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RESEARCH ARTICLE

Characterization of disinfectant susceptibility profiles among clinical isolates of *Acinetobacter baumannii* in Ardabil, Iran

MALEK NAMAKI KHELJAN[1], MALIHE HASSANZADEH[2], MEHRAN SRDARI JABEDAR[1], ALI MOHAMMADI GOLLOU[3], PARASTOO ASHOURI[1], ROGHAYEH TEIMOURPOUR[1] and MOHSEN ARZANLOU[1,4*]

[1] Department of Microbiology, School of Medicine, Ardabil University of Medical Sciences, Ardabil, Iran

[2] Department of Biology, Ardabil Branch, Islamic Azad University, Ardabil, Iran

[3] Department of Biology, Faculty of Basic Sciences, University of Maragheh, Maragheh, Iran

[4] Zoonoses Research Center, Ardabil University of Medical Sciences, Ardabil, Iran

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ABSTRACT

Antimicrobial disinfectants have been extensively used to control hospital-acquired infections worldwide. Prolonged exposure to bacteria could promote resistance to antimicrobial disinfectants. This study evaluated the antimicrobial activity of four commonly used disinfectants; triclosan, chlorhexidine digluconate, benzalkonium chloride, and formaldehyde against *Acinetobacter baumannii* clinical isolates. This study also determined the prevalence and association of efflux pumps encoding genes *qacE*, *qacED1*, *emrA*, and *aceI* with tolerance to disinfectants. A total of 100 *A. baumannii* isolates were included in the current study. The antimicrobial disinfectants' minimum inhibitory concentration (MIC) was determined using an agar dilution method. Genes involved in resistance to disinfectants were investigated by PCR method. The benzalkonium chloride MICs ranged between 32 and 128 μg mL^{-1}, chlorhexidine digluconate 8–64 μg mL^{-1}, triclosan 1–32 μg mL^{-1}, and formaldehyde 128 μg mL^{-1}. Overall, the highest MIC_{90} value was identified for formaldehyde (128 μg mL^{-1}), followed by benzalkonium chloride and chlorhexidine digluconate (64 μg mL^{-1}, each one) and triclosan (4 μg mL^{-1}). In the present study, the *qacE, qacED1, emrA*, and *aceI* genes were found in 91%, 55%, 100%, and 88% of isolates, respectively. The *qacG* gene was not identified in our *A. baumannii* isolates. The *qacED1* gene was associated with higher MICs for all disinfectants tested ($P < 0.05$), while the *qacE* and *aceI* genes were associated with higher MICs for benzalkonium chloride and chlorhexidine. This study indicated that triclosan is the most effective disinfectant against *A. baumannii* isolates.

KEYWORDS

Acinetobacter baumannii, disinfectant, resistance, efflux pump genes

*Corresponding author. Medical Microbiology, Department of Microbiology, School of Medicine, Ardabil University of Medical Sciences, Ardabil, 5166614711, Iran.
E-mail: m.arzanlou@arums.ac.ir, arzanlo53@gmail.com

INTRODUCTION

Hospital-acquired infections (HAIs) are a pervasive challenge accounting for a significant burden of infectious diseases worldwide [1]. It has been estimated that 7% of patients in high- and 15% in low-/middle-income countries (LMIC) will acquire at least one HAI in acute care hospitals [2]. HAIs are usually associated with increased mortality, as infected patients are mostly debilitated individuals with multiple predisposing factors [1]. Of note, HAIs caused by drug-resistant organisms are associated with higher mortality [1]. *Acinetobacter baumannii* is one of the most drug-resistant bacteria, along with others such as methicillin-resistant

Staphylococcus aureus (MRSA), vancomycin-resistant *Enterococcus* (VRE), extended-spectrum beta-lactamase (ESBL) producing *Enterobacterales*, carbapenem-resistant *Enterobacterales* and multi-drug resistant *Pseudomonas aeruginosa*, responsible for HAIs [3–5]. *A. baumannii* causes a variety of HAIs, including pneumonia, bacteremia, urinary tract infections, meningitis, and wound infections [6]. Infections caused by *A. baumannii* are usually associated with significant mortality, ranging from 8% to 35% [7]. Ventilator-associated pneumonia and bloodstream infections are the most critical *A. baumannii* nosocomial infections associated with the highest mortality rates [8]. In addition to being resistant to antibiotics, it can withstand dry conditions and is resistant to antimicrobial disinfectants. This makes it a persistent and successful hospital pathogen. Extensive use of antibiotics in hospitals, particularly ICUs, creates a selective advantage for Multidrug-resistant (MDR) *A. baumannii* to persist in the hospital environment [9]. Globally, resistant strains of *A. baumannii* have become more prevalent. They are responsible for the majority of hospital outbreaks [6]. The ability to resist desiccation enhances the prolonged viability of organisms on healthcare equipment surfaces. This could serve as a secondary reservoir and a transmission route in hospital outbreaks of MDR-*A. baumannii* [10, 11]. Therefore, to successfully control the MDR-*A. baumannii* HAIs, healthcare workers' compliance with hand hygiene and environment disinfection to prevent endemic strains is particularly critical [12]. There are plenty of chemicals used as disinfectants and antiseptics. Quaternary ammonium compounds (QACs), hydrogen peroxide, biguanides, chlorine-releasing agents, peroxygenase, alcohols, and phenolic compounds are the most commonly used agents [13]. While disinfectants are essential for controlling HAIs in the healthcare sector [12], they are also widely used for disinfection in households, veterinary and industrial environments [14]. Disinfectants usage increased intensively during the Covid-19 pandemic in the healthcare and public sectors worldwide [13]. It has been shown that increased bacteria exposure to disinfectants could result in tolerance or resistance [15–17]. The reduced susceptibility of antibiotic-resistant *A. baumannii* strains to detergents and alcohol disinfectants has been reported [18]. Disinfectants have been linked to the *A. baumannii* HAI outbreak [19]. On the other hand, cross-resistance to antibiotics was observed in disinfectant non-susceptible bacteria frequently [15, 20]. Resistance to disinfectants is mainly mediated by efflux pumps which expel disinfectants from bacterial cells [21, 20]. Efflux pumps are rather unspecific and can extrude a variety of structurally different compounds [22]. Therefore, antibiotics may also be co-extruded with disinfectants from bacterial cells, thereby leading to the co-occurrence of resistance to antibiotics and even facilitating the selection of higher levels of antibiotic resistance. It is crucial to monitor the susceptibility of *A. baumannii* to disinfectants in order to choose the right agents and use them effectively in controlling nosocomial infections [19]. There is no information about *A. baumannii* isolates' susceptibility to common disinfectants and antiseptics in Iran, and scarce information is available worldwide. This study aimed to investigate the susceptibility of *A. baumannii* isolates collected from patients in Iran to common antiseptic/disinfectant compounds used in hospitals. These compounds included benzalkonium chloride (BZC), chlorhexidine di-gluconate (CHDG), triclosan (TRE), and formaldehyde (FOR). Moreover, the frequency of disinfectant tolerance-associated (BTA) genes, *qacA/B*, *qacED1*, *emeA,* and *aceI* were investigated.

MATERIALS AND METHODS

Bacterial isolates

In this study, 100 clinical isolates of *A. baumannii* were included. The isolates were collected and characterized during 2017–2019 from patients admitted to hospitals affiliated with Ardabil University of Medical Sciences, previously [23]. According to ERIC-PCR analysis, the isolates belonged to 24 heterogeneous clusters. More than half (57%) of the isolates were from tracheal specimens, while the remaining were from specimens such as wound, urine, blood, urinary catheter, pleural fluid, lung secretions, and synovial fluid. The isolates were resistant to various antibiotics, including cefazolin, ciprofloxacin, imipenem, cefotaxime, meropenem, ceftazidime, cefepime, gentamicin, and amikacin. The resistance rates ranged from 78% to 100%. However, all strains were sensitive to polymyxin B (100%). Furthermore, 99% of the isolates were multidrug-resistant (MDR).

Determination of disinfectant susceptibility

The minimum inhibitory concentrations (MICs) of 4 antimicrobial disinfectant agents; formaldehyde (Acros, Belgium 38%), Benzalkonium chloride (Sigma Aldrich, USA 95%), triclosan (BioBasic, Canada 98% <), and chlorhexidine digluconate (Sigma Aldrich, USA 20%) were determined using an agar dilution method according to the CLSI guideline. Briefly, serial 2-fold dilutions of disinfectants in a concentration range between 0.125 and 1,024 μg mL^{-1} were prepared on Mueller–Hinton agar (Himedia, India) medium. Then, 1–2 μL of each bacterial suspension containing 10^4 CFU was spotted on the agar surface. The plates were incubated at 37 °C for 18–24 h. The MIC was considered the lowest concentration of antimicrobial disinfectants that inhibit the growth of the organisms [24]. The MIC_{50} and MIC_{90} were scored as the lowest concentrations of compounds that inhibit the growth of 50% and 90% of isolates, respectively.

Screening for disinfectant tolerance-associated genes

The presence of disinfectant tolerance-associated genes, including *qacE, qacED1, emrA,* and *aceI* which encode efflux pumps in *A. baumannii* was investigated using PCR testing. Template DNA was extracted using the boiling method according to previous reports [25]. The genes were amplified using specific primer nucleotides designed in this study (Table 1) in a 25 μL PCR reaction mixture. The reaction

Table 1. Primer sequences and PCR conditions

Gene name	Primer name	Primer sequence (5′–3′)	Annealing °C	Product size
qacED1	qacED1 F	AATCCATCCCTGTCGGTGTT	53	190
	qacED1 R	CGCAGCGACTTCCACGATGGGGAT		
qacE	qacE F	TTAGGATGGAGACGAAATTTTCA	59	240
	qacE R	CGCTTAACACCTAGTATTATTACCGT		
fabI	FabI F	ATGCTGAAAATTGTTTTGAGTGAGA	59	830
	FabI R	TTCATCATCCTTCATAGATTGGCTC		
aceI	AceI F	ATGTTGATTTCCAAGAGAAGACTCA	59	420
	AceI R	TGCTTTAGCATTTGGGAAAAACTTA		
qacG	qacG F	TTGAATAATTGGTTATTTCTGGCT	59	333
	qacG R	TTAGTGAACACTTGCCTTAGATAG		
emrA	EmrA F	TTAAACATCGATATTAGAGTATTGCGC	59	354
	EmrA R	ATGTTCGCATTAATCTCAAAAATAAAT		

mixture consisted of 12.5 µL of Premix Taq® mix, 2 µL of extracted template DNA, l µL (10 µmol) of each forward and reverse primers, and 9.5 µL deionized nuclease-free water. The amplification program comprised following steps: initial denaturation: 4 min at 94 °C, denaturation: 1 min at 94 °C, annealing: 1 min (temperatures are shown in Table 1), extension: 1 min at 72 °C for 30 cycles, and final extension: 1 min at 72 °C. PCR products were visualized with 1.5% agarose gel electrophoresis. PCR products were electrophoresed at 100 V over 1 h in a 1% agarose gel (Sinaclon, Tehran, Iran) in a 0.5X TBE buffer. Then, the samples were stained with DNA safe stain (Sinaclon, Tehran, Iran), and DNA bands were inspected by UV illumination (Uvi Tec, Cambridge, UK).

The identity of the representative amplified genes was confirmed by sequencing technique (Microsynth Company, Switzerland). Genomic DNA from isolates containing target genes was used as a positive control for PCR testing.

Statistical analysis

The association between disinfectant tolerance-associated genes and MICs of antimicrobial disinfectants in *A. baumannii* isolates were evaluated using the Chi-square test. A *p*-value of <0.05 was used to indicate statistically significant results.

RESULTS

Table 2 shows the MICs of disinfectant agents against *A. baumannii* isolates. The MIC range for disinfectant compounds was as follows: formaldehyde 128 µg mL^{-1}, benzalkonium chloride 32–128 µg mL^{-1}, chlorhexidine digluconate 8–64 µg mL^{-1}, and triclosan 1–32 µg mL^{-1}. Overall, the highest MIC_{90} value was identified for formaldehyde (128 µg mL^{-1}), followed by benzalkonium chloride and chlorhexidine digluconate (64 µg mL^{-1}, for each one) and triclosan (4 µg mL^{-1}).

In the present study, the *qacE, qacED1, emrA, and aceI* genes were found in 91% (n = 91), 55% (n = 55), 100% (n = 100) and 88% (n = 88) of isolates, respectively. The *qacG* gene was not identified in our *A. baumannii* isolates.

As shown in Table 3, the *qacED1* gene was found to be associated with higher MICs for all disinfectants tested (P < 0.05). In contrast, the presence of the *qacE* and *aceI* genes was associated with increased MICs for only benzalkonium chloride and chlorhexidine.

Overall, 6 tolerance-associated gene profiles were identified. The isolates simultaneously harboring *aceI* + *emrA* + *qacE* + *qacED1* genes (46%) and *aceI* + *emrA* + *qacE* genes (34%) were more prevalent among *A. baumannii* isolates in the present study (Table 4).

Table 2. Minimum inhibitory concentration of antimicrobial disinfectants against clinical isolates of *A. baumannii.*

Antimicrobial disinfectant	MICs (µg mL^{-1})										
	1	2	4	8	16	32	64	128	256	MIC_{50}	MIC_{90}
Formaldehyde N = 100 n (%)	-	-	-	-	-	-	-	93 (93)	7 (7)	128	128
Benzalkonium chloride N = 100 n (%)	-	-	-	-	-	14 (14)	81 (81)	5 (5)	-	64	64
Triclosan N = 100 n (%)	28 (28)	55 (55)	8 (8)	-	-	9 (9)	-	-	-	2	4
Chlorhexidine digluconate N = 100 n (%)	-	-	-	4 (4)	21 (21)	1 (1)	74 (74)	-	-	64	64

Table 3. Association between antimicrobial disinfectant MICs and antimicrobial disinfectant tolerance associated genes in clinical isolates of *A. baumannii*

	Disinfectant (MICs μg mL^{-1})							
	Formaldehyde		Benzalkonium chloride		Triclosan		Chlorhexidine	
Gene	≤128	256 ≤	≤64	128 ≤	≤4	8 ≤	≤32	64 ≤
qacED1+	48	7	50	5	47	8	8	47
qacED1-	45	0	45	0	44	1	18	27
p value	0.013		0.037		0.032		<0.01	
qacE+	84	7	86	5	82	9	17	74
qacE-	9	0	9	0	9	0	9	0
p value	0.38		0.47		0.32		<0.01	
aceI+	83	5	84	4	79	9	14	74
aceI-	10	2	11	1	12	0	12	0
p value	0.16		0.57		0.24		<0.01	
emrA+	93	7	95	5	91	9	26	74
emrA-	0	0	0	0	0	0	0	0
p value	-		-		-		-	

Table 4. Combination pattern of efflux pump encoding genes among *A. baumannii* isolates

Genes profile	No. (%) of isolates $N = 100$
aceI + emrA	2 (2)
emrA + qacE	8 (8)
emrA + qacED1	7 (7)
aceI + emrA + qacE	34 (34)
aceI + emrA + qacED1	3 (3)
aceI + emrA + qacE + qacED1	46 (46)

DISCUSSION

A. baumannii is a prevalent hospital pathogen responsible for HAIs worldwide [23]. To maintain hand hygiene and disinfect medical equipment and the hospital environment, it is essential to use antimicrobial disinfectants such as antiseptics and disinfectants [26]. Triclosan was found to be the most effective disinfectant against *A. baumannii* clinical isolates in this study, with an MIC_{90} value of 4 μg mL^{-1}. In contrast, previous studies in China reported a wide range of MIC_{90} values for triclosan against *A. baumannii* isolates, ranging from 0.5 μg mL^{-1} to 128 μg mL^{-1} [27–29]. Currently, there are no established standard breakpoints for interpreting the results of the disinfectants' susceptibility tests. However, some studies provide a provisional resistance breakpoint as ≥ 64 μg mL^{-1} for triclosan in *A. baumannii* isolates [28]. In our isolates, the MIC_{90} value of triclosan is less than 64 μg mL^{-1} and falls within the current in-use concentration range of triclosan (2,000–20,000 μg mL^{-1}). It has been shown that triclosan is stable in the environment and remains on surfaces, and releases slowly [27]. So, exposure of bacteria to sub-MIC concentrations may induce triclosan resistance encoding genes and causes the development of isolates with elevated MIC levels [28]. Studies have shown that the antibacterial action of triclosan is due to the inhibition of enoyl-acyl carrier protein reductase (ENR) (involved in type II fatty acid synthesis). Four isoenzymes FabL, FabV, FabI, and FabK were described for ENR. Only FabI is targeted by triclosan. Genes encoding FabI and FabV are common among bacteria, including *A. baumannii*. Mutations and increased expression levels in the *fabI* gene and the presence of the *fabV* gene may lead to elevated triclosan MIC [16].

In this study, benzalkonium chloride and chlorhexidine digluconate were found to be the second most effective antimicrobial agents against *A. baumannii* isolates (MIC_{90} = 64 μg mL^{-1}). Reduced susceptibility of *A. baumannii* isolates to benzalkonium chloride and chlorhexidine digluconate was reported previously [19, 30, 28, 31, 32]. As antiseptics, chlorhexidine and benzalkonium have been widely used in healthcare settings for a long time [33]. Therefore, the higher MICs of benzalkonium chloride and chlorhexidine digluconate against *A. baumannii* isolates could be attributed to constant exposure to these agents. It has been shown that exposure of *A. baumannii* to chlorhexidine sub-MIC concentrations increases the expression of *A. baumannii* efflux pumps [34]. In *A. baumannii* isolates, reduced susceptibility to chlorhexidine and benzalkonium chloride has been linked to the activity of several efflux pumps [28, 34]. In this study, the efflux pump encoding genes *qacE*, *qacED1*, and *qacG* were present in 91%, 55%, and 0% of the isolates, respectively. The *qacE* gene and its modified variant, the *qacED1* gene, are primarily found in gram-negative bacteria and are located on plasmids. [35]. In similar studies, the frequency of *qacE* ranges from 40% to 52%, while the frequency of *qacED1* ranges from 68% to 93% in *A. baumannii* isolates [36–38]. Akin to our study, a study conducted in China reported the absence of the *qacG* gene in *A. baumannii* isolates. The correlation of *qacG* genes and reduced susceptibility to biguanides has been observed in methicillin-resistant *S. aureus* isolates [32]. In this study,

the presence of the *qacE* gene showed a positive correlation ($P \leq 0.05$) with reduced susceptibility to benzalkonium chloride and chlorhexidine digluconate in *A. baumannii* isolates. Our results are supported by previous reports indicating the diminished antimicrobial activity of chlorhexidine and benzalkonium in the presence of *qac* genes in *A. baumannii* isolates [36–38]. The *aceI,* chlorhexidine-specific efflux pump AceI, encoding gene was identified in 88% of our isolates. AceI contributed to adoptive tolerance to chlorhexidine in *A. baumannii* [39]. In this study, a positive correlation was observed between the presence of *aceI* and increased MIC to benzalkonium chloride and chlorhexidine digluconate. This finding potentiates previous studies showing that inactivation or inhibition of the AceI efflux pump reduces the MICs of chlorhexidine and benzalkonium in *A. baumannii* standard isolates [28, 34].

The highest MICs (MIC_{90} = 128 μg mL^{-1}) were obtained for formaldehyde against *A. baumannii* isolates compared to other disinfectants investigated in the current study. Similar results were previously reported in Iran for *Enterococcus* spp and *P. aeruginosa* isolates, with an MIC_{90} value of 512 μg mL^{-1} [15, 16]. This substance is used to disinfect or sterilize medical equipment in hospitals [40]. Formaldehyde mediates its toxic effects by the creation of DNA-DNA and DNA-protein cross-links, as well as covalent DNA mono-adducts [41]. Increased tolerance of bacteria to formaldehyde has been reported due to inactivation by formaldehyde dehydrogenase and alterations in cell envelope proteins in bacteria [40]. Our data show a correlation between the presence of the *qacED1* gene and increased formaldehyde MICs in *A. baumannii* isolates.

CONCLUSION

In summary, high MIC levels were detected for all antimicrobial disinfectants against *A. baumannii* isolates. Triclosan was the most effective agent in this study. Furthermore, disinfectant tolerance genes were observed in isolates. We recommend the prudent use of disinfectants and continuous monitoring of the changes in their antimicrobial activity to slow the development and spread of disinfectant resistance and better infection control practices in hospitals.

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Data availability: The datasets generated and analyzed during the current study are available in the NCBI GenBank repository, under the accession numbers: MW390506.1, MW384875.1, MW486166.1, and MW486167.1.

Conflict of interest: The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Author contributions: MNK conceived the study, carried out the experiments, analyzed the results, and prepared the manuscript draft. PA collected the isolates. MSJ carried out the experiments. AMG analyzed the statistical data. MH revised the manuscript. RT analyzed the results and revised the manuscript. MA conceived the study, supervised and led the project.

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