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## ORIGINAL RESEARCH PAPER



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# Antibiotic induced biofilm formation of novel multidrug resistant *Acinetobacter baumannii* ST2121 clone

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## ABSTRACT

The aim of this study was to identify antimicrobial resistance and virulence factor genes exhibited by multidrug resistant (MDR) *Acinetobacter baumannii*, to analyze biofilm formation and to investigate clonal subtypes of isolate. Whole genome sequencing was done by Illumina NovaSeq 6,000 platform and multilocus sequence typing (MLST) was performed by Oxford and Pasteur typing schemes. Influence of imipenem and levofloxacin on biofilm formation was investigated in 96-well plates at 3 replicates. The strain was found to carry OXA-23, OXA-51-like, AmpC and TEM-1 beta-lactamases. The sequence of the *bla*<sub>OXA-51-like</sub> gene has been identified as a *bla*<sub>OXA-66</sub>. According to Pasteur MLST scheme the strain displayed ST2 allelic profile. However, based on Oxford MLST scheme this strain represents the new ST2121, as the *gdhB* gene has a single allelic mutation namely, the *gdhB*-227. It was determined that MDR isolate carried *bap*, *bas*ABCDGHIJ, *csuA*/BABCDE, *bau*ABCDEF, *plcD*, *pga*ABCD, *entE*, *barAB*, *ompA*, *abaIR*, *piT2EAFTE/AUBI*, *fim*ADT, *cvaC*, *bfmR*, *bfmS* virulence genes. In our study imipenem induced the highest biofilm formation at a concentration of 32 µg/ml and levofloxacin at a concentration of 16 µg/ml. In conclusion, we detected a new MDR *A. baumannii* ST2121 clone harboring *bla*<sub>OXA-66</sub> gene that has been reported for the first time in Turkey.

## KEYWORDS

Biofilm, MLST, OXA-66, ST2121, Virulence genes, WGS

## INTRODUCTION

*Acinetobacter baumannii* is a worldwide hospital-acquired and opportunistic Gram-negative bacterial pathogen that is included in the WHO's list of antibiotic-resistant priority [1] (critical) pathogens [2, 3]. In multidrug resistant (MDR) isolates, carbapenem resistance is associated with carbapenem-hydrolysing class D β-lactamase (CHDLs) production [4–6]. Of these, OXA-23 is the primary mediator of resistance to last resort carbapenems [7, 8], which is the first choice for most infections [9]. Because of its ability to develop many resistance mechanisms, isolates of *A. baumannii* are also included in the extensively drug resistant (XDR) class [10]. The increasing number of MDR and XDR counterparts of such pathogens and inadequate antibiotic development encouraged the re-use of old antibiotics [11–14].

Efflux pumps can be effective in cell signaling, metabolism, homeostasis and different roles such as bacterial virulence. There are five main efflux pumps in bacteria; i. the resistance-nodulation-division (RND) family, ii. the multidrug and toxic compound extrusion (MATE) family iii. the adenosine triphosphate (ATP)-binding cassette (ABC) superfamily, iv. the major facilitator superfamily (MFS), v. the small multidrug resistance (SMR) family [15]. RND family is specific to Gram-negative bacteria, while MFS, ABC, SMR and MATE families are commonly found in both Gram-negative and Gram-positive [15]. AdeABC which is most

Table 1. MLST clonal subtypes of *A. baumannii* isolate in this study

Pasteur ST	<i>cpn60</i>	<i>fusA</i>	<i>gltA</i>	<i>pyrG</i>	<i>recA</i>	<i>rplB</i>	<i>rpoB</i>
ST2	2	2	2	2	2	2	2
Oxford ST	<i>gltA</i>	<i>gyrB</i>	<i>gdhB</i>	<i>recA</i>	<i>cpn60</i>	<i>gpi</i>	<i>rpoD</i>
ST2121	1	3	227	2	2	102	3

common, AdeFGH, AdeIJK belonging to RND family in *A. baumannii* MDR isolates are efflux systems that mediate resistance [16]. While each of these systems is multi-membered and mediates resistance against antibiotics in various groups, each system is tightly regulated with different control mechanisms [17].

Biofilm formation is critical because it resists significant killer processes such as antimicrobial agents and desiccation, and allows to live on almost all surfaces in the intensive care unit (ICU). Its survival in the ICU, where it poses the greatest threat, depends on its ability to form biofilms on biotic or abiotic surfaces and is the origin of its pathogenicity. Knowing the positive correlation between biofilm formation and antibiotic resistance and 5%–24% more biofilm formation capacity than other species supports this idea [18, 19]. Biofilm associated protein (Bap), two-component system (BfmS/BfmR), chaperon-usher pilus (Csu), extracellular exopolysaccharide (EPS), outer membrane protein A (OmpA), poly- $\beta$ - (1,6) -N -acetyl glucosamine (PNAG) and quorum sensing system are some of the genes that are effective in biofilm formation in *A. baumannii*, they also act as virulence factors [20, 21].

The development of resistance encourages large-scale research at *A. baumannii*. Therefore, whole genome sequence (WGS) studies are a susceptible method for obtaining more comprehensive and detailed results. Our goal is to reveal resistance-associated and virulence factor genes by performing WGS, to determine the clonal subtypes of local clinical isolate *A. baumannii* and to analyze biofilm formation and induction of biofilm formation in the presence of antibiotics.

## MATERIALS AND METHODS

### Bacterial isolate

*A. baumannii* strain was isolated from sputum culture in 2017 in Trabzon Fatih State Hospital in Turkey and antibiotic susceptibility was evaluated using imipenem, meropenem, piperacillin, piperacillin/tazobactam, amikacin, gentamicin, tobramycin, netilmicin ciprofloxacin and levofloxacin. 16S rDNA sequencing was performed for molecular identification. The sequence information of 16S rDNA gene was analyzed by BLAST search using the NCBI GenBank database.

### Detection of antibiotic resistance genes

PCRs were performed using primers of *bla*<sub>OXA-51</sub>, *bla*<sub>OXA-23</sub>, *bla*<sub>OXA-24</sub>, *bla*<sub>OXA-58</sub>, *bla*<sub>VIM</sub>, *bla*<sub>NDM</sub>, *bla*<sub>AMP</sub>, *bla*<sub>KPC</sub> and *mcr-1* genes. PCR mixture contained: 5  $\mu$ l of genomic DNA, 20 pM of each primer, 10  $\mu$ l reaction buffer, 3  $\mu$ l 25mM

MgCl<sub>2</sub>, 200mM of dNTPs and 1.5 of U Taq DNA Polymerase (GeneON) in a final volume of 50  $\mu$ l. All PCR results were analyzed on 1% agarose containing 0.5  $\mu$ g/mL ethidium bromide and subsequently visualized under ultraviolet light.

### DNA isolation

Bacterial genomic DNA was isolated using Zymo Research “Quick-DNA TM Fungal/Bacterial Miniprep Kit, Cat. No. D6005” according to the manufacturer’s instructions. DNA quality and quantity were measured spectrophotometrically and fluorometrically.

### NGS sequencing

Sequencing library was prepared using Nextera XT DNA Library Preparation Kit and sequencing was done by Illumina NovaSeq 6,000 platform as paired-end (PE) 2x150 bases reads.

### Bioinformatics analysis

After sequencing, quality of raw reads were checked, and adapter sequences, contamination and low quality reads were removed. Putative genes in the consensus genome was extracted based on the reference genome annotation file using CLC genomics workbench tool and these genes were functionally annotated by using OmicsBox tool. Virulence factor genes (*bap*, *bas*, *csu*, *bau*, *plc*, *pga*, *entE*, *bar*, *omp*, *abaI*, *pil*, *fim*, *cvaC*) were determined using the VFDB database.

### MLST sequence types

The Oxford and Pasteur typing schemes were used to characterize the clonal subtypes of the strain (Table 1).

### Accession numbers

Draft genome sequence data of *A. baumannii* (A73) strain have been submitted to the NCBI BioProject as accession PRJNA600804 and GenBank accession number CP047930 in this study.

### Determination of imipenem and levofloxacin minimum inhibitor concentrations (MIC) by broth microdilution method

It was previously established that the strain was resistant to different antibiotic groups. In this direction, minimum inhibitory concentrations were determined by liquid microdilution method by choosing levofloxacin from fluoroquinolone group and imipenem representing the carbapenem group. The experiments were done in triplicate using 96-well plates. Antibiotic concentrations were prepared as

128-1 µg/ml. *Escherichia coli* DH5α was used as a control. Results were evaluated according to EUCAST. The obtained MIC values were used as a reference in the investigation of biofilm formation.

### Quantitative determination of biofilm formation

Quantitative analysis of the strain was done using semi-quantitative crystal violet staining. The isolate was cultured overnight in Mueller-Hinton Agar (MHA) medium and a single colony was mixed with the Luria-Bertani (LB) broth medium to prepare 0.5 colony forming units (cfu) of bacterial suspension. 200 ml of bacterial suspension was added to each well of the 96-well plate, and the plate was then incubated for 24 hours at 37 °C. After incubation, the bacterial suspension in the wells was poured and the plate was then washed three times with distilled water every 30 seconds. After adding 200 µl of 1% crystal violet dye to each well, it was left at room temperature for 20 minutes and then the dye was removed. After removing the dye, the plate was washed again with distilled water and dried at room temperature for 15 minutes. 200 µl of 95% ethanol was added to each well and the optical absorbance (A) value was read on the spectrometer at 620 nm (Thermo Scientific, USA) after the crystal violet adhering to the well wall was completely dissolved. As a negative control, the OD value of Ac. (2) weak positive (+),  $Ac < A \leq 2Ac$ ; (3) moderately positive (++) ,  $2Ac < A \leq 4Ac$ ; and (4) strong positive (+++) ,  $A > 4Ac$  [22].

### Antibiotic induced biofilm formation assay

The above-mentioned imipenem and levofloxacin antibiotics were used to investigate the formation of biofilms by antibiotic induction. Biofilm formation experiments were performed on 96-well plates and were performed in 3 replicates. *A. baumannii* A73 strain was incubated at 37 °C for 24 hours by inoculating with different sublethal imipenem and levofloxacin concentrations (1/2, 1/4, 1/8 \* MIC). Quantitative determination method of biofilm formation mentioned above was performed after incubation [23].

### Motility experiment

For all mobility experiments LB agar was used. To test the mobility of the strain, the culture was adjusted to the same optical density by adding sterile LB broth and a 1 ml drop was placed in the center of the plate. After inoculation, it was incubated at 37 °C for 48 hours. Collection of positive isolate was defined as strains showing  $a > 10$  mm region around the inoculation site. Agar was thrown for twitch mobility. The plates were stained with 0.2% crystal violet and photographed. Experiments were carried out three times [24].

## RESULTS

Biochemical and molecular identification of the isolate was performed and the strain was determined to be

*A. baumannii* according to both methods. According to the antibiotic susceptibility test, the isolate was resistant to carbapenems (imipenem, meropenem), penicillins (piperacillin, piperacillin/tazobactam), aminoglycosides (amikacin, gentamicin, tobramycin, netilmicin) and fluoroquinolones (ciprofloxacin, levofloxacin) group antibiotics. According to the susceptibility profile of the isolate to these antibiotic groups, the strain was found to be MDR.

The isolate was found to carry OXA-23, OXA-51, Amp and TEM-type beta lactamases. The WGS result revealed that the OXA-51-like gene was an OXA-66 variant. Additional prevalent β-lactamases such as *bla*<sub>OXA-24</sub> and *bla*<sub>OXA-58</sub> were absent in the genome of isolate. In addition to the *bla*<sub>OXA-23</sub> gene, the presence of *carO* gene was observed. CarO is an outer membrane protein associated with carbapenem and is one of the main factors supporting carbapenem resistance in *A. baumannii*.

Isolate contained other drug resistance genes, including; aminoglycoside resistance genes (*aac*(3), *aac*6', *aph*(6)-I, *strA*), chloramphenicol resistance genes (*cat*, *catB*), tetracycline resistance genes (*tetR*; repressor). A mutation in position 638 of the *gyrA* gene was detected namely, Met638Leu, that is responsible for fluoroquinolone resistance. Multilocus sequence typing (MLST) was performed using Oxford typing schemes (*gdhB*, *gltA*, *gpi*, *gyrB*, *cpn60*, *recA* and *rpoD*) and Pasteur system (*cpn60*, *fusA*, *gltA*, *pyrG*, *recA*, *rpIB* and *rpoB*) for characterization of the clonal subtype of the strain. The strain displayed ST2 allelic profile based on Pasteur typing. However, the strain possessed a single allelic alteration in the *gdhB* [allelic alteration in 189th nucleotide (G→A)] resulting in *gdhB*-227 variant, thus indicating the new ST2121 based on Oxford typing scheme. ST218 is the closest clone of this novel ST2121 (<https://pubmlst.org/>). According to WGS analysis, RND (*adeA*, *adeB*, *adeC*, *adeF*, *adeG*, *adeH*, *adeI*, *adeJ*, *adeK*), MFS (EmrA/B), SMR (EmrE) and DMT (permease) identified efflux pumps family (Table 2).

Virulence factor genes were evaluated by bioinformatics programs. According to results, strain was found to carry *bap*, *bas* (*basA*, *basB*, *basC*, *basD*, *basG*, *basJ*, *basF*, *basH*, *basI*), *csu* (*csuA*, *csuB*, *csuC*, *csuD*, *csuE*, *csuA/csuB*), *bau* (*bauA*, *bauB*, *bauC*, *bauD*, *bauE*, *bauF*), *plc* (*plc*, *plcD*), *pga* (*pgaA*, *pgaB*, *pgaC*, *pgaD*), *entE*, *bar* (*bara*, *barB*), *ompA*, *pil* (*pilT2*, *pilE*, *pilA*, *pilF*, *pilT*, *pilE/A*, *pilU*, *pilB*), *aba* (*abaR*, *abaI*), *cvaC*, *fim* (*fimA*, *fimT*, *fimD*) virulence genes.

MIC values of imipenem and levofloxacin were determined by broth microdilution method. According to the obtained results MIC values of imipenem and levofloxacin were determined to be 64 µg/ml.

*A. baumannii* twitch motility was analyzed based on the cell's ability to spread to petri dishes. The isolate exhibits an average of 16.6 mm diameter movement area.

Biofilm formation ability which is related virulence, is one of the most important ability of *A. baumannii*. Therefore, the biofilm forming capacity of the isolate was measured. It was seen that the isolate has the biofilm formation ability. The quantitative value of biofilm formation was found to be 0.1775 in the OD620, with the average value



Table 2. *A. baumannii* A73 strain antibiotic resistance genes and efflux pump family

Antibiotic Class	Gene Name	Efflux pump family	Gene name
Beta-lactam	<i>ampC</i>	MFS family	MFS-ShiA-like
	<i>bla<sub>OXA-65</sub></i>		AraJ
	<i>bla<sub>OXA-23</sub></i>		MFS transporter
	<i>bla<sub>TEM-1</sub></i>		MFS_NepI_like
Aminoglycosides	<i>aac(3)</i> <i>aac6'</i> <i>aph(6)-I</i>	DMT family transporter	MFS transporter NreB
			MFS
			EmrA/B
			ABC transporter permease
Chloramphenicol	<i>strA</i> <i>cat</i> <i>catB</i>	RND Family	DMT family transporter
			multidrug DMT transporter
			drug/metabolite DMT transporter
			permease
Fluoroquinolones	Mutation at position 638 in <i>gyrA</i> : Met → Leu	LysE family transporter	<i>adeA</i>
			<i>adeF</i>
			<i>adeG</i>
			<i>adeH</i>
Tetracyclines	<i>tetR</i>	SMR	<i>adeI</i>
			<i>adeJ</i>
			<i>adeK</i>
			<i>adeT</i>
			<i>adeT2</i>
			OEP
			tolC
			LolA_like permease

obtained by three replicate readings. It was seen that the value obtained as a result of reading the negative control with three replicates was 0, 0450. In line with this result, it was determined that the strain formed a moderate biofilm. Figs. 1 and 2 show the biofilm formation profile of the strain in the absence of antibiotics (0 µg/ml) and in the presence of imipenem and levofloxacin in 1/8, 1/4 and 1/2 MICs.

Biofilm forming capacity of this strain was significantly increased in the presence of imipenem, with the highest biofilm induction at a concentration of 32 µg/ml (1/2 of MIC) was found (approximately 1.5 times). Again, it seems that the ability of the strain to generate biofilm increased compared to antibiotic-free biofilm formation at the concentration of 1/4\*MIC (OD620 value 0.2216) and 1/8\*MIC (OD620 value 0.2002). Quantitative biofilm formation values were observed as Levofloxacin concentration at

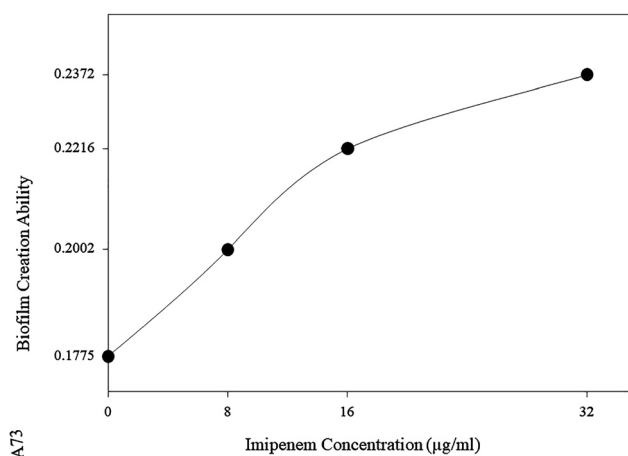


Fig. 1. The ability of A73 to form biofilms at different imipenem concentration

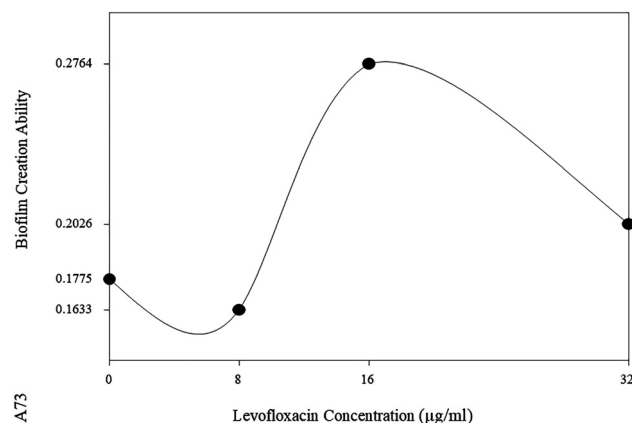


Fig. 2. The ability of A73 to form biofilm at different Levofloxacin concentration



0,2026 at 32 µg/ml, 0,2764 at 16 µg/ml and 0,1633 at 8 µg/ml. In the presence of levofloxacin at concentrations of 16 µg/ml (1/4 of MIC), a significant induction was observed in biofilm formation, resulting in a 1.66-fold increase in strain. In the concentration of 8 µg/ml levofloxacin, the strain was found to have reduced ability to form biofilm. According to these results, the most induction of biofilm formation was at the levofloxacin concentration at 16 µg/ml. Biofilm formation of *A. baumannii* was significantly induced in the presence of levofloxacin and imipenem. According to the data obtained, it was determined that the strain, which has a moderate biofilm formation ability, has a strong biofilm ability in the presence of both imipenem and levofloxacin.

## DISCUSSION

The most common infections caused by *A. baumannii* are ventilator-associated pneumonia, bacteremia, meningitis and wound infections in ICUs [3]. Many studies have shown that *A. baumannii* has a high rate of multiple drug resistance and *Acinetobacter* infections are associated with increases morbidity or mortality [3].

OXA-type carbapenemases are one of the main mechanisms of carbapenem resistance in *A. baumannii* [25–26]. *A. baumannii* has five families of OXA carbapenemase [27]. The presence of OXA-23-like enzymes is one of the most common carbapenem resistance mechanisms in *A. baumannii*. In this work, both OXA-23 and OXA-66 type class D beta lactamases were detected in *A. baumannii*. To our knowledge, in this study OXA-66 is reported in *A. baumannii* clinical isolate for the first time in Turkey. The presence of OXA and ESBL genes such as TEM provides resistance to almost all beta-lactam antibiotics [28]. In this study, it was determined that *A. baumannii* carried beta-lactamase type TEM-1. Both OXA and TEM-type beta lactamase carrying strain is seen to show resistance to beta-lactams. We found that the strain produces ADC-type *ampC*. A study was conducted to determine the prevalence and related drug resistance of AmpC  $\beta$ -lactamases in *A. baumannii* in China and showed that resistance to most broad-spectrum  $\beta$ -lactam antibiotics in *A. baumannii* strains may be associated with the ADC-type *ampC* [29].

Efflux pumps contribute to multidrug resistance in Gram-negative bacteria. In *A. baumannii*, the four efflux pump categories RND, MF, MATE, SMR family of transporters are associated with antimicrobial resistance [30]. Many studies have shown that overexpression of *adeABC* and mutations provide an important mechanism for multidrug resistance in the *A. baumannii* isolates [31]. Cooperation between *bla*<sub>OXA</sub> and *adeABC* genes can cause high carbapenem resistance in *A. baumannii* isolates. Some studies have shown that the *adeFGH* operon may be partially related to resistance to the antimicrobial agent. The AdeFGH efflux pump probably mediates resistance to fluoroquinolones in *A. baumannii* [32]. In our study, it was observed that isolate had high resistance against fluoroquinolones. Some researchers have shown a correlation

between overexpression of AdeG and increase in ciprofloxacin resistance [32]. The studies have reported that overexpression of AdeG is associated with *A. baumannii* pathogenicity. Another efflux pump found in *A. baumannii* is the AdeIJK pump. This pumps out various antibiotic groups such as beta-lactams, chloramphenicol, fluoroquinolones, rifampin, trimethoprim. Researchers have shown that the loss of AdeIJK in *A. baumannii* has an effect on antibiotic susceptibility profiles [33]. The small multidrug resistance (SMR) family is a drug resistance determinant like the RND efflux pump family. *E. coli*'s EmrE protein is a well-known member of the SMR family and contributes to resistance to toxic compounds [34]. In this study, EmrA/B, EmrE, AdeABC, AdeFGH and AdeIJK efflux pumps were detected in *A. baumannii*.

Mutations in *gyrA*, *gyrB*, *parC*, and *parE* genes are frequently identified in *A. baumannii* and these confer high level fluoroquinolone resistance. Studies have shown that the amino acid change of Ser83Leu in GyrA causes high resistance to ciprofloxacin and nalidixic acid [35]. In this study, a new mutation in the *gyrA* gene was detected (Met638Leu). This mutation can be shown as one of the causes of high fluoroquinolone resistance of the isolate.

Previously, ST2 clone has been reported in *A. baumannii* in Mediterranean countries including Turkey [36, 37]. The ST2 clone is known to be associated with production of OXA-23 carbapenemase [36]. Dagher et al. showed that isolates have the most common ST2 clone in Lebanon [36]. A study by the SENTRY Antimicrobial Surveillance Program in China, Hong Kong, India, Korea, Singapore, and Thailand showed that there was a high *A. baumannii* distribution producing OXA-23. The fact that similar results are obtained in different countries is related to the spread of international I and II clones of outbreaks worldwide. Also, in the Mediterranean countries such as Turkey, *A. baumannii* outbreaks have resulted from the spread of ST2 strains in particular [36]. According to the Oxford scheme clones such as ST218 is responsible for antimicrobial resistance dissemination. ST218 clones in clinical isolates of *A. baumannii* has not yet been reported in Turkey. To date, the ST218 clone has been reported in Denmark, Japan, China and Taiwan [38, 39]. In this study, new ST2121 clone, similar to ST218 clone was first detected in an *A. baumannii* isolate in Turkey.

Biofilm is an important virulence factor of *A. baumannii*. Although the importance of MDR *A. baumannii* in hospital infections is known, the role and mechanism of virulence factors for human infections remain largely uncertain in the pathogenesis of *A. baumannii*. In a study in 2020 in Turkey, *csaA*, *csuE*, *fimH*, *ompA* and *bla*<sub>PER-1</sub> virulence genes were investigated by PCR. They determined that 60% of the isolates studied constitute biofilm [40]. Various reports have shown that *A. baumannii*'s biofilm-related genes, including *csuE*, *ompA*, *bap*, *epsA*, *bfpS*, are responsible for biofilm development and antibiotic resistance [41]. Also, *pgaABCD* genes are important structures for the formation of biofilm in *A. baumannii*. Among the clinical isolates of *Acinetobacter* spp., biofilm formation was found to be under the

control of the quorum sensing system (abaI, abaR) molecule. The relationship between drug resistance and the effect of *abaR* on *A. baumannii*'s biofilm formation was investigated and *abaR* in *A. baumannii* was found to be related to biofilm formation [42].

In a study, three representative strains called *A. baumannii* ABR2, ABR11 and ABS17 were used to evaluate the biofilm inducibility. In the absence of antibiotics, they observed that the greatest ability in biofilm formation was in the ABS17 strain followed by ABR11 and ABR2 [23]. Similarly, in our study, it was found that the formation of biofilm was induced in the presence of *A. baumannii* A73 carbapenem group antibiotics, imipenem and levofloxacin from fluoroquinolone group. The highest biofilm induction was determined at a concentration of 16 µg/ml (1/4 MIC) levofloxacin. This result highlights the importance of molecular epidemiology before choosing antibiotic therapy against biofilms created by *A. baumannii*.

## CONCLUSION

Biofilm formation, antimicrobial resistance and desiccation resistance are important interrelated factors [43]. While biofilm formation is an important virulence factor in developing antibiotic resistance, resistance to cationic polypeptides such as colistin is known to mediate desiccation tolerance and increase it [44]. Based on all this, research studies and analysis of relationship between virulence factors, biofilm formation and antibiotic resistance will be an important tool in eliminating *A. baumannii*-related problems.

In conclusion, the cause of multidrug-resistance in isolated *A. baumannii* was investigated. In this study, new amino acid changes in *gyrA* gene was determined by WGS. A new STOXf, ST2121, clone was also detected and investigated. In addition, ST2Pas/ST2121 strain harboring *bla*<sub>OXA-66</sub> gene has been reported for the first time in Turkey in *A. baumannii*.

**Conflict of interest:** The authors declare no conflicts of interest.

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