Antiseptikler ve dezenfektanlar, nozokomiyal infeksiyonlarının önlenmesinde ve infeksiyon kontrol çalışmalarında önemli bir rol almaktadır. Gram-negatif bakterilerin bir kısmı antiseptik ve dezenfektanlara direnç göstermektedir. Bu nedenle, bu dirençli mikroorganizmalar tarafından oluşturulan nozokomiyal infeksiyonları önlemek için, uygun antiseptik ve dezenfektanların seçimi önemlidir. Bu çalışmada *Pseudomonas aeruginosa* ve *Acinetobacter baumannii* izolatlarında antimikrobiyal duyarlılığı ve hastanemizde kullanılan antiseptik ve dezenfektan maddelerin etkinliği ve biyofilm üretiminin dirençle ilişkisi araştırıldı.

Yöntemler: Bu çalışmaya Ocak - Aralık 2014 tarihleri arasında çoklu ilaç dirençli 50 *Pseudomonas aeruginosa* ve 50 *Acinetobacter baumannii* suşu alındı. Biyofilm üretimi, mikroplak yöntemi ile spektrofotometrik olarak saptandı. Sodyum hipoklorit, klorhekzidin, orto-fitalaldehit (OPA), perasetik asit (PA) ve perasetik asit/hidrojen peroksit için aktivite süspansiyon yöntemi ile tayin edildi.

Bulgular: Çoklu ilaç dirençli *A. baumannii* ve *P. aeruginosa* suşlarında, hastanemizde sıklıkla kullanılan antiseptik ve dezenfektanlardan klorhekzidin %98, sodyum hipoklorit %90, OPA %96, PA %94, perasetik asit/hidrojen peroksit %96 oranında etkili bulundu. Antibiyotik duyarlı *A. baumannii* ve *P. aeruginosa* suşlarında ise, klorhekzidin, OPA ve PA %100, sodyum hipoklorit %98, perasetik asit/hidrojen peroksit ise %94 oranında etkin olduğu saptandı. Biyofilm üretimi ile biyosidal direnç ilişkisi incelendiğinde, biyofilm üreten *A. baumannii* suşlarında herhangi bir dezenfektan-antiseptik direnci %22 iken, bu oran *P. aeruginosa* suşlarında %2'dir. Biyofilm negatif suşlarda dezenfektan-antiseptik direnci *A. baumannii* için %2, *P. Aeruginosa* için ise %6 idi. Biyofilm üretimi ve dezenfektan direnci, *P. aeruginosa*'ya kıyasla, *A. baumannii* grubunda anlamlı düzeyde yüksek bulundu ($p<0.05$).

Sonuç: İncelenen antiseptik/dezenfektanların hastanemiz yoğun bakım ünitesinden izole edilen nonfermentatif Gram-negatif bakterilere karşı %90 ve üzeri oranda etkin olduğu saptandı. Periyodik aralıklarla, izole edilen patojenler üzerinde kullanılan antiseptik/dezenfektanların etkinliğinin test edilmesi akılcı olacaktır.

Anahtar sözcükler: *Acinetobacter baumannii*; biyofilm; dezenfektan; direnç; *Pseudomonas aeruginosa*.

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