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Research article

Antimicrobials profiling, biofilm formation, and clonal lineage of methicillin-resistant *Staphylococcus aureus* isolated from cockroaches

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ABSTRACT

Cockroaches are widely recognized as vectors for transmitting pathogenic microorganisms in hospital and community environments due to their movement between contaminated and human-occupied spaces. *Staphylococcus aureus* (*S. aureus*), particularly methicillin-resistant *Staphylococcus aureus* (MRSA), is a primary global health concern because of its capacity to cause a wide range of infections and its resistance to many antibiotics. Despite efforts to control nosocomial infections, the role of cockroaches in disseminating antibiotic-resistant bacteria has not been fully explored. This study aims to investigate the antibiotic resistance patterns, biofilm formation, and genetic characteristics of *S. aureus* isolated from cockroaches in hospital environments. Understanding the role of cockroaches as vectors of drug-resistant *S. aureus* can contribute to developing more effective infection control strategies in healthcare settings. This study examined 386 cockroaches, including 230 American and 156 German cockroaches. Antibiotic sensitivity, inducible resistance, and biofilm formation were evaluated. The presence of *mecA*, *ermA*, *ermB*, *ermC*, *msrA*, *icaA*, *icaB*, *icaC*, *icaD*, SCC*mec*, *mupA*, *mupB*, and *iles-1* genes was determined. Randomly amplified polymorphic DNA typing was performed to determine genetic relatedness. Fifty *S. aureus* isolates were identified, with 48 % confirmed as MRSA. No isolate exhibited constitutive resistance to clindamycin. However, 96 % of the isolates displayed inducible clindamycin resistance (iMLSB phenotype) when tested using the D-test. The prevalence of *icaA*, *icaB*, *icaC*, and *icaD* genes were 34 %, 8 %, 0 %, and 0 %, respectively. So, 29.1 %, 16.6 %, 12.5 %, and 8.3 % of isolates had SCC*mec* gene cassettes of types I, II, III, and IV, respectively. The prevalence of *ermA*, *ermB*, *ermC*, and *msrA* genes was found to be 18 %, 16 %, 58 %, and 4 %, respectively. Seven different clusters were found in the RAPD-PCR, with cluster A (5 isolates) being the most common. These results show that cockroaches are important in transmitting resistance factors as mechanical vectors. Therefore, taking sanitary measures to control the insect population is unavoidable.

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1. Introduction

Among the most populous arthropods are insects, which play a crucial role in economics, health, and medicine [1]. Blattaria order contains 4000 species of cockroaches, of which less than 1 % are pests. They can be found in warehouses, bakeries, hospitals, and ships. During the day, they are concealed in dark, deep crevices; they leave their shelters to feed at night [2].

Periplaneta americana and *Blattella germanica* are the two cockroach species commonly found in urban environments, including hospitals [3]. Their presence in healthcare settings is not limited to any particular country. Still, it has been reported globally in regions such as Malaysia, Singapore, China, Indonesia, India, Iran, and Pakistan, where they have been linked to the transmission of pathogens [4–6]. Cockroaches are mechanical vectors capable of spreading a wide variety of bacteria, including *Staphylococcus aureus* (*S. aureus*), through their movement between contaminated environments and areas where human contact occurs [7]. Their ability to thrive in unsanitary conditions makes them particularly concerning in healthcare facilities, where they can carry and disseminate drug-resistant pathogens [8].

The prevalence of cockroaches in hospitals indicates poor hygienic conditions, as these pests can transport bacteria on their external surfaces and within their digestive systems [9]. Studies have shown that cockroaches can harbor numerous multi-drug-resistant (MDR) bacteria, including *S. aureus*, which causes a wide range of infections, from superficial skin infections to life-threatening conditions such as sepsis and pneumonia [10]. The morbidity and mortality associated with hospital-acquired *S. aureus* infections have increased dramatically worldwide. A major global health issue is methicillin-resistant *Staphylococcus aureus* (MRSA) [11,12]. Cockroaches can indirectly transmit *S. aureus* to humans by contaminating hospital surfaces, medical equipment, or food, leading to human infections, particularly in immunocompromised patients [13]. Although eradicating cockroaches is essential in improving hospital hygiene, it is equally crucial to understand the genetic characteristics of the pathogens they carry [14].

S. aureus's ability to form biofilms is critical to its pathogenicity. Biofilm-associated *S. aureus* infections are resistant to antibiotic treatment [15]. Polysaccharide intercellular adhesin (PIA) is required for biofilm formation, and its synthesis is controlled by the intercellular adhesion (icaADCB) operon [16]. Staphylococcal strains resistant to erythromycin may be susceptible to clindamycin and display a D-shaped inhibition zone around the clindamycin disk with flattening towards erythromycin (an inducible MLSB phenotype). A constitutive resistance MLSB (cMLSB) isolate has a spherical inhibition zone and is resistant to erythromycin and clindamycin [17]. Staphylococci that were resistant to erythromycin but sensitive to clindamycin showed a spherical inhibition zone around clindamycin and were classified as MS (resistant to erythromycin and sensitive to clindamycin) [17]. Staphylococcal chromosomal cassette *mec* (SCC*mec*) is associated with the *mecA* gene, causing virulent characteristics. The size and genetic content of the 11 main SCC*mec* types vary. They also contain multiple resistance genes. SCC*mec* types I, II, and III are usually associated with healthcare-related MRSA (HA-MRSA), While SCC*mec* types IV and V are usually found in community-related MRSA (CA-MRSA) [18,19].

Randomly amplified polymorphic DNA PCR (RAPD-PCR) is a quick and straightforward method characterized by low annealing temperatures. This method uses primers composed of random short oligonucleotides to amplify multiple DNA regions. The resulting fragments differ in number and size, reflecting variations in the distance between primer binding sites unique to each bacterial isolate [20].

This study aims to investigate the antibiotic resistance profiles, biofilm production capabilities, and molecular typing of *S. aureus* isolated from cockroaches in a hospital setting. By understanding the role of cockroaches as carriers of antibiotic-resistant pathogens, we can better inform strategies for infection prevention and control in healthcare environments.

2. Materials and methods

2.1. Cockroach collection

This cross-sectional study was conducted in a large teaching hospital (Ayatollah Rouhani), affiliated with Babol University of Medical Sciences, Iran, between November 2022 and September 2023. Using a sterile glove, we captured 386 cockroaches. Each sample was placed in a separate bottle to prevent cross-contamination with other cockroaches. Each sample was placed at −4 °C for 5 min to anesthetize. A standard taxonomic key and cockroach characteristics were used to identify the cockroach species [21].

2.2. Isolation and identification of S. aureus

In order to dislodge microorganisms from the cuticle surface, each cockroach was rinsed in 5 ml of sterile physiological saline for 20 s, followed by vortexing. The resulting solution was serially diluted and plated onto appropriate culture media to examine microbial growth.

To decontaminate the internal surface of the cockroaches, they were first washed with 70 % ethyl alcohol for 2 min to eliminate surface contaminants. After this initial wash, the cockroaches were immersed in sterile physiological saline for 2–3 min to facilitate the isolation of bacteria from their internal tissues. This method ensured that the isolated bacterial strains were from the cockroaches, while minimizing the risk of external contamination [14].

The cockroaches' digestive systems were aseptically transferred into sterile physiological saline vials using sterile forceps to isolate bacteria from the internal tissues. The homogenates were then mechanically homogenized for 5 min. After inoculating the homogenates onto blood agar (Merck, Germany) and mannitol salt agar (MSA) (Merck, Germany) plates, they were incubated at 37 °C for 24 h. Gram staining confirmed colonies suspected of containing *S. aureus*, followed by differential tests such as catalase, coagulase, and DNase assays. DNA samples were examined using PCR to detect the *S. aureus*-specific *nucA* nuclease genes for previously defined

colonies. These tests confirmed the presence of *S. aureus* [22–25]

2.3. Antimicrobial susceptibility test

Antimicrobial susceptibility testing was performed using the Kirby–Bauer disk diffusion method on the Mueller–Hinton agar (MHA) (Condalab, Spain). The antibiotics (Padtan Teb Co, Iran) tested include erythromycin (15 μg), clindamycin (2 μg), gentamicin (10 μg), ciprofloxacin (5 μg), tetracycline (30 μg), cefoxitin (30 μg), and trimethoprim sulfamethoxazole (SXT; 1.25–23.75 μg). *S. aureus* ATCC 25923 was used as a control strain to validate the susceptibility testing according to Clinical and Laboratory Standards Institute (CLSI, 2023) guidelines [19,26].

2.4. Quantitative biofilm production assay

Biofilm production was determined in 96-well microtiter plates using the Stepanović et al. methods [27]. Control strains known for biofilm production, such as *S. aureus* ATCC 29213, were included in the assay to validate the results. Therefore, *S. aureus* isolates were cultured overnight and diluted to 0.5 McFarland turbidity. After that, 1: 100 dilutions of this suspension in fresh Tryptic soy broth (TSB) (Merk, Germany) supplemented with 1 % glucose were conducted, and 200 μl of the diluted suspension was placed into each well of a microtiter plate, which was then incubated at 37 °C for 24 h. The connected cells were then fixed for 15 min in 200 μl of 99 % methanol (Merk, Germany). After that, 200 μl of 2 % crystal violet was added to each well, and the plates were incubated at room temperature for 15 min. Finally, each well received 125 μl of 30 % acetic acid, and the OD570 was determined using a microtiter-plate reader (Bio-Rad, USA). The biofilm formation test was performed three times for each sample, and measurements were made. The average OD of the negative controls plus (3 ×) their standard deviation (SD) was used to determine the optical density cut-off value (ODc). The isolate with OD ≤ ODc was considered as a non-biofilm producer, ODc < OD ≤ 2ODc as a weak biofilm producer, 2ODc < OD ≤ 4ODc as a moderate biofilm producer, and OD > 4ODc as a strong biofilm producer.

Table 1
Sequences of primers used in the study.

	Genes	Primer Sequence 5' -3'	PCR annealing temperature (∘C)	Amplicon Size (bp)	Reference
Antibiotic resistance and biofilm formation encoded genes	***nucA***	F: GCGATTGATGGTGATACGGTT R: ACGCAAGCCTTGACGAACTAAAGC	51 °C	279	[50]
	mecA	F: AGTTCTGCAGTACCGGATTTGC R: AAAATCGATGGTAAAGGTTGGC	51 °C	533	[51]
	ermA	F: TCTAAAAAGCATGTAAAAGAA R: CTTCGATAGTTTATTAATATTAGT	48 °C	645	[52]
	ermB	F: TAACGACGAAACTGGCTAAAA R: ATCTGTGGTATGGCGGGTAAG	51 °C	416	[53]
	ermC	F: AATCGTCAATTCCTGCATGT R: TAATCGTGGAATACGGGTTTG	48 °C	299	[54]
	msrA	F: GGCACAATAAGAGTGTTTAAAGG R: AAGTTATATCAGAATAGATTGTCCTGTT	52 °C	940	[55]
	icaA	F: ACACTTGGCGCAGTCAA R: TCTGGAACCATCCAACA	52 °C	188	[33]
	icaB	F: ATGGCTTAAAGCACACGACGC R: TATCGGCATCTGGTGTGACAG	56 °C	527	[56]
	icaC	F: TAACTTTAGGCGCATATGTTT R: TTCCAGTTAGGCTGGTATTG	52 °C	400	[57]
	icaD	F: GCTTGACCATGTTGCGTAACC R: GAACCGCTTGCCATGTGTTG	52 °C	483	[58]
	mupA	F: TATATTATGCGATGGAAGGTTGG R: AATAAAATCAGCTGGAAAGTGTTG	53 °C	458	[59]
	mupB	F: CTAGAAGTCGATTTTGGAGTAG R: AGTGTCTAAAATGATAAGACGATC	54 °C	674	[60]
	iles-1	F: ATAAAGGTAAAAAGCCAGTTTATTGGT R: CAACATACTCCAATTCCTTAC	52 °C	360	[61]
SCC *mec* Types	***ccrA2-B***	*β*: ATTGCCTTGATAATAGCCTCT *α3*: TAAAGGCATCAAATGCACAAACACT	54 °C	937	[30]
	ccrC	*ccrCF*:CGTCTATTACAATGCACAAACAAT *ccrCR*: CCTTTATAGACTGGATTATTCAAAATAT	54 °C	518	[30]
	IS1272	*1272F1*: GCCACTCATAACATATGGAA *1272R1*: CATCCGAGTGAAACCCAAA	54 °C	415	[30]
	mecA-IS431	*5RmecA*: TATACCAAACCCGACAACTAC *5R431*: CGGCTATAGTGATAACATCC	54 °C	359	[30]

2.5. Genomic DNA extraction

Bacterial colonies were extracted using the method described previously [28]. The purity and concentration of the isolated DNA was measured using the Nanodrop spectrophotometer (Thermo Scientific Wilmington, USA) [29].

2.6. PCR amplification

The PCR method detected isolates harboring resistance determinants, including *mecA*, *ermA*, *ermB*, *ermC*, *msrA*, *mupA*, *mupB*, and *ileS-1*. The genes related to biofilm formation, including *icaA*, *icaB*, *icaC*, and *icaD*, were also determined. Table 1 details the primers

Table 2
Characteristics of *S. aureus* isolated from hospital cockroaches.

Isolates number	Cockroach species	Cockroaches surfaces	Resistance profiling	Biofilm formation	D-test	Genes	Sccmec types
1	*P. americana*	External	FOX/TE	Moderate	–	*ermC/icaA/mupA/*	I
2	*P. americana*	External	FOX	Strong	–	*msrA/mupA*	II
3	*P. americana*	External	FOX	Weak	–	*ermA/ermC/icaA/mupA*	IV
4	*B. germanica*	External	FOX	Weak	–	*ermC/icaA/icaB/mupA*	I
5	*P. americana*	External	FOX/TE/CP/GM/	Weak	–	*ermB/ermA/ermC/icaA/ mupA*	II
6	*P. americana*	External	TE/CP/SXT	Weak	–	*ermB/ermC/icaA/mupA*	II
7	*B. germanica*	External	–	Weak	–	*msrA/ermC/mupA*	–
8	*P. americana*	External	FOX/TE/CP/GM/	Weak	–	*ermBA/ermC/icaA/icaB/ mupA*	II
9	*P. americana*	External	FOX/TE/SXT	Weak	–	*ermA/ermC/icaB/mupA*	III
10	*B. germanica*	External	FOX/CP	Weak	–	*ermA/icaA*	II
11	*P. americana*	External	FOX/TE	Moderate	–	*icaA*	–
12	*P. americana*	External	–	Moderate	–	*ermC*	–
13	*B. germanica*	External	–	None biofilm	–	*ermC/icaA*	–
14	*P. americana*	External	–	Weak	–	-	–
15	*P. americana*	External	–	Weak	–	*icaA*	–
16	*P. americana*	External	–	Strong	–	*ermC/icaA*	–
17	*B. germanica*	External	–	Weak	–	*icaA*	–
18	*P. americana*	External	–	Strong	–	*ermC*	–
19	*P. americana*	External	–	Strong	–	*ermC/icaA*	–
20	*P. americana*	External	FOX/TE	Strong	–	*ermC/icaA/icaB*	–
21	*B. germanica*	External	–	Weak	–	*ermC*	–
22	*P. americana*	Internal	–	Moderate	–	*ermC*	–
23	*P. americana*	Internal	TE	Strong	–	*ermC*	–
24	*P. americana*	External	–	Strong	–	-	–
25	*B. germanica*	External	FOX/CP	Weak	–	*icaA*	I
26	*P. americana*	External	–	Strong	–	-	–
27	*P. americana*	External	–	Weak	–	-	II
28	*P. americana*	External	FOX/SXT/GM	Weak	–	*icaA/mupA*	I
29	*P. americana*	External	FOX	Weak	–	-	III
30	*B. germanica*	External	TE/CP/SXT	Weak	–	*ermB/ermC*	II
31	*P. americana*	External	FOX/TE/CP	Strong	–	*ermC*	II
32	*B. germanica*	External	–	Weak	–	-	–
33	*B. germanica*	External	–	Weak	–	*ermB/ermC*	II
34	*B. germanica*	Internal	TE	Moderate	–	*ermB*	–
35	*P. americana*	Internal	FOX	Moderate	–	*ermC*	III
36	*P. americana*	Internal	TE/CP	Weak	–	*ermB/icaA*	–
37	*B. germanica*	Internal	–	Moderate	–	*ermB/ermC*	III
38	*P. americana*	Internal	–	Weak	–	*ermB/ermC*	III
39	*P. americana*	Internal	FOX/TE/E	Weak	+	*ermC*	I
40	*B. germanica*	Internal	FOX/CP	Weak	–	*ermA/ermC*	IV
41	*B. germanica*	Internal	CP	Weak	–	*ermA/ermC*	–
42	*P. americana*	Internal	–	Strong	–	-	–
43	*B. germanica*	Internal	FOX/CP	Weak	–	-	–
44	*P. americana*	Internal	FOX	Strong	–	*ermA*	I
45	*P. americana*	Internal	FOX/TE/E	Weak	+	*ermA/ermC*	I
46	*P. americana*	Internal	TE/CP/E	Weak	+	*ermA/ermC*	–
47	*P. americana*	Internal	FOX/TE/CP	None biofilm	–	-	–
48	*P. americana*	Internal	FOX	Weak	–	-	–
49	*P. americana*	Internal	FOX/TE/CP/GM/	Weak	–	-	–
50	*P. americana*	Internal	FOX/TE/CP/SXT/ GM	Weak	–	-	–

S: Susceptible; R: Resistant; I: Intermediate.
E: Erythromycin, CC: Clindamycin, FOX: Cefoxitin, TE: Tetracycline, CP: Ciprofloxacin, SXT: Trimethoprim-Sulfamethoxazole, GM: Gentamicin.

used to amplify these genes, providing each primer's sequences and annealing temperature. PCR amplification was conducted using 5 μL of Master Mix (Ampliqon, Denmark), 5.2 μL of DNase-free distilled water, 0.3 μL of each primer (at 10 pmol/L), and 1.2 μL of DNA template. In this study, Amplified products were visualized by electrophoresis in 1.5 % agarose gels stained with prepared in 1X TBE (Tris/Borate/EDTA) buffer and then visualized under ultraviolet light following staining with safe stain load dye (SinaClon, Iran).

SCC*mec* gene cassette typing revealed that the amplification of *mecA-IS431* indicated cassette type V, while the presence of *IS1272* was found in cassette types I and IV. The *ccrC* gene was detected in cassette types III and V, and the *ccrA2-B* gene complex indicated cassette types II and IV [30]. The PCR was performed using 12 μl reactions containing 5 μl of Master Mix (Ampliqon, Denmark), 3.4 μl of DNase-free distilled water, 0.3 μl of each primer (10 pmol/μl) (synthesized by metabion, Germany), and 1.2 μl of DNA template. PCR included an initial denaturation step at 94 °C for 5 min, followed by 30 cycles of denaturation at 94 °C for 45 s, annealing at 54 °C for 30 s, extension at 72 °C for 1 min, followed by a final extension cycle at 72 °C for 5 min. PCR amplicons were visualized under ultraviolet light after electrophoresis on a 1.5 % agarose gel (SinaClon, Iran).

2.7. RAPD-PCR

RAPD-PCR was performed to assess the twenty-four MRSA *S. aureus* isolates using a specific primer (5′-CCGCAGCCAA-3′) according to the previous method described [31]. Amplifications were carried out in a total volume of 25 μl containing 10 μl of Master Mix (Ampliqon, Denmark), 10 μl of DNase-free distilled water, 2.5 μl of primer (10 pmol/μl) (synthesized by metabion, Germany), and 2.5 μl of DNA template. Thermal cycles included one cycle of 5 min at 94 °C, 40 cycles at 94 °C, 60 s at 35 °C, and 60 s at 72 °C, followed by a 7 min extension at 72 °C. Amplified products were assessed by electrophoresis on 1.5 % agarose gels and observed DNA bands using ultraviolet light after staining with safe stain load dye (SinaClon, Iran). As previously mentioned, the GelJ program was utilized to assess RAPD patterns. Isolates with a DNA similarity value of 80 % or above were classified as identical genotypes.

2.8. Sequence supporting data

In this study, a subset of key genes involved in antibiotic resistance and biofilm formation was selected for sequencing based on their clinical relevance and the results obtained from PCR amplification. Specifically, the genes *icaA*, *icaB*, *ermA*, *ermB*, *ermC*, *msrA*, and *mupA* were sequenced, as these genes play a crucial role in biofilm formation and resistance to macrolides, lincosamides, and mupirocin, which are important in the context of hospital-acquired infections.

Table 3
Antibiotic resistance, biofilm formation, and SCC *mec* types in *S. aureus* isolated from hospital cockroaches.

Parameters	Total (n = 50) No.	External surface (n = 31) No. (%)	Internal surface (n = 19) No. (%)	*P* value	*P. americana* (n = 35) No. (%)	*B. germanica* (n = 15) No. (%)	*P* value
Antibiotics							
E	3	0 (0)	3 (15.8)	0.049	3(8.6)	0(0)	0.174
FOX	24	14 (45.2)	10 (52.6)	0.608	19(54.3)	5 (33.3)	0.608
TE	18	9 (29)	9 (47.4)	0.318	16(45.7)	2(13.3)	0.002
CP	16	8 (25.8)	8 (42.1)	0.422	10(28.6)	6(40.0)	0.218
SXT	5	4 (12.9)	1 (5.3)	0.331	4(11.4)	1(6.7)	1.000
GM	4	2 (6.5)	2 (10.5)	0.411	4(11.4)	0(0)	0.509
MRSA	24	14 (45.2)	10 (52.6)	0.608	19(54.3)	5(33.3)	0.174
D-test	3	0 (0)	3 (15.8)	0.049	3(8.6)	0(0)	0.545
Biofilm formation							
Strong	11	8 (26.7)	3 (16.7)	0.520	11(32.4)	0 (0)	0.027
Moderate	7	3 (10)	4 (22.2)		5 (14.7)	2 (14.3)	
Weak	30	19 (63.3)	11 (61.1)		18 (52.9)	12 (85.7)	
Antibiotic resistance genes							
msrA	2	2 (6.5)	0 (0)	0.519	1 (2.9)	1 (6.7)	0.514
ermA	9	4 (12.9)	5 (26.3)	0.273	7 (20)	2 (13.3)	0.220
ermB	8	4 (12.9)	4 (21.1)	0.459	4 (11.4)	4 (26.7)	0.705
ermC	29	19 (61.3)	10 (52.6)	0.547	19 (54.3)	10 (66.7)	0.416
mupA	10	10 (32.3)	0 (0)	0.008	8 (22.9)	2 (13.3)	0.702
mupB	–	–	–	–	–	–	–
iles-1	–	–	–	–	–	–	–
Biofilm formation genes							
icaA	17	16 (51.6)	1(5.3)	<0.001	12 (34.3)	5 (33.3)	0.948
icaB	4	4 (12.9)	0 (0)	0.284	3 (8.6)	1 (6.7)	1.000
icaC	–	–	–	–	–	–	–
icaD	–	–	–	–	–	–	–
SCC *mec* type							
I	7	4 (12.9)	3 (15.8)	0.033	5 (14.3)	2 (13.3)	0.912
II	9	9 (29)	0 (0)		6 (17.1)	3 (20)	
III	5	2 (6.5)	3 (15.8)		4 (11.4)	1 (6.7)	
IV	2	1 (3.2)	1 (5.3)		1 (2.9)	1 (6.7)	

E: Erythromycin, CC: Clindamycin, FOX: Cefoxitin, TE: Tetracycline, CP: Ciprofloxacin, SXT: Trimethoprim-Sulfamethoxazole, GM: Gentamicin.

The DNA sequences for each gene were analyzed at (https://www.ncbi.nlm.nih.gov/blast/). A total of 7 genes were sequenced in this study, and the sequences were submitted to GenBank. The accession numbers assigned to these sequences are PP474972, PP501544, OR876252, OR921084, OR921285, PP501545, and PP437203, respectively.

2.9. Data analysis

Data analysis was conducted with SPSS 22.0 (SPSS Inc., Chicago, USA). Chi-square and Fisher's exact tests were applied. *P*-values less than 0.05 were considered significant.

3. Results

Table 2 presents the characteristics of *S. aureus* isolated from hospital cockroaches. Of the 386 analyzed samples, 230 (60 %) were identified as *P. americana* and 156 (40 %) as *B. germanica*. Among the *P. americana*, in internal surfaces and external surfaces, *S. aureus* was isolated in 8.7 % (20/230) and 6.5 % (15/230), respectively. In the *B. germanica* samples, *S. aureus* was isolated from 7.05 % (11/156) internal surfaces and 2.6 % (4/156) external surfaces.

3.1. Bacterial isolation and identification

All *S. aureus* isolates were Gram-positive cocci, catalase-positive, and exhibited the characteristic golden-yellow colonies on mannitol salt agar. Biochemical characterization confirmed that all isolates were coagulase-positive, identifying them as *S. aureus*. Additionally, PCR amplification of the *nuc* gene confirmed the identity of all isolates as *S. aureus*.

3.2. Antimicrobial susceptibility pattern

According to Table 3, the highest antibiotic resistance among *S. aureus* isolates from the external and internal surfaces of hospital cockroaches was observed with cefoxitin, showing resistance frequencies of 45.2 % (14/31) and 52.6 % (10/19), respectively. Erythromycin resistance was significantly more prevalent in isolates from the internal surface than those from the external surface ($P < 0.05$).

The highest rates of antibiotic resistance in *S. aureus* isolated from hospital cockroaches were observed with cefoxitin (54.3 %) in *P. americana* and ciprofloxacin (40 %) in *B. germanica*. Conversely, the lowest resistance in *S. aureus* isolates from *P. americana* was to erythromycin (8.6 %). Notably, no resistance to erythromycin and gentamicin was detected in any of the *S. aureus* isolated from *B. germanica* hospital cockroaches. The statistical analysis showed that the resistance to tetracycline was significantly higher among *P. americana* and *B. germanica* hospital cockroaches ($P < 0.05$).

Among the 50 *S. aureus* isolated from hospital cockroaches, 24 (48 %) were confirmed as MRSA through PCR analysis. The frequency of MRSA among *S. aureus* isolated from hospital cockroaches was 45.2 % (14/31) for external surfaces and 52.6 % (10/19) for internal surfaces. Additionally, the frequency of MRSA isolates was 54.3 % (19/35) among *P. americana* and 33.3 % (5/15) among *B. germanica* hospital cockroaches.

Phenotypic analysis for inducible clindamycin resistance using the D-test revealed that 15.8 % (3/19) of *S. aureus* isolates from internal surfaces exhibited inducible resistance. In contrast, none of the isolates from external surfaces showed this resistance. The frequency of inducible resistance was significantly higher among internal surface isolates compared to external surface isolates ($P < 0.05$). Furthermore, 8.6 % (3/35) of the isolates from *P. americana* cockroaches displayed inducible resistance, while no inducible resistance was detected in isolates from *B. germanica* cockroaches.

3.3. Biofilm formation

Among the *S. aureus* isolates, 96 % (48/50) exhibited biofilm formation. For isolates from external surfaces, 26.7 % (8/31) showed strong biofilm formation, 10 % (3/31) moderate, and 63.3 % (19/31) weak biofilm formation. In isolates from internal surfaces, 16.7 % (3/19) displayed a strong biofilm phenotype, 22 % (4/19) moderate, and 61.1 % (11/19) weak biofilm formation. No significant difference in biofilm formation ability was observed between isolates from hospital cockroaches' external and internal surfaces ($P>0.05$).

S. aureus isolated from *P. americana* cockroaches, 32.4 % (11/35) exhibited strong biofilm formation, 14.7 % (5/35) moderate, and 52.9 % (18/35) weak biofilm formation. Among the *S. aureus* isolated from *B. germanica* cockroaches, 14.3 % (2/15) showed moderate biofilm formation, while 85.7 % (12/15) exhibited weak biofilm formation, with none demonstrating strong biofilm formation. A significant difference in biofilm formation ability was observed between isolates from *P. americana* and *B. germanica* cockroaches ($P < 0.05$).

3.4. Antibiotic resistance genes

The study detected *ermA* in 18 % (9/50), *ermB* in 16 % (8/50), *ermC* in 58 % (29/50), and *msrA* in 4 % (2/50) of the *S. aureus* isolates. The *mupB* and *ileS-1* genes were not detected among the isolates. The *ermC* gene was consistently the most prevalent among isolates from the cockroaches' external and internal surfaces and isolates from *P. americana* and *B. germanica* cockroaches. Notably, the

abundance of the *mupA* gene differed significantly between strains isolated from hospital cockroaches' external and internal surfaces ($P < 0.05$).

3.5. Genes encoding biofilm formation

Among the *S. aureus* isolated from hospital cockroaches, the *icaA* gene was the most frequently detected biofilm formation gene, present in 34 % (17/50) of the isolates. In contrast, the *icaC* and *icaD* genes were not detected in any of the isolates. The *icaA* gene was consistently the most prevalent across isolates from both the external and internal surfaces of cockroaches, as well as in isolates from *P. americana* and *B. germanica* cockroaches. Notably, the prevalence of the *icaA* gene differed significantly between strains isolated from the external and internal surfaces of hospital cockroaches ($P < 0.05$).

3.6. SSCmec typing

The frequencies of SCC*mec* types among the *S. aureus* isolated from hospital cockroaches were as follows: type I was present in 14 % (7/50) of the isolates, type II in 18 % (9/50), type III in 10 % (5/50), and type IV in 4 % (2/50). SCC*mec* type II was the predominant type among *S. aureus* isolated from external surfaces (29 %) and from *P. americana* (17.1 %) and *B. germanica* (20 %) hospital cockroaches in our study. Notably, none of the strains isolated from the internal surfaces of hospital cockroaches carried SCC*mec* type II. A significant difference in SCC*mec* types was observed between *S. aureus* isolates from hospital cockroaches' external and internal surfaces ($P < 0.05$). However, no significant difference was found between isolates from *P. americana* and *B. germanica* cockroaches ($P > 0.05$).

3.7. Association between biofilm formation with antibiotic resistance, resistance genes, biofilm-associated genes, and SCCmec typing

Table 4 shows the relationship between biofilm formation and antibiotic resistance, resistance genes, biofilm-associated genes, and SCC*mec* types in *S. aureus* isolated from hospital cockroaches. The highest rates of antibiotic resistance across all biofilm production types were observed against cefoxitin, with resistance rates of 36.4 % (4/11) in strong biofilm producers, 42.9 % (3/7) in moderate producers, and 53.3 % (16/30) in weak biofilm producers. However, it is essential to note that no significant correlation was found between cefoxitin resistance and biofilm-forming typ ($P>0.05$). Among the antibiotics tested in *S. aureus* isolated from hospital cockroaches, only resistance to ciprofloxacin showed a significant association with the type of biofilm produced ($P < 0.05$).

The most common MRSA and D-test positive isolates had weak biofilm formation, accounting for 53.3 % (16/30) and 10 % (3/30), respectively. Among isolates with strong, moderate, and weak biofilm formation, the *ermC* gene was the most prevalent induced

Table 4
Association between biofilm formation with antibiotic resistance, resistance genes, biofilm-associated genes, and SCC *mec* typing in *S. aureus* isolated from hospital cockroaches.

Parameters	Total (n = 50) No.	Strong biofilm (n = 11) No. (%)	Moderate biofilm (n = 7) No. (%)	Weak biofilm (n = 30) No. (%)	*P* value
Antibiotics					
E	3	0 (0)	0 (0)	3 (100)	0.756
FOX	23	4 (36.4)	3 (42.9)	16 (53.3)	0.820
TE	17	3 (27.3)	3 (42.9)	11 (36.7)	0.237
CP	15	1 (9.1)	0 (0)	14 (46.7)	0.014
SXT	5	0 (0)	0 (0)	5 (16.7)	0.566
GM	4	0 (0)	0 (0)	4 (13.3)	0.452
MRSA	23	4 (36.4)	3 (42.9)	16 (53.3)	0.820
D-test	3	0 (0)	0 (0)	3 (10)	0.723
Antibiotic resistance genes					
msrA	2	1 (9.1)	0 (0)	1(3.3)	0.614
ermB	8	0 (0)	2 (28.6)	6 (20)	0.195
ermA	9	1 (9.1)	0 (0)	8 (26.7)	0.310
ermC	28	6 (54.5)	5 (71.4)	17 (56.7)	0.834
mupA	10	1 (9.1)	1 (14.3)	8 (26.7)	0.523
mupB	0	–	–	–	–
iles-1	0	–	–	–	–
Biofilm formation genes					
icaA	16	3 (27.3)	2 (28.6)	11 (36.7)	0.908
icaB	4	1 (9.1)	0 (0)	3 (10)	1.000
icaC	0	–	–	–	–
icaD	0	–	–	–	–
SCC *mec* type					
I	7	1 (9.1)	1 (14.3)	5 (16.7)	0.634
II	9	2 (18.2)	0 (0)	7 (23.3)	
III	5	0 (0)	2 (28.6)	3 (10)	
IV	3	0 (0)	1 (14.3)	2 (6.7)	

E: Erythromycin, CC: Clindamycin, FOX: Cefoxitin, TE: Tetracycline, CP: Ciprofloxacin, SXT: Trimethoprim-Sulfamethoxazole, GM: Gentamicin.

resistance gene in 54.5 % (6/11), 71.4 % (5/7), and 56.7 % (17/30) of cases, respectively. However, no significant associations were observed between antibiotic resistance genes and biofilm production type ($p > 0.05$).

The most prevalent biofilm-associated gene was *icaA*, found in 27.3 % (3/11) of strong, 28.6 % (2/7) of moderate, and 36.7 % (11/30) of weak biofilm-forming isolates. However, no significant associations were observed between biofilm formation genes and the biofilm production type ($p > 0.05$). SCC*mec* type II was the most frequent among isolates with strong (18.2 %) and weak (23.3 %) biofilm formation, while SCC*mec* type III was most common in isolates with moderate biofilm formation (28.6 %). However, no significant associations were observed between SCC*mec* type and biofilm production type ($p > 0.05$).

3.8. RAPD-PCR analysis

RAPD-PCR analysis of the 24 MRSA *S. aureus* isolates revealed that the isolates could be categorized into seven distinct clusters: A (5 isolates), B (4 isolates), C (4 isolates), D (3 isolates), E (3 isolates), F (2 isolates), and G (2 isolates). One additional isolate did not cluster with others, indicating a unique genotype. The presence of multiple genetic clusters suggests that several distinct clones of MRSA were circulating within the hospital environment at the time of the study (Fig. 1).

4. Discussion

Cockroaches are notorious pests due to their nocturnal and unsanitary habits. Through excessive fecal deposition, they contaminate food and spread bacteria and other pathogenic microorganisms in the environment. Cockroaches are prevalent in human dwellings, particularly those that store, process, prepare, or serve food. They can also be found in hospitals in wards, operating rooms, intensive care units, and laboratories. Their omnivorous nature enables them to carry pathogenic bacteria, including *S. aureus*, notorious for its malignancy and resistance [32]. *S. aureus* is a highly successful opportunistic pathogen capable of colonizing humans and animals' skin and mucous membranes. Its repertoire of virulence factors and possession of antibiotic-resistance genes render it one of the most significant pathogens acquired in hospitals [33].

Isolates from the external surface of cockroaches likely represent transiently colonizing or contaminating bacteria picked up from the environment. These bacteria may not establish long-term colonization but can still be transferred to surfaces, medical equipment, or food in hospital settings. In contrast, isolates from the internal surface (digestive tract) suggest more permanent colonization, where the bacteria survive within the cockroach and can be spread through their secretions or feces, indicating a potential for more prolonged dissemination.

This study identified *S. aureus* in 50 of the 386 cockroaches collected from the hospital environment. Among these, 48 % of the isolates were confirmed as MRSA, highlighting the significant role of cockroaches in harboring antibiotic-resistant bacteria. A study by Kassiri et al. at Vali-e-Asr Hospital in Khorramshahr, Iran, reported that *S. aureus* was identified in 2 out of 20 collected cockroach isolates (10 %) [34]. The findings of the current study, showing a 13 % contamination rate with *S. aureus*, align with Kassiri's results.

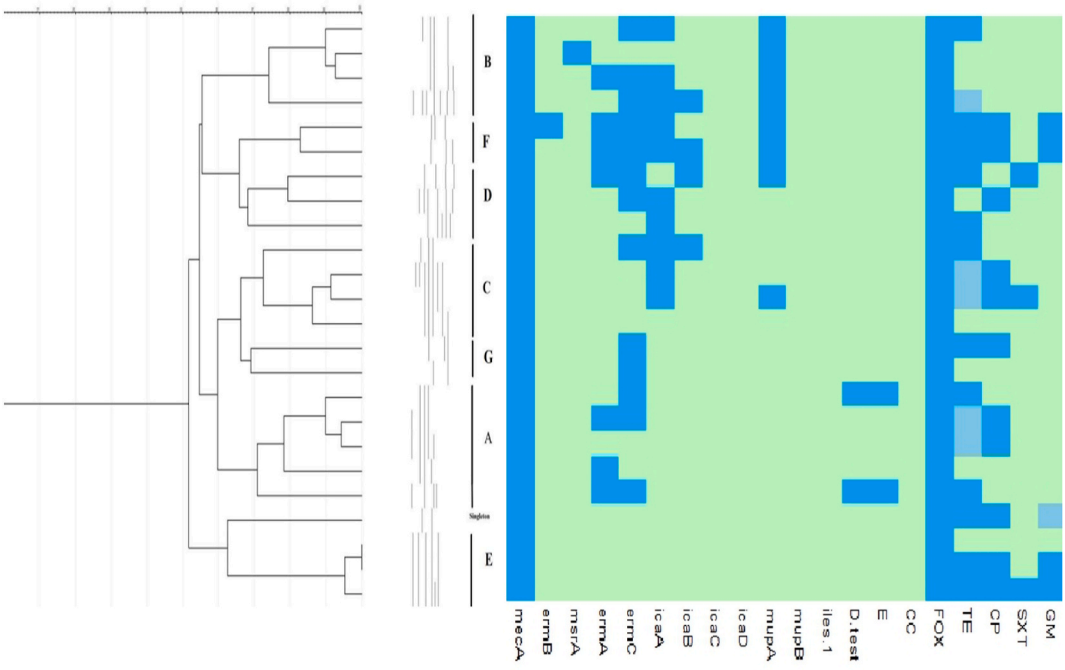

Fig. 1. RAPD-PCR dendrogram of MRSA isolates from cockroaches.

However, the total number of samples collected from cockroaches in the latter study was significantly lower. Heidari et al. in Shahrekord found that out of 100 collected cockroach samples, 44 isolates were contaminated with *S. aureus*, of which 8 (18.18 %) were contaminated with MRSA [35]. In a cross-sectional study by Abdolmaleki et al., conducted among 530 *P. americana* and *B. germanica* cockroaches at tertiary hospitals in Tehran Province, Iran, the prevalence of MRSA was reported to be 52.7 % and 43.3 %, respectively. External wash samples of *P. americana* cockroaches showed the highest prevalence of MRSA isolates at 57.6 % [22] The current study, with 48 % MRSA isolates, shares similarities with the study by Abdolmaleki et al. However, unlike the study by Abdolmaleki et al., the current study found the highest percentage of MRSA in isolates derived from the internal surface.

In this study, 6 % of the isolates were D-test positive, and 48 % were resistant to methicillin. Hashemi et al. in Tehran found that, out of 80 *S. aureus* isolates from patients hospitalized in Tehran, 70 % were resistant to erythromycin and 45 % to clindamycin. The D-Zone test identified 15 positive samples, differing from the current study. These discrepancies could be due to variations in the source of isolates, sampling locations, and the origins of the isolates [36]. Furthermore, a study by Adhikari et al. on 270 clinical samples from the Microbiology Laboratory of Nepal Medical College and Teaching Hospital in Kathmandu, Nepal, found that 25.1 % of *S. aureus* isolates were methicillin-resistant, with 54.4 % resistant to erythromycin and 41.8 % to clindamycin. The resistance to erythromycin and clindamycin in MSSA compared to MRSA was higher, which contrasts with the current study's findings, potentially due to differences in sample origin, sampling timeframe, geographic area, and sample size [37]. Abdolmaleki et al. also investigated the antibiotic resistance pattern among MRSA isolates, revealing the highest frequency of resistance against penicillin (100 %), ceftriaxone (100 %), tetracycline (100 %), gentamicin (83.33 %), and trimethoprim-sulfamethoxazole (80.55 %). MRSA isolated from internal gut samples have the highest frequency of resistance against penicillin (100 %), ceftriaxone (100 %), tetracycline (100 %), trimethoprim-sulfamethoxazole (80 %), and gentamicin (73.33 %). However, in the current study, of the 50 isolates examined, resistance to ciprofloxacin, trimethoprim-sulfamethoxazole, tetracycline, gentamicin, cefoxitin, erythromycin, and clindamycin was 32 %, 10 %, 36 %, 2 %, 48 %, 6 %, and 0 %, respectively. The differences between these studies may be attributed to Abdolmaleki's focus on MRSA isolates, whereas the current study included both MRSA and MSSA.

In research by Ebrahimzadeh et al. on 40 *S. aureus* isolated from patients at Motahari Hospital, it was determined that 100 % of the isolates carried the *mecA* gene, contrasting with 48 % in the current study [38]. This discrepancy could stem from a smaller sample size than the current study and differences in sample origin and geographical conditions. The current study reported the frequency of *ermA*, *ermB*, *ermC*, and *msrA* genes as 18 %, 16 %, 58 %, and 4 %, respectively. Furthermore, the frequency of *icaA*, *icaB*, *icaC*, and *icaD* genes was 34 %, 8 %, 0 %, and 0 %, respectively. Additionally, the prevalence of mupirocin resistance genes, including *mupA*, *mupB*, and *iles-1*, was reported as 20 %, 0 %, and 0 %, respectively. Since this study is the first to examine antibiotic resistance genes in isolates derived from cockroaches, no previous studies are available for comparison. Therefore, comparisons were made with studies conducted on clinical isolates. A study by Haddadi et al. in Shiraz reported that among 120 MRSA isolates, 45.8 % harbored the *mupA* gene, compared to 20 % in the current study [39]. This difference could be due to variations in sampling conditions, the origin of samples, and geographic location.

Hashemi et al. in Iran indicated among 80 clinical isolates, *ermC*, *ermB*, and *ermA* genes were detected in 8 (10 %), 6 (7.5 %), and 4 (5 %) isolates respectively. In contrast, the current study found the prevalence of *ermA*, *ermB*, *ermC*, and *msrA* genes to be 18 %, 16 %, 58 %, and 4 %, respectively [36]. The discrepancies could be due to the type of sample origin, the year of sampling, and geographic location.

In research by Noorbakhsh et al. in Iran, among 250 *S. aureus* isolates derived from various hospital infections, the presence of *icaA*, *icaB*, *icaC*, and *icaD* genes was reported as 58.3 %, 63.2 %, 67.3 %, and 59.4 % [40], respectively. The difference in findings could be attributed to the samples' origin, the sampling year, and geographic conditions.

The current study determined that the prevalence of strong, moderate, weak, and no biofilm formation was 22 %, 14 %, 60 %, and 4 %, respectively. A study by Mansouri et al. reported that out of 80 *S. aureus* isolates from 502 milk samples collected from bovines with subclinical mastitis in Boyerahmad and Dena townships, Iran, 68.7 % were capable of forming biofilms [41]. However, the current study's results indicated that out of 50 isolates, 48 (96 %) could form biofilms. This discrepancy may be attributed to the origin of the sample. In a study by Arbab Soleimani et al., 80 *S. aureus* isolates were identified based on biochemical tests in 100 wound samples collected from hospitals in Shahroud, Iran. According to the results examining resistance to the antibiotic methicillin, 65 samples were resistant, of which 66.6 % could produce robust biofilms, and the remaining 33.3 % had a moderate capability to form biofilms [42]. However, the current study reported that 22 % could form robust biofilms, and 14 % had a moderate ability to form biofilms. The difference could be due to the sample's origin, geographic conditions, and size.

In the study, the frequency percentages for SCC *mec* types I, II, III, IV, and untypable were 29.1 %, 16.6 %, 12.5 %, 8.3 %, and 33.5 %, respectively. The prevalence of SCC *mec* types I, II, and III, accounting for 58.2 % of the isolates, suggests an association with community-acquired infections. A study by Alagely et al. analyzed 143 clinical *S. aureus* isolates obtained from patients in various hospitals in Baghdad, all of which carried the *mecA* gene. SCC *mec* typing revealed the following distribution: Type I (23.7 %), Type II (5.8 %), Type III (16 %), Type IV (38.6 %), and Type V (62.7 %), with the highest percentage attributed to Type V [43]. The difference in results could be due to the sample origin, a higher sample volume than the current study, and the easy transmission of genes through mobile genetic elements like plasmids and transposons.

This study found that cefoxitin resistance rates varied across biofilm-producing categories, with resistance rates of 36.4 % in strong biofilm producers, 42.9 % in moderate producers, and 53.3 % in weak producers. Comparatively, Gaire et al., in a study of different clinical specimens at the Microbiology laboratory of Sukraraj Tropical and Infectious Disease Hospital, found that moderate biofilm producers had an 85.7 % resistance rate to cefoxitin, suggesting that while some studies report high resistance across all biofilm types, others indicate a more pronounced resistance in specific categories of biofilm producers [44]. This study did not observe significant associations between antibiotic-resistance genes and biofilm production types. However, in the study by Sun et al. in China,

ermA-positive strains were associated with medium to strong biofilm formation in erythromycin-resistant MRSA. In contrast, no significant relationship between *ermB*- and *ermC*-positive isolates and biofilm production type was identified [45].

In this study, the most prevalent biofilm-associated gene was *icaA*, detected in 27.3 % of strong, 28.6 % of moderate, and 36.7 % of weak biofilm-forming isolates. However, no significant association was observed between biofilm-associated genes and the degree of biofilm production. In contrast, the study by Piechota et al. found that *S. aureus* isolates from hospitalized patients in Poland with *icaABCD* or *icaABD* produced significantly more biofilm than those with only *icaAD* among strong biofilm-forming strains. No statistically significant differences in biofilm production were observed in moderate or weak biofilm-producing strains carrying these genes [46].

In this study, SCC*mec* type II was the most prevalent among isolates with strong (18.2 %) and weak (23.3 %) biofilm formation. In comparison, SCC*mec* type III was most common among isolates with moderate biofilm formation (28.6 %). However, no significant associations were identified between SCC*mec* type and biofilm production level. In contrast, the study by Taherrirad et al. in Northern Iran reported that 71.8 % of SCC*mec* type III isolates exhibited biofilm production capabilities, and all isolates with other SCC*mec* types were biofilm producers, showing statistically significant differences [47]. Similarly, Naicker et al. in South Africa found that SCC*mec* type IV isolates produced the strongest biofilms (though only three isolates were tested), while the single SCC*mec* III isolates formed a weak biofilm [48].

This study used RAPD-PCR typing to classify 24 MRSA isolates into 7 RAPD types. The isolates were grouped into clusters A (5 isolates), B (4), C (4), D (3), E (3), F (2), and G (2), with one isolate not fitting into any category. Hakimi et al. Studied *S. aureus* isolates from food, bovine, and human sources using RAPD-PCR. From 208 isolates, a total of 57 polymorphic bands were generated. Accordingly, all samples were classified into 9 clusters (A to I) with over 80 % similarity. Some clusters contained isolates from a single source, like clusters A, B, C, E, and H, whereas others, like D, F, G, and I, included isolates from different sources. The results demonstrated that specific isolates, especially MRSA ones, are transmissible between different sources, highlighting the importance of strict hygiene practices to break the chain of transmission [49].

The genetic diversity observed among the *S. aureus* isolates, as indicated by RAPD-PCR, suggests that multiple distinct clones are circulating within the hospital environment. This implies that the hospital may have been exposed to several different sources of MRSA, possibly through staff, patients, or visitors, with cockroaches acting as carriers perpetuating these resistant strains' spread. The presence of MRSA on cockroaches' external and internal surfaces further supports the idea that these insects can transfer bacteria across different areas of the hospital, potentially contaminating surfaces, medical equipment, or food.

5. Conclusion

This study indicates that cockroaches can significantly contribute to pathogenic bacteria transmission. Additionally, the resistance pattern profile of the isolates revealed that antibiotic resistance is relatively high in *S. aureus* isolated from cockroaches compared to other research studies. In conclusion, our study emphasizes the critical need for effective pest management strategies in hospitals to reduce the risk of MRSA transmission through cockroaches. Further research should focus on exploring the specific mechanisms by which cockroaches contribute to the spread of antibiotic-resistant bacteria and assessing the impact of targeted interventions on reducing this risk. Enhanced surveillance of cockroach populations in healthcare environments could also provide valuable data for mitigating the spread of resistant pathogens.

CRediT authorship contribution statement

Yasin Saberi: Software, Methodology, Investigation, Formal analysis. **Mehrdad Halaji:** Validation, Software, Formal analysis, Data curation. **Mohsen Karami:** Writing – review & editing, Visualization, Conceptualization. **Jalal Jafarzadeh:** Methodology, Investigation. **Kasra Javadi:** Writing – review & editing, Writing – original draft. **Hoda Shirafkan:** Visualization, Validation, Data curation. **Abazar Pournajaf:** Writing – review & editing, Supervision, Resources, Project administration.

Data availability statement

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Ethics and consent

The ethical committee of the Research Center at Babol University of Medical Science approved this study (accepted number: IR.MUBABOL.HRI.REC.1401.141).

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Declaration of Competing 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.

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