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Biofilm Formation of *Acinetobacter baumannii* Under *in vitro* and *in vivo* Colistin Exposure

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ABSTRACT

Objective: We aimed to find the alterations in biofilm formation of *Acinetobacter baumannii* (*A. baumannii*) during adaptation to colistin resistance under colistin stress.

Materials and Methods: Eighteen patients with an isolation of *A. baumannii* (nine colistin resistant and nine colistin susceptible) and additionally two patients that develop colistin resistant *A. baumannii* infection during hospital stay were included the study. For *in vitro* adaptation study, four colistin susceptible strains of one of the patients were sub-cultured onto Mueller Hinton agar containing sub-MIC concentrations of colistin for 40 serial passages. The colistin resistance was determined by broth dilution. Biofilm production was measured by crystal violet assay and images were taken with confocal microscopy. Genotyping of selected isolates was done by MLST. The thirty-three per cent of ColR *A. baumannii* was isolated from respiratory tract.

Results: The biofilm formation in ColR *A. baumannii* isolates was 78%, and it was 54% in ColS *A. baumannii*. In the adaptation study, we did not find a difference in biofilm levels of laboratory-induced colistin resistant generations. On the other hand, clinical ColR isolate was found to be 2.6-3.4 times more biofilm producer than laboratory induced generations.

Conclusion: We suggest that *A. baumannii* may develop adaptation mechanisms to constitute colistin-resistance in the presence of host-dependent factors and environmental stress conditions in order to gain stronger biofilm production to enhance virulence.

Keywords: *Acinetobacter baumannii*, colistin resistance, biofilm, adaptation, virulence

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INTRODUCTION

Acinetobacter baumannii is one of the most pathogenic nosocomial infection agents because of its extreme resistance to almost all known antibiotics and host immune responses (1). The emergence of colistin-resistance in *A. baumannii* has been reported throughout the world (2, 3).

Biofilm formation ability enhances virulence of *A. baumannii* by increasing survival of cells in unfavourable environmental conditions, such as underexposure of disinfectants, antibiotics or attack of immune cells (4, 5). Antibiotic-resistant phenotypes

strongly influence biofilm forming capacity of *A. baumannii* (6). The expression of biofilm-associated virulence genes and biofilm thickness in MDR (multidrug-resistant) strain was found to be higher than drug-sensitive strain (7).

A. baumannii acquires colistin resistance by rapid induction of resistance mechanisms in the presence of colistin (8). Simultaneously, bacteria display an adaptation to colistin exposure with significant changes in transcriptome profile and cell membrane structure (9). Biofilm-related outer membrane lipoprotein (*pgaB*) was found to be upregulated in colistin resistant strains (10). However, another study showed that mutations involved in colistin resistance phenotype downregulate the expression of biofilm-associated genes of colistin-resistant isolates (11).

The understanding of bacterial pathogenesis has lead to the development of many potential strategies and novel drugs to treat MDR bacteria. Anti-virulence treatment is one of the promising therapy approaches (12). The aim of this study is to explore the alterations in biofilm formation capacity of *A. baumannii* during adaptation to colistin resistance under colistin stress.

MATERIALS AND METHODS

Study population and bacterial strains

We included 18 patients with isolation of *A. baumannii* between October 2014 and September 2018 from different centres in Turkey. The gender, age, source, carbapenem resistance, carbapenemase type, colistin exposure, and colistin resistance data were recorded. For all patients one representative *A. baumannii* (nine colistin resistant and nine colistin susceptible) isolate was included. Additionally, we selected two patients that developed colistin resistant *A. baumannii* infection during their hospital stay. The colistin susceptible and resistant pairs of *A. baumannii* isolated during the patients' hospital course were studied.

Antibiotic susceptibility testing

Colistin minimum inhibitory concentrations (MICs) were determined by broth microdilution method. The isolates were grown on Tryptic Soy Agar (TSA) (Becton, Dickinson, U.S.) overnight. Then, the turbidity of each isolate was adjusted to 0.5 MacFarland by cation-adjusted Mueller Hinton broth (MH) using nephelometer (Becton, Dickinson, U.S.). Serial dilution was performed with final concentrations between 256 µg/mL and 0.25 mg/L. The samples were incubated at 37°C overnight and MICs were determined by measuring absorbance values at 540 nm in addition to reading by the naked eye. Escherichia coli ATCC 25922 standard strain was used as a reference, and resistance breakpoint was set as >2 µg/ mL based on Clinical and Laboratory Standards Institute (CLSI) guideline (13).

Assessment of biofilm formation

Biofilm production was measured by Crystal Violet Assay. Thus, all isolates were grown on TSA overnight. Then, a single colony was inoculated into 5 mL of Tryptic Soy Broth (TSB) and incubated at 37°C on a shaker (125 rpm) until turbidity reached 10-13 MacFarland. The cultures were diluted to 1:50 using TSB containing 0.1% glucose. 100 µL of diluted cultures in 96-well plate was incubated at 37°C for 24 h. After removal of non-adherent bacteria, adherent bacteria were fixed by incubation at 60°C for 40 min and stained with 125 µL of crystal violet. Bound crystal violet was dissolved by 95% ethanol. Optic Density (OD) values of wells were measured at 540 nm. The isolates with ODs between 0.12> and £0.5 were considered weak, >0.5 were strong biofilm producers (14).

For imaging of biofilms, bacterial cells were fixed with 3.5% formaldehyde solution overnight. The fixed cells were stained using the Live/Dead Backlight viability kit with following the manufacturer's protocol. Samples were examined with Leica DMI8 laser scanning confocal microscope (15, 16).

HIGHLIGHTS

- Colistin resistant *Acinetobacter baumannii* has increased biofilm production capacity.
- The biofilm production might be a part of adaptation response of bacteria to colistin resistance.

Table 1. The demographic and laboratory data of study population.

		Total n=18 (%)	Colistin resistant (n=9) (%)	Colistin susceptible (n=9) (%)
Median age (min-max)		71 (51-84)	74 (51-84)	77 (57-82)
Female gender		11 (61.1)	8 (88.9)	3 (33.3)
Source	Blood	12 (67)	3 (33)	9 (100)
	Respiratory tract	3 (17)	3 (33)	-
	Wound	1 (6)	1 (11)	-
	Rectal swap	2 (11)	2 (22)	-
Carbapenamase resistance		18 (100)	9 (100)	9 (100)
OXA-23 Carbapenamase type		18 (100)	9 (100)	9 (100)
Colistin exposure		15 (83)	9 (100)	6 (67)

Table 2. The overview of ColR-ColS *A. baumannii* pairs isolated from two patients.

Patient No	Isolate	Colistin Resistance	Colistin MIC (mg/L)	Duration of colistin therapy at the day of isolation	ST Type	Source
1	K411	S	1	0	ST2	Sputum
	K412	S	1	0	ST2	IAF*
	K408	S	0.5	9	ST2	IAF
	K399	S	1	15	ST2	Sputum
	K409	R	16	25	ST2	IAF
2	K1007	S	2	0	ST2	Respiratory tract
	K1006	R	16	4	ST2	Respiratory tract

IAF*: Intra abdominal fluid; **S**: susceptible; **R**: resistant

In vitro adaptation study

For *in vitro* adaptation, four colistin susceptible strains (K399, K408, K411 and K412) of one of the patients with both colistin resistant and susceptible *A. baumannii* isolation were selected. The selected susceptible isolates were sub-cultured onto Mueller Hinton agar (MHA) containing 1 µg/mL colistin (Sigma) by 40 serial passages. Biofilm production and colistin MICs of each generation were determined.

Genotyping of isolates

MLST was performed by amplifying seven house-keeping genes, namely, *cpn60*, *fusA*, *gltA*, *pyrG*, *recA*, *rplB*, and *rpoB* according to the protocol developed

by University of Oxford on *A. baumannii* MLST website (https://pubmlst.org/abaumannii/). Allelic profiles and sequence types (STs) were determined using Applied Math Bionumerics V7.6 software.

RESULTS

Among 18 patients, seven of them were male. All of the patients with colistin resistant *A. baumannii* isolation received colistin therapy. Twelve of the isolates (67%) were obtained from blood cultures. All isolates were carbapenem resistant and OXA-23 producers (Table 1).

Data of the two patients that develop colistin resistant *A. baumannii* infection during hospital stay was presented in Table 2. Both patients received colistin therapy, and the patient 2 died after four days of *A. baumannii* isolation. All isolates belonged to global ST2 clone.

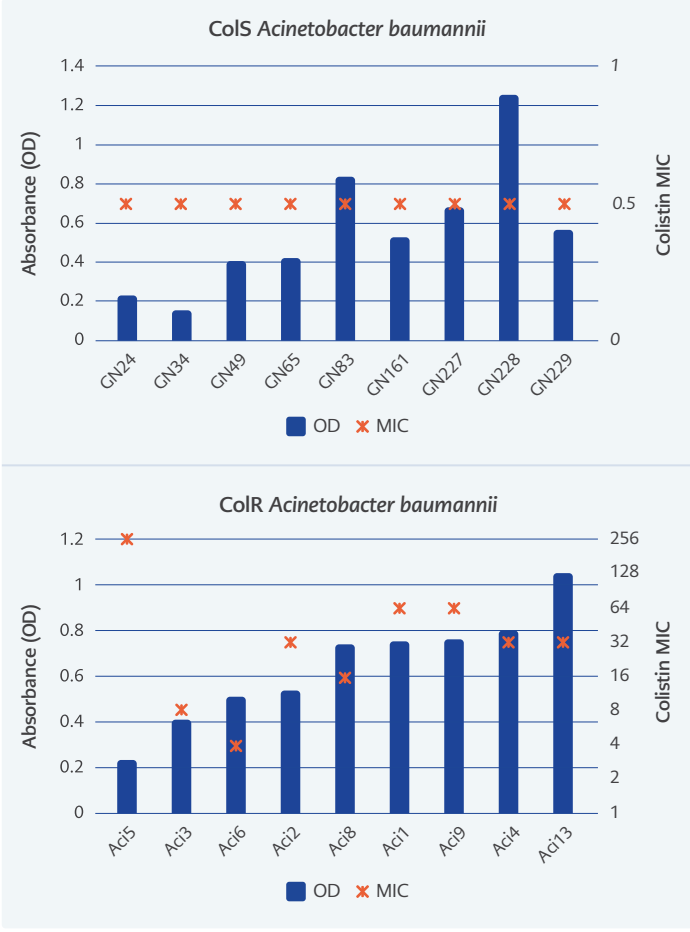

Figure 1. The biofilm and MIC levels of ColS and ColR *A. baumannii* isolates. OD: Optic Density; MIC: Minimum inhibitory concentration.

Biofilm formation of *A. baumannii* isolates

The biofilm production of ColR and ColS isolates were shown in Figure 1. Among all *A. baumannii*, 54% (5/9) of ColS isolates and 78% of ColR (7/9) were found to be strong biofilm producers. The median of OD values for ColS isolates was 0.53 (0.23-1.24), median for ColR isolates was 0.73 (0.12-1.05). In seven of the nine ColR isolates, the elevated colistin MICs were found to be accompanied to high levels of biofilms.

The biofilms and MICVs of *A. baumannii* from two selected patients were shown in Figure 2. In patient 1, biofilm production of ColR isolate was higher than ColS original strain; however, a slight decrease was observed in biofilm production of ColR strain than ColS counterpart in patient 2 (Figure 2A). Also, microscope images showed that biofilm productivity of ColR strain was stronger than ColS in patient 1, but there was a decrease in biofilm formation of ColR isolate compared to ColS strain in patient 2. These results were consistent with the crystal violet results.

Biofilm production of *A. baumannii* generations during *in vitro* adaptation to colistin exposure

The biofilm production and colistin MICs of selected generations of four colistin susceptible isolates (K399, K408, K411, and K412) and ColR isolate (K409) from patient 1 were shown in Figure 3. The Colistin MICs were increased to resistance breakpoint (2 mg/mL) at the first generation.

The results showed no considerable difference in biofilm formation between the generations. The K409 ColR clinical isolate had 2.6-3.4 fold stronger biofilm production capacity than laboratory induced colistin resistant generations.

DISCUSSION

The emergence of colistin resistance in A. *baumannii* has increased the need for the development of new therapeutic approaches. Inhibition of virulence factors is becoming one of the most popular strategies for the treatment of infections (12, 17). Biofilm for-

mation of *A. baumannii* is a significant virulence determinant that because of the strong interaction of bacteria with host cells (7).

Here we presented 18 cases with an isolation of *A. baumannii* from different specimens. 17 % of total isolates and 33 % of ColR isolates were from respiratory specimens. The biofilm production rate of all isolates was 67%. In 2008, a multicentered cohort study showed 63% biofilm formation among *A. baumannii* isolated from various sources. They also reported that respiratory isolation was associated with non-biofilm production (18). Controversially, in another study, high-level biofilm formation was observed in respiratory specimens of 61 patients in 2013 (19).

In this study, we detected strong biofilm production (OD>0.5) in 78% of ColR, and in 54% of ColS *A. baumannii* isolates. Recent studies reported a significant association between high biofilm formation capacity and multidrug-resistant profile of *A. baumannii* (20). A multi-centric hospital based study showed that more than 90% of the biofilm producer *A. baumannii* isolates were multidrug-resistant (6). Additionally, MDR and biofilm producer *A. baumannii* strains were reported from outbreaks in hospitals especially in intensive care units (21, 22). However, there are controversial reports about the colistin resistance with biofilm production. Farshadzadeh et al. (11) found that the biofilm-forming ability of ColR *A. baumannii* was not significantly different from their ColS counterparts. They claimed that biofilm formation capacity could be related to the change in growth rate. In two different studies, Pournaras and Dafopoulou revealed that the acquisition of colistin resistance via a single pmrB mutation was associated with an impaired biofilm formation capacity because of the decrease in growth rate (23, 24). Another important finding of our study was the association of high colistin MICs with high biofilm values among ColR A. baumannii. A very re-

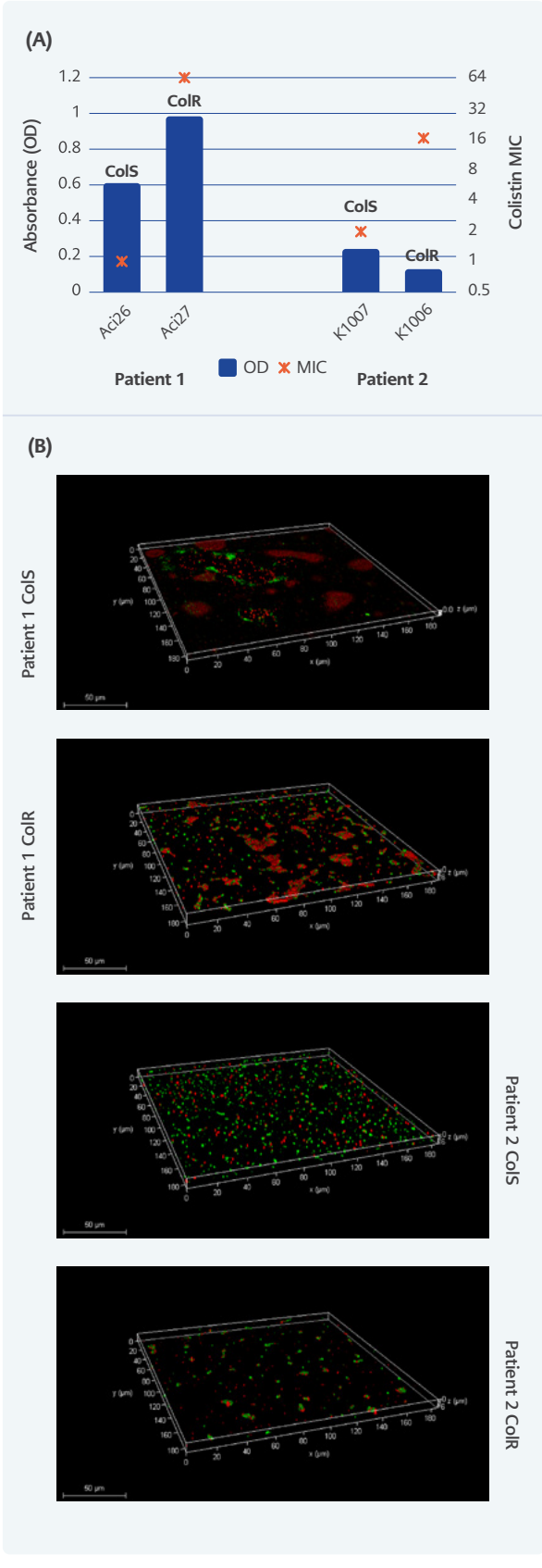

Figure 2. The biofilm and MIC levels of ColS-ColR *A. baumannii* pairs from two selected patients. Crystal violet assay and MICs (A); confocal images of biofilms (B). In confocal images, red color indicates dead, green color indicates live cells. OD: Optic Density; MIC: Minimum inhibitory concentration.

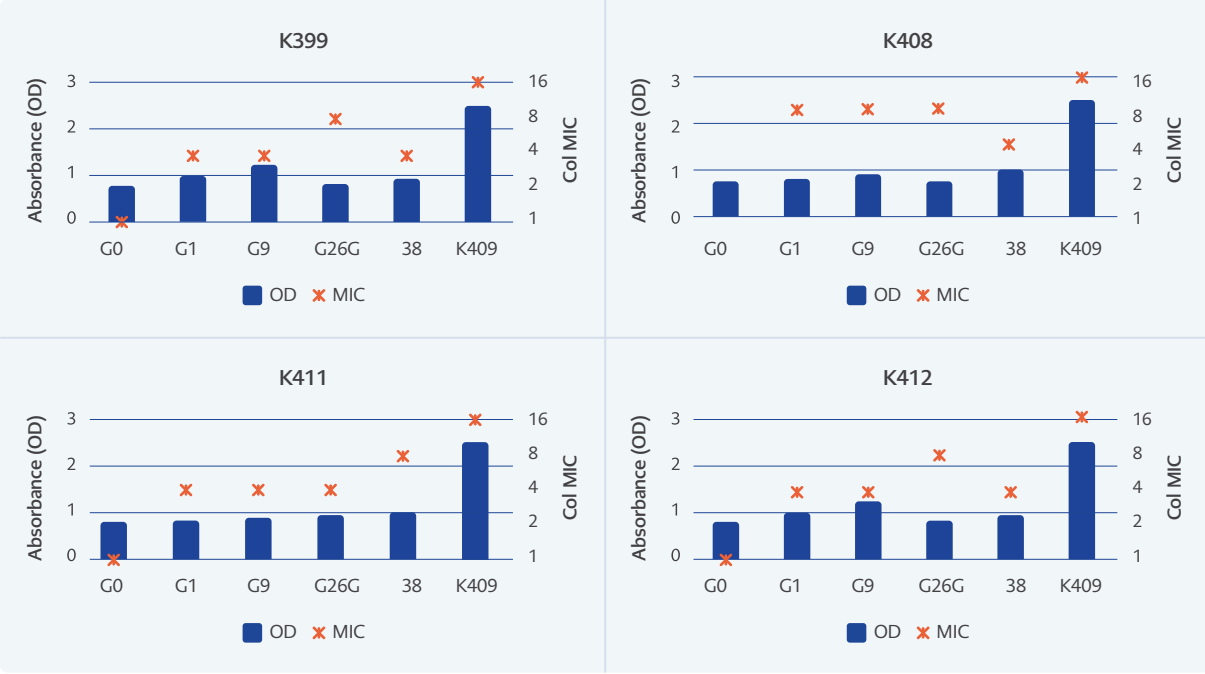

Figure 3. Biofilm production and colistin MICs of selected generations during *in vitro* adaptation to colistin exposure. OD: Optic Density; MIC: Minimum inhibitory concentration. K399, K408, K411 and K412 are the numbers of each isolates.

cent study reported overexpression of biofilm-associated genes underexposure of colistin (25). In our study, all patients received colistin therapy before isolation of ColR strains.

In this study, we also examined the alterations in biofilm ability of *A. baumannii* during the *in vitro* adaptation period to colistin resistance and compared the *in vivo* and *in vitro* results. The ColR (K409) isolate was detected on the 25th day of the colistin therapy. This isolate was found to be a strong biofilm producer revealed 2.6-3.4 times more biofilm levels than ColS first pair. When we mimicked *in vivo* colistin use in the laboratory, the exposure of sub-MIC concentration of colistin (1mg/L) did not alter biofilm production of the generations. Even the 25th generation that corresponds to the duration of colistin therapy at the isolation day of clinical ColR *A. baumannii* did not show a difference than ColS first pair. These results suggested that colistin exposure is not the only factor that affects the *in vivo* biofilm formation ability of *A. baumannii* under colistin stress. Other contributors might have a role in biofilm production of bacteria during the development of colistin resistance. Similarly, a study published in 2018 declared that colistin exposure did not have an effect on the biofilm-forming capacity of laboratory-induced *A. baumannii* (26). Besides, recent *in vitro* studies reported the biological cost of adaptation to colistin resistance in *A. baumannii*. The loss of LPS leads fitness cost and low biofilm formation capacity so consequently reduces virulence (11, 27, 28).

In conclusion, the biofilm forming capacity of ColR *A. baumannii* isolates is higher than ColS *A. baumannii*. On the other hand, we did not find a difference in biofilm levels of laboratory-induced colistin resistant generations. We suggest that *A. baumannii* may develop adaptation mechanisms to constitute colistin-resistance in the presence of host-dependent factors and environmental stress conditions in order to gain stronger biofilm production capacity to enhance virulence.

Ethics Committee Approval: The regulation on clinical research entered into force on 19 August 2011 (Official Gazette number 28030) in Turkey and ethics committees within the scope of this regulation began to be formed thereafter. Present study was conducted before related regulation and therefore ethics committee approval was not provided.

Informed Consent: Written informed consent was obtained after explaining to them the details of our study from the patients who participated in this study.

Peer-review: Externally peer-reviewed

Author Contributions: Concept - B.Ö., F.C., Ö.D.; Supervision - F.C., Ö.E.; Resources - Ş.K., Ö.E., C.V.; Materials - B.Ö., C.V.; Data Collection and/or Processing - B.Ö., C.V.; Analysis and/or Interpretation - B.Ö., Ö.D., C.V.; Literature Search - B.Ö., Ö.D.; Writing Manuscript - B.Ö., Ö.D.; Critical Reviews - F.C., Ö.E.

Conflict of Interest: The authors have no conflict of interest to declare.

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