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Detection and Evaluation of Multidrug Resistance and the Presence of Virulence Genes Related to the Formation of *ompA*, *epsA*, and *Bap* Biofilms in Clinical Isolates of *Acinetobacter baumannii* in Tehran, Iran

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Abstract

Background: *Acinetobacter baumannii* is a prevalent pathogenic bacterium that causes nosocomial infections globally. A multitude of *A. baumannii* strains have acquired a broad spectrum of antibiotic resistance in recent years, primarily due to the influence of genes associated with the production of biofilms.

Methods: Two hundred clinical isolates were acquired and described from Shahid Mostafa Khomeini, Tohid, and Shahid Motahari hospitals in Tehran, Iran, in 2018. The disk diffusion method was then used to determine whether genes related to the formation of outer membrane protein A (*ompA*), exopolysaccharide (*epsA*), and biofilm-associated protein (Bap) by polymerase chain reaction were present or not, as per the 2020 Clinical and Laboratory Standards Institute (CLSI) guidelines.

Results: We found 60 different types of *A. baumannii*, all confirmed by blaOXA-51-like gene area sequencing and polymerase chain reaction (PCR) *16S rRNA*. It was found that *A. baumannii* isolates were completely unaffected by piperacillin, meropenem, cefotaxime, ceftazidime, ceftriaxone, and ciprofloxacin. We also found that 96.6% of the *A. baumannii* isolates had genes related to making *ompA* biofilms, 85% had genes related to making *epsA* biofilms, and 75% had genes related to making *Bap* biofilms.

Conclusion: After examining the elevated level of antibiotic resistance among *A. baumannii* isolates and the existence of biofilm-associated genes in clinical isolate, this study showed that virulence genes linked to the formation of *epsA*, *Bap*, and *ompA* biofilms are a major cause of antibiotic resistance.

Keywords: *Acinetobacter baumannii*, Biofilms, *Bap* gene, *epsA* gene, Multidrug resistance, *ompA* gene, Virulence genes

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Introduction

Gram-negative *Acinetobacter baumannii*, often known as *A. baumannii*, is a non-fermenting bacillus that can take the form of cocci or coccobacilli (1-3). *A. baumannii* is rarely recognized as a typical component of the microbial population in healthy individuals and rarely results in severe infections in those with strong immune systems (3,4). *A. baumannii* is a pathogen that may be obtained in hospital environments. It can cause various illnesses, such as pneumonia, urinary tract infections, sepsis, and skin and wound contamination (2-4).

A. baumannii exhibits multidrug resistance (MDR) (4,5), which is characterized by many antibiotic resistance mechanisms, including the upregulation of efflux pumps, the synthesis of beta-lactamase enzymes, and a decrease in the permeability of outer membrane proteins (OMPs). The production of enzymes, like phosphoryl transferases and acetyltransferases, makes aminoglycoside antibiotics less effective (3,6-8). Other mechanisms of antibiotic resistance include the acquisition of genetic elements with resistance factors such as plasmids, integrons, transposons, and resistance islands (8,9). Gram-negative bacteria typically express various purine proteins that affect cell permeability on the surface of their outer membrane. Among these proteins, outer membrane protein A (*ompA*) is highly prevalent (8,10,11).

The *epsA* gene produces the OMP called exopolysaccharide (EPS) in *A. baumannii*. EPS accumulates on the cell surface, protecting cells from the hostile external environment (12). In addition to the aforementioned processes, *A. baumannii* exhibits another mechanism of antibiotic resistance known as biofilm

formation (13). Complex bacterial communities known as biofilms form in an extracellular matrix that bacteria produce. This matrix comprises polysaccharides, DNA, and proteins (6,14). Bacterial biofilms pose significant challenges to medicine, industry, and the environment. The attachment of bacteria to surfaces is a significant issue in ecology, biotechnology, biological contamination, and wastewater treatment (15). Multiple proteins contribute to the formation of this resistance, including those that create and enhance the biofilm structure (6,11). The initial member of this protein family, referred to as biofilm-associated protein (*BAP*), has been identified in *A. baumannii*. The ability of bacteria to form biofilms, which are essential for pathogen infectivity, is provided by these proteins on their outer surface (6,11,16).

Biofilm formation is an important mode of pathogenicity in numerous bacteria since it leads to drug resistance and enables evasion from the host immune system in the natural habitat. The existence of the *Bap* gene (associated biofilm protein) in this specific pathogen has been discovered through extensive research on the variables that lead to the production of *A. baumannii* biofilms (17). Bacteria that develop biofilm exhibit a markedly higher level of resistance to antibiotics as compared to bacteria in the planktonic state. As a result, biofilm-forming bacteria are able to tolerate antimicrobial drugs at concentrations up to 1000 times higher than concentrations that bacteria in the planktonic state can withstand. Iran and other nations have conducted many studies on the processes involved in developing biofilms by various *A. baumannii* strains and the frequency of significant genes (5,11,18,19). Nevertheless, few studies have been conducted in Tehran, Iran, on the connection between biofilm development and biofilms' unique resistance to antimicrobial drugs in *A. baumannii* clinical samples. This investigation aimed to characterize and investigate drug resistance trends and the presence of the *ompA*, *epsA*, and *Bap* genes linked to biofilm development in clinical isolates of *A. baumannii* from Tehran, Iran.

Methods

For this study, 200 clinical samples were obtained from Tehran's Shahid Mostafa Khomeini, Tohid, and Shahid Motahhari hospitals. A transfer medium was then used to deliver these samples to the lab. The species of *Acinetobacter* were identified using standard biochemical techniques, including the oxidase test, mechanical agar culture, 37 °C and 42 °C incubation, TSI medium culture, citrate test, urea test, gelatin hydrolysis, Dnase test, motion test, and culture on glucose-containing OF medium (4). The detection of *16S rRNA* and *blaOXA-51*-like genes confirmed the isolates of *A. baumannii* (Table 1).

The standard strain of *A. baumannii* ATCC: 19606 was used as a positive control in the study. The disk diffusion method, commonly called the Kirby-Bauer test, was utilized to ascertain the degree of antibiotic resistance in *A. baumannii* isolates. The 2020 Clinical and Laboratory Standards Institute's (CLSI's) requirements were followed in doing this. The development of antibiotic resistance is significantly influenced by the presence of virulence genes associated with the creation of *epsA*, *Bap*, and *ompA* biofilms, which are resistant to these antibiotics. To target and test the effectiveness against bacteria, the study used a variety of antibiotics, including piperacillin, ampicillin-sulbactam, ceftazidime, cefepime, cefotaxime, ceftriaxone, cholestin, polymyxin, gentamicin, amikacin, tetracycline, imipenem, meropenem, levofloxacin, ciprofloxacin, norfloxacin. All of these antibiotics were produced by MAST (UK Limited).

Boiling was used to extract DNA from clinical samples, and PCR was performed with specific primers to find genes linked to the synthesis of *ompA*, *epsA*, and *Bap* biofilms (Table 1) (12). The sensitivity and specificity of the chosen primers were evaluated on the NCBI website after their BLAST analysis. For each gene, polymerase chain reaction (PCR) was then carried out following the protocols in Table 2.

Results

Sample collection

This study discovered 60 isolates of *A. baumannii* in wounds, pus, sputum, and blood samples (Table 3). Standard biochemical assays were used to identify them, and the identities of all 60 types were validated by PCR sequencing of the *16S rRNA* and *blaOXA-51*-like gene areas.

Table 1. The study primer sequences

Gene	Primer sequence	Product size	Tm (°C)
16S rRNA	F: AGAGTTTGATCCTGGCTCAG R: ACGGCTACCTTGTTACGACTT	1500	58
blaOXA-51-like	F: TAATGCTTTGATCGGCCTTG R: TGGATTGCACTTCATCTTGG	353	52
epsA	F: AGCAAGTGGTTATCCAATCG R: ACCAGACTCACCCATTACAT	207	50
ompA	F: CGCTTCTGCTGGTGCTGAAT R: CGTGCAGTAGCGTTAGGGTA	468	50
Bap	F:TACTTCCAATCCAATGCTAGGGAGGGTACCAATGCAG R: TTATCCACTTCCAATGATCAGCAACCAAACCGCTAC	130	65

Table 2. Temperature protocol relates to the cycle of the thermocycler for the *ompA*, *epsA* genes, and the *Bap* gene

Gene	Pre-denaturation	Denaturation	Annealing	Extension	Final extension
Gene *ompA*	94 °C 5 min	94 °C 45 seconds ←	55 °C 4° seconds 32 cycles	72 °C 45 seconds →	72 °C 5 min
Gene *epsA*	94 °C 5 min	94 °C 45 seconds ←	55 °C 4° seconds 32 cycles	72 °C 45 seconds →	72 °C 5 min
Gene *Bap*	94 °C 5 min	94 °C 45 seconds ←	65 °C 4° seconds 32 cycles	72 °C 45 seconds →	72 °C 5 min

Table 3. Features pertaining to patient sex and the quantity and proportion of *A. baumannii* isolates in the sample

Sample isolation place	Number of isolates divided from patients	Percentage	Number of *Acinetobacter baumannii* isolates	Percentage
Blood	89	44.5%	25	41.7%
Sputum	48	24%	15	25%
Wounds	36	18%	12	20%
Urine	27	13.5%	8	13.3%
Total	200	100%	60	100%

Based on differential biochemical testing, 60 of the 200 isolates delivered to the laboratory were identified as *A. baumannii*. Each sample was cultivated on MacConkey agar and blood agar medium in the laboratory and incubated for 24 hours at 37 °C. It was proven that *Acinetobacter* gram-negative coccobacilli were present by a direct test (gram staining) using a microscope 24 hours later. Then, growth at 37 °C and 42 °C and biochemical tests, including Indole, Methyl Red, Voges Proskauer, Citrate Utilization (IMVIC), urease, Triple Sugar Iron (TSI), Oxidative Fermentative (OF), methyl red voges proskauer (MRVP), Sulfur Indole Motile (SIM), catalase , and oxidase, were used to determine the different *Acinetobacter* types. Immobile, oxidase negative, catalase positive, indole negative, pigment negative, urease positive, citrate positive, S2H negative, methyl red (MR) negative, and voges proskauer (VP) negative lactose insensitive isolates might yield *A. baumannii*.

Determination of antibiotic resistance pattern

Based on the CLSI 2020 table, the antibiotic resistance assessment showed that all isolates of *A. baumannii* were completely resistant (100%) to cefotaxime, ciprofloxacin, piperacillin, meropenem, and ceftazidime. Of the 60 isolates of *A. baumannii* collected from the wound, pus, sputum, and blood samples, 50 cases (more than 80%) exhibited significant resistance to the selected antibiotics. These cases were identified using standard biochemical tests, and their identities were confirmed by amplifying *16S rRNA* and the *blaOXA-51*-like gene using the PCR method. The present investigation found that *A. baumannii* isolates exhibited the least resistance to polymyxin B and colistin, with only 3.3% of the antibiotics being ineffective. The data suggest that biofilm-related genes, specifically *ompA*, *epsA*, and *Bap*, are crucial in developing resistance against these antibiotics. Using the disk diffusion technique, Table 4 shows the antibiotic resistance rate of *A. baumannii* strains.

PCR detection of virulence genes related to the formation of ompA, epsA, and Bap biofilms

Following testing for antibiotic sensitivity and resistance in the samples, PCR was used to examine the frequency of genes linked to *ompA*, *epsA*, and *Bap* biofilm production (Figure 1).

Additionally, 96.6%, 85%, and 75% of the genes linked to *ompA*, *epsA*, and *Bap* biofilm formation were found in isolates of *A. baumannii*, according to molecular PCR analysis. Among the available antibiotics and genes, piperacillin (PIP), ceftazidime (CAZ), cefotaxime (CTX), colistin (CI), ciprofloxacin (CIP), ceftriaxone (CRO), and gene *ompA*, in order, were excluded from the analysis because they had a fixed value in all samples. We used the contingency table and chi-square test to examine the relationship between antibiotic resistance and selected genes. As shown in Table 3, people with *espA* and *Bap* genes displayed resistance against amikacin (AN), tetracycline (TE), levofloxacin (LEV), imipenem (IPM), norfloxacin (NOR), cefepime (FEP), and gentamicin (GM) and were sensitive to polymyxin (PB) antibiotics (Table 5).

The association between genes and antibiotic resistance was investigated using a chi-square test. The table indicates a strong positive connection ($P < 0.05$) between the *Bap* gene and resistance to AN, IPM, and NOR. Furthermore, a notable correlation was seen between resistance to AN and the existence of the *espA* gene (Table 6).

Discussion

Acinetobacter baumannii, a common nosocomial pathogen, has both intrinsic and acquired resistance

to antibacterial drugs, making it a significant cause of infections in intensive care units. The ability to acquire resistance genes is responsible for forming MDR strains (20-23). In this study, most *A. baumannii* isolates had virulence genes linked with the production of *ompA*, *epsA*, and *Bap* biofilms, which was either consistent or discordant with prior findings. As a result, Zeighami et al analyzed 100 clinical specimens of *A. baumannii* and found that all isolates were resistant to most antibiotics, and some had MDR. Thirty-two isolates were resistant to all antibiotics tested, and 91% of them showed high levels of drug resistance. All *A. baumannii* isolates had at least one gene associated with biofilm development, with *csuE* being the most common. Of all isolates, 98% showed the presence of more than four simultaneous genes associated with biofilm formation (24). The outcomes of this investigation were consistent with our discoveries about the expression of several genes linked to the production of biofilms and the pattern of antibiotic resistance.

Table 4. The proportion of *A. baumannii* isolates that are resistant to various drugs

Antibiotic drug	*n* (R%)	*n* (I%)	*n* (S%)
PIP	60 (100%)	0 (0%)	0 (0%)
SAM	20 (33.3%)	28 (46.7%)	12 (20%)
CAZ	60 (100%)	0 (0%)	0 (0%)
FEP	54 (90%)	6 (10%)	0 (0%)
CTX	60 (100%)	0 (0%)	0 (0%)
CL	2 (3.3%)	0 (0%)	58 (96.7%)
PB	2 (3.3%)	0 (0%)	58 (96.7%)
GM	55 (91.7%)	2 (3.3%)	3 (5%)
AN	45 (75%)	12 (20%)	2 (3.3%)
TE	51 (85%)	5 (8.3%)	4 (6.7%)
LEV	53 (88.3%)	7 (11.7%)	0 (0%)
CIP	60 (100%)	0 (0%)	0 (0%)
IPM	59 (98.3%)	0 (0%)	1 (1.7%)
MEN	60 (100%)	0 (0%)	0 (0%)
CRO	60 (100%)	0 (0%)	0 (0%)
NOR	59 (98.3%)	0 (0%)	1 (1.7%)

PIP: piperacillin, SAM: ampicillin-sulbactam, CAZ: ceftazidime, FEP: Cefepime, CTX: cefotaxime, CL: colistin, PB: polymyxin, GM: gentamicin, AN: amikacin, TE: tetracycline, LEV: levofloxacin, CIP: ciprofloxacin, IPM: imipenem, MEN: meropenem, CRO: ceftriaxone, NOR: norfloxacin; R: resistant; I: intermediate; S: sensitive.

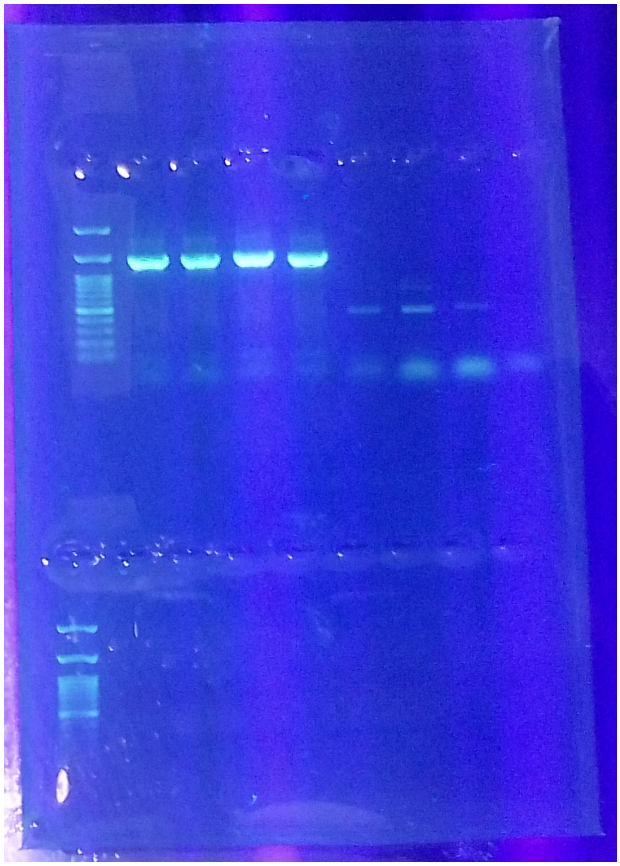

Figure 1. PCR amplification of the *16S rRNA* gene. Lane 1: Ladder (100 bp), lane 2: positive control (1500 bp), lane 3–5: positive results; PCR amplification of the *ompA* gene, lane 2: positive control (207 bp), lane 6–8: positive results (468 bp), lane 9: negative control

Table 5. Antimicrobial resistance pattern in clinical isolates of *A. baumannii* as determined by diffusion disk testing

A-B	Gene											
	Bap						*espA*					
	Positive			Negative			Positive			Negative		
	S	I	R	S	I	R	S	I	R	S	I	R
SAM	11 (23.4%)	23 (48.9%)	13 (27.7%)	1 (7.7%)	5 (38.5%)	7 (53.8%)	10 (19.2%)	24 (46.2%)	18 (34.6%)	2 (25%)	4 (50%)	2 (25%)
FEP	0 (0%)	5 (10.6%)	42 (89.4%)	0 (0%)	1 (7.7%)	12 (92.3%)	0 (0%)	6 (11.5%)	46 (88.5%)	(0%)0	0 (0%)	8 (100%)
PB	45 (95.7%)	0 (0%)	2 (4.3%)	13 (100%)	0 (0%)	0 (0%)	50 (96.2%)	0 (0%)	2 (3.8%)	8 (100%)	0 (0%)	0 (0%)
GM	3 (6.4%)	1 (2.1%)	43 (91.5%)	0 (0%)	1 (7.7%)	12 (92.3%)	3 (5.8%)	2 (3.8%)	47 (90.4%)	0 (0%)	0 (0%)	8 (100%)
AN	0 (0%)	11 (23.4%)	36 (76.6%)	2 (15.4%)	2 (15.4%)	9 (69.2%)	0 (0%)	9 (17.3%)	43 (82.7%)	2 (25%)	4 (50%)	2 (25%)
TE	2 (4.3%)	4 (48.5%)	41 (87.2%)	2 (15.4)	1 (7.7%)	10 (76.9%)	4 (7.7%)	5 (9.6%)	43 (82.7%)	0 (0%)	0 (0%)	8 (100%)
LEV	0 (0%)	6 (12.8%)	41 (87.2%)	0 (0%)	1 (7.7%)	12 (92.3%)	0 (0%)	7 (13.5%)	45 (86.5%)	0 (0%)	0 (0%)	8 (100%)
IPM	0 (0%)	0 (0%)	47 (100%)	1 (7.7%)	0 (0%)	12 (92.3%)	0 (0%)	1 (1.9%)	51 (98.1%)	0 (0%)	0 (0%)	8 (100%)
NOR	0 (0%)	0 (0%)	47 (100%)	0 (0%)	1 (7.7%)	12 (92.3%)	0 (0%)	1 (1.9%)	51 (98.1%)	0 (0%)	0 (0%)	8 (100%)

Table 6. Resistance to specific antibiotics and the presence of genes are significantly correlated

Agent	Gene					
	Bap			*espA*		
	Value	df	Asymptotic Significance (2-sided)	Value	df	Asymptotic significance (2-sided)
SAM	3.591	2	0.166	0.330	2	0.848
FEP	0.098	1	0.754	1.026	1	0.311
PB	0.572	1	0.449	0.318	1	0.573
GM	1.777	2	0.411	0.839	2	0.657
AN	7.607	2	0.022 *	19.497	2	0.000 *
TE	2.028	2	0.363	1.629	2	0.443
LEV	0.254	1	0.614	1.219	1	0.270
IPM	3.677	1	0.055 *	0.156	1	0.692
NOR	3.677	1	0.055 *	0.156	1	0.692

Azizi et al conducted a study on 65 MDR *A. baumannii* isolates. The results showed that 23 (35.4%) isolates had strong biofilm activity, 18 (27.7%) had moderate activity, 13 (20%) had weak activity, and 11 (16.9%) had no biofilm activity. All of the selected isolates also had the *ompA* and *csuE* genes. Our findings on the production of some biofilm-related genes supported the outcomes of this investigation (25).

Amin et al conducted a study on 64 isolates of *A. baumannii* in Ahvaz, Iran. They discovered that most of these isolates exhibited resistance to all antibiotics except for tigecycline and colistin. The study revealed that genes associated with the creation of biofilms were present in both biofilm-producing and non-biofilm-producing strains. Nevertheless, it was discovered that the strains that produced biofilms had stronger gene expression. This aligns with earlier research on biofilm formation and antibiotic resistance patterns (11). Regarding the expression of genes linked to the production of biofilms and the pattern of resistance to numerous antibiotics, the results of this analysis were consistent with our research.

Thummeepak et al investigated the presence of virulence genes in the biofilm formation of 225 clinical isolates of *A. baumannii* from three hospitals in Thailand. The prevailing virulence genes were *ompA*, with a prevalence of 84.4%, and *bfmS*, with a prevalence of 84%. The prevalence of the *Bap*, *blaPER-1*, and *epsA* genes in the isolates was 48%, 30.2%, and 22.2%, respectively. There was a total of nine isolates that had five virulence genes. Additionally, the *ompA* and *Bap* genes were associated with MDR in various strains, as reported in a study involving 12 cases (12). The results of this study supported our conclusions concerning the production and frequency of specific virulence genes during the creation of biofilms, as well as the pattern of resistance to several antibiotics.

The study conducted by Mahmoudi Monfared et al demonstrates a significant inclination of clinical *A. baumannii* isolates to develop biofilms associated with the *blaPER-1* and *Bap* genes. Most samples from Isfahan exhibited susceptibility to colistin, underscoring the necessity for novel approaches to prevent and manage infections caused by strains that form biofilms (26).

Tigecycline and colistin are The only treatments for *A. baumannii* infections that show extensively drug resistant (XDR) or MDR. When used together with tigecycline or colistin, carbapenems are regarded as the most efficacious therapy for infections caused by multidrug-resistant *A. baumannii*. In addition, these combination drugs exhibit higher efficacy and lower toxicity compared to tigecycline or colistin, which are used as single therapies. According to the antibiotic susceptibility test results, the bulk of *A. baumannii* isolates showed colistin sensitivity, which is consistent with earlier findings from Iran and other countries (27-29). Hazhirkamal and colleagues' research in Hamedan, Iran, found that 95% of *A. baumannii* isolates were MDR and 32.5% could form a strong biofilm. The most frequently studied genes were *VIM* (81%), *SPM* (45.2%), and *IMP* (35.7%). The enterobacterial repetitive intergenic consensus (ERIC)-PCR method can genetically classify *A. baumannii* isolates (30).

The *bfmR* and *pbpG* genes were shown to be present in all 50 clinical isolates of *A. baumannii* by Mozafari et al. They also noticed that 49 samples included the *Bap*, *plD*, *surA*, and *csuA* genes. In *A. baumannii* strains, virulence factor genes predominate over 90% of the time, suggesting that most clinical strains can form biofilm structures because biofilm-formation-related genes are very prevalent (31).

Biofilm is crucial for protecting bacteria from immune system cells and antimicrobial agents. Most *A. baumannii* strains express genes related to biofilm formation, which directly correlates with resistance to most antimicrobial agents. Antibiotic-resistant strains have more biofilm formation genes. MDR strains also have a greater ability to express biofilm formation genes (32-34).

Conclusion

Eighty percent of the fifty isolates examined in the research

showed resistance to the selected antibiotics. Furthermore, genes associated with forming *Bap*, *ompA*, and *epsA* biofilms were observed at frequencies of 75%, 96.6%, and 85%, respectively, in *A. baumannii* isolates. The study indicates that virulence genes, including *ompA*, *epsA*, and *Bap*, play a major role in forming MDR *A. baumannii* isolates. This is supported by the predominance of these genes in creating *ompA*, *epsA*, and *Bap* biofilms.

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Competing Interests
There are no conflicts of interest.

Ethical Approval
The writers have carefully followed ethical guidelines that address issues like plagiarism, informed consent, wrongdoing, fabricating or manipulating data, publishing or submitting works more than once, redundancy, and other related issues. This work has been approved by the Shahed University Faculty of Medicine Ethics Committee (IR.SHAHED.REC.1397.101).

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