Original Article

Antimicrobials Resistance Profiling and Clonal Lineages of *Staphylococcus aureus* Isolated from Cockroaches in University-Affiliated Hospitals, 2023

Mohsen Karami[1], Mehdi Safari[2], Yasin Saberi[1], Seyedeh Zohre Mirbagheri[1,3], Mehrdad Halaji[1], Jalal Jafarzadeh[1], *Abazar Pournajaf[1]

[1]Infectious Diseases and Tropical Medicine Research Center, Health Research Institute, Babol University of Medical Sciences, Babol, Iran
[2]Student Research Committee, Babol University of Medical Sciences, Babol, Iran
[3]Cancer Research Center, Health Research Institute, Babol University of Medical Sciences, Babol, Iran

***Corresponding author:** Dr Abazar Pournajaf, E-mail: abazar_pournajaf@yahoo.com

(Received 08 Sep 2024; accepted 18 Mar 2025)

Abstract

Background: Cockroaches, recognized as mechanical vectors, play a crucial role in transmitting microbial pathogens. *Staphylococcus aureus* (*S. aureus*), particularly antibiotic-resistant strains, poses a significant threat as a nosocomial pathogen. This study aimed to investigate the resistance profiles to gentamicin, vancomycin, and antiseptics in *S. aureus* strains isolated from cockroaches in hospitals affiliated with Babol University of Medical Sciences.

Methods: In this cross-sectional study, 60 *S. aureus* strains were isolated from 376 cockroaches in three university-affiliated hospitals. Antibiotic susceptibility to gentamicin and vancomycin was tested by disk diffusion and agar dilution. PCR was used to detect resistance and antiseptic genes, and MLVA typing determined the genetic relatedness of resistant isolates.

Results: Among the 60 bacterial isolates, 46.7% (28) displayed resistance to gentamicin. The frequencies of aminoglycoside resistance coding genes (*AMEs*) for the *aac*(6')-*Ie*+*aph*(2″), *ant*(4')-*Ia*, *aph*(3')-*IIIa* and *ant*(6)-*Ia* genes were 64.3%, 42.8%, 17.8%, and 46.4%, respectively. Only 3.3% (2 isolates) exhibited vancomycin resistance, with one isolate (1.7%) carrying the *vanA* gene. The frequencies of genes encoding the antiseptic resistance genes *qacA/B*, *qacC*, *qacD*, *psmA*, *sasX*, and *smr* were 5%, 20%, 18.3%, 26.4%, 1.2%, and 31.7%, respectively. Analysis of *agr* gene types showed that *agr type I* was the most prevalent. In addition, the multiple-locus variable number tandem-repeat analysis (MLVA) identified 29 unique type sequences among the identified antibiotic-resistant isolates.

Conclusion: The high genetic diversity among antibiotic-resistant *S. aureus* isolates, as revealed by MLVA, underscores the importance of controlling hospital cockroach populations to curb the spread of antibiotic resistance.

Keywords: Gentamicin; Vancomycin; Molecular typing; Cross resistant, MLVA

Introduction

Staphylococcus aureus is a prominent pathogen associated with a spectrum of diseases, ranging from skin infections to life-threatening conditions such as pneumonia, endocarditis, and bacteremia (1). Over the past few decades, a worrisome surge in the prevalence of pathogens and the emergence of antibiotic-resistant strains has been observed in severe infections (2).

Aminoglycoside antibiotics remain pivotal for managing severe staphylococcal infections, despite global reports of increasing resistance to these drugs (3). As potent bactericidal agents, aminoglycosides exert their effects by binding to the 30S ribosomal subunit, thereby inhibiting protein synthesis (4). Notably, gentamicin and tobramycin exhibit high effectiveness against staphylococci and are often used in conjunction with a β-lactam or glycopeptide, particularly in treating staphylococcal endocarditis, due to their synergistic effects (4). Gentamicin was chosen for this study because it is

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Published Online: March 31, 2025

widely used in clinical practice and surveillance programs as the sentinel aminoglycoside for monitoring resistance trends. In addition, gentamicin resistance genes, such as those encoding aminoglycoside-modifying enzymes, are frequently located on mobile genetic elements that also carry genes conferring reduced susceptibility to disinfectants (e.g., qac efflux pumps), creating a potential for co-selection. Tobramycin, while clinically important, is less widely used in general surveillance and does not serve as a standard marker for global resistance monitoring, which makes gentamicin the more informative choice for investigation.

On the other hand, global concerns have arisen due to the emergence of vancomycin-intermediate and vancomycin-resistant *S. aureus* (VISA and VRSA) strains (5). The increasing prevalence of vancomycin resistance in *S. aureus* is now a significant issue and has been reported in various parts of the world (5). The rationale for including vancomycin in this study is directly linked to these global threats: understanding whether strains carrying QAC resistance genes also exhibit vancomycin resistance could reveal potential co-selection mechanisms and inform infection control strategies in hospital settings. Disinfection and decolonization efforts within hospitals may be compromised due to potential resistance to antibiotics and antiseptic agents, contributing to an escalation in the severity of staphylococcal infections and the dissemination of related clinical and epidemiological challenges.

Studies have consistently demonstrated that employing infection control management strategies, including the disinfection of colonized body parts using agents such as chlorhexidine and quaternary ammonium compounds (QACs), effectively reduces the risk of invasive nosocomial infections (6, 7). However, reduced susceptibility to QACs in *S. aureus* strains is associated with common genes such as *qacA*, *qacB*, *qacC*, *qacD* and *smr* (8). Moreover, various mechanisms contribute to the survival and transmission of microorganisms in the environment (9).

Cockroaches, which are prevalent in industrial and residential settings, including hospitals, are among the most common insects. Not only are they persistent pests, but their omnivorous feeding habits and indiscriminate defecation make them notorious for harboring, transmitting, and spreading human pathogens, thereby posing a significant risk to public health (9). More than 100 species of resistant microbial pathogens are carried by cockroaches (10).

Microbiological and epidemiological investigations have indicated that *S. aureus* is commonly found in *Periplaneta americana* and *Blattella germanica*, which are prevalent in public spaces, particularly in hospital environments (11). The transmission of *S. aureus* isolates between humans and animals has gained recent attention. However, there is limited understanding of the broader global diversity of *S. aureus* isolates originating from insects. This knowledge gap hinders our ability to pinpoint the origin of strains responsible for human infections. To implement effective control measures targeting reservoirs and transmission routes, it is imperative to enhance our understanding of *S. aureus* associated with cockroaches (12).

The agr operon, which consists of the *agrA*, *agrB*, *agrC* and *agrD* genes, is crucial for regulating over 70 genes in *S. aureus*, 23 of which are specifically involved in the bacterium's pathogenicity and its capacity to cause invasive infections. Additionally, *S. aureus* can be classified into four distinct groups: agr type I, agr type II, agr type III and agr type IV. It is proposed that agr types have unique characteristics and are found in varying prevalence across different geographical areas. Therefore, recognizing the dominant types in each particular region is practically beneficial (13).

In addition, a valuable source of genetic polymorphisms lies in tandem repeat sequences known as variable numbers of tandem repeats (VNTRs). These sequences exhibit varying repeat numbers at different loci and al-

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leles, offering insights into genetic diversity (14). Molecular typing, employing multilocus VNTR analysis (MLVA), involves the analysis of repeat copy numbers at multiple VNTR loci. This genotyping method is utilized for strain comparison and provides valuable insights into population structure (15).

Cockroaches are recognized as potential sources of diverse antibiotic-resistant bacteria that act as carriers for various diseases affecting human residents, particularly in public places such as hospitals (9). Therefore, the primary objective of this study was to identify gentamicin, vancomycin, and antiseptic resistance genes in *S. aureus* strains isolated from cockroaches within educational and therapeutic hospitals affiliated with Babol University of Medical Sciences. Specifically, we hypothesize that hospital-associated cockroaches carry *S. aureus* strains exhibiting co-resistance to critically important antibiotics and disinfectants.

Materials and Methods

This descriptive and analytical study, conducted in 2023 at Babol University of Medical Sciences, received ethical approval from the Ethics Committee of the university and was registered under the code IR.MUBABOL.HRI.REC.1402.013.

Sample Collection

A total of 376 cockroaches were collected from three hospitals: Shahid Beheshti (A), Ayatollah Rouhani (B), and Yahyainejad (C). Each cockroach was captured directly from its active site using manual methods and sterile gloves for each specimen and then transferred into separate sterile 50 ml Falcon tubes with lids. Subsequently, the live adult cockroaches were transported to the laboratory at room temperature for further analysis. Before washing, the adult cockroaches were immobilized in a refrigerator for 5 minutes. All specimens were identified to the species level using diagnostic keys (16).

Isolation and Identification of *Staphylococcus aureus*

Each cockroach was rinsed in 5 mL of sterile physiological serum for 20 seconds, followed by vortexing to dislodge microorganisms from the cuticle surface. The resulting solution was then serially diluted and plated onto appropriate blood agar media (Merck, Germany) for microbial examination.

For decontamination of the inner surface, the cockroaches were subjected to a 2-minute wash with 70% ethyl alcohol to remove surface contaminants. Afterward, the samples were immersed in sterile physiological serum for 2 to 3 minutes to eliminate residual alcohol.

To isolate bacteria from internal tissues, the digestive systems of the cockroaches were aseptically dissected and transferred into sterile physiological serum vials using sterile forceps and mechanically homogenized for 5 minutes. The resulting homogenates were then inoculated onto blood agar and mannitol salt agar plates, followed by incubation at 37 °C for 24 hours. Then, Gram staining was employed to evaluate cell morphology, followed by differential tests, including catalase, coagulase, and DNase assays, according to standard microbiological protocols. Positive results in these tests confirmed the presence of *S. aureus* (11, 17).

Gentamicin (GM) Resistance Testing

A 10-µg gentamicin disc (Condalab, Spain) was used to assess gentamicin resistance. *Staphylococcus aureus* was inoculated as a lawn on Mueller--Hinton agar media, with the bacterial concentration adjusted to 0.5 (1.5×10^8 CFU/mL) of the McFarland standard. After the incubation period (37 °C for 24 hours), a halo diameter of 12 mm or less was indicative of gentamicin-resistant *S. aureus* (18).

Determination of the Minimum Inhibitory Concentration (MIC) of Vancomycin

Vancomycin (Condalab, Spain) resistance was determined following M100-S22-CLSI standards (19). The agar dilution method was

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Published Online: March 31, 2025

employed using Brain Heart Infusion (BHI) agar medium supplemented with vancomycin at a concentration of 16 µg/mL. After the incubation period (37 °C for 24 hours), the growth of bacteria on this medium confirmed the resistance of *S. aureus* to vancomycin.

DNA Extraction

For the extraction of DNA from *S. aureus* strains, a Qiagen kit (QiaAmp DNA Mini Kit, Cat. No: 51304, Germany) was used. Isolated colonies were inoculated into LB broth culture medium (Merck, Germany, Cat Number: 11285) and incubated for 24 hours at 24 °C. The resulting culture was then centrifuged at 12,000 rpm.

Following the kit protocol, 200 µl of lysing buffer (for enhancing DNA purification) was added to the lysostaphin enzyme (Sigma, St Louis, USA)-treated samples, which were subsequently heated for 1 h at 37 °C. Subsequently, proteinase K was added, followed by the addition of cold absolute ethanol and centrifugation. After the addition of 70% ethanol and removal of the supernatant, the extracted DNA was precipitated.

Investigation of Gentamicin, Vancomycin, and Antiseptic Resistance Genes in *Staphylococcus aureus* Isolates

For the PCR analysis, a 25-microliter reaction mixture was prepared, consisting of 12 microliters of water and 8 microliters of Master Mix (Bioneer, South Korea) with a concentration of X 2 and 1 microliter each of forward and reverse primers (Table 1) provided by Sinaclon (Tehran, Iran) with a concentration of 10 pmol. Subsequently, 3 microliters of extracted DNA at a concentration of 50 nanograms was added to the mixture. The thermal cycling program for PCR included an initial denaturation step at 95 °C for 5 minutes, followed by 35 cycles of denaturation at 94 °C for 30 seconds, annealing at the appropriate temperature and time for each gene (Table 2), extension at 72 °C for 30 seconds, and a final extension cycle at 72 °C for 7 minutes. The *vanA* gene was amplified by PCR using specific primers, and the purified amplicons were sequenced using the Sanger sequencing method. The obtained nucleotide sequence was submitted to the GenBank database and assigned an accession number.

Following PCR, the resulting products were electrophoresed on a 1% agarose gel containing DNA safe stain (SinaClon, Iran) and visualized under a UV Tech system (England).

***Agr* Genotyping**

*Agr t*yping was performed by multiplex PCR to determine the *agr* allele types I to IV using agr group-specific primers, reaction mixtures, and amplification conditions as described by Gilot et al. (32). Finally, PCR products were analyzed by electrophoresis through a 1.5% agarose gel containing DNA safe stain (SinaClon, Iran) and visualized under a UV Tech system (England).

Molecular Typing of *Staphylococcus aureus* using the MLVA Method

The MLVA was applied to 29 antibiotic-resistant *S. aureus* isolates obtained from cockroaches. This method utilized 5 loci, as specified in Table 3 from a previous study (33). For the PCR assays, the reaction mixture consisted of 1 µL of each primer (Cinacloon, Iran) as outlined in Table 3, 8 µL of Master Mix with a 2X concentration (Bioneer, South Korea), 40 ng of extracted DNA, and distilled water up to a final volume of 25 µL using a specific thermal cycling program (33). PCR products were analyzed by electrophoresis through a 1% agarose gel containing DNA safe stain (SinaClon, Iran) and visualized under a UV Tech system (England). Additionally, manual reading was employed to calculate the number of replicates based on amplicon size.

To determine repeat values, the following formula was used: repeat number (bp) = PCR product size (bp) - flanking regions (bp)/repeat size (bp). Additionally, Simpson's diversity in-

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Published Online: March 31, 2025

dex (D) and the 95% confidence interval (CI) for each VNTR locus were calculated using PhyloViz software version 2.0 (http://www.phyloviz.net). A Hamming-distance-based phylogenetic tree was used to reveal the genetic distances between individuals with an overall parsimony score per site.

Statistical Analysis of Data

The results were analyzed using SPSS version 26 software. Descriptive statistics, including averages and percentages, were reported to summarize the evaluated data. The potential statistical relationships between the data were assessed using chi-square and Fisher's exact tests. A p-value$\leq$ 0.05 was considered to indicate statistical significance.

Results

A total of 376 cockroaches were collected from three university-affiliated hospitals in 2023. Morphological identification revealed two predominant species: *Periplaneta americana* (58.2 %) and *Blattella germanica* (41.8%). Among the collected specimens, 61.7% were female and 38.3% male; most were adults (72.6%), while immature nymphs comprised 27.4%. Bacteriological analysis confirmed 60 strains of *S. aureus* using phenotypic and biochemical methods. Of these isolates, 47 (78.3%) were recovered from internal organs and 13 (21.7%) from external body surfaces. The bacterial load varied between groups, with adult *P. americana* harboring higher loads on both niches compared to *B. germanica*. Furthermore, among the *S. aureus* strains, 46.7% (28 isolates) exhibited resistance to gentamicin. PCR revealed varying frequencies of aminoglycoside resistance coding genes (AMEs) *aac*(6')-*Ie*+*aph*(2''), *ant* (4')-*Ia*, *aph*(3')-*IIIa* and *ant* (6)-*Ia* with proportions of 64.3%, 42.8%, 17.8%, and 46.4%, respectively (Table 4). Comprehensive details related to isolates are presented in Table 5.

According to CLSI guidelines (version 2023), the vancomycin MIC interpretation criteria classified strains as susceptible (S) with MIC $\geq$ 2 µg/mL, semisusceptible (I) with MIC 4-8 µg/mL, and resistant (R) with MIC $\leq$ 15 µg/mL. Among the 60 isolates, only 2 strains (3.3%) exhibited vancomycin resistance. Molecular testing revealed the vanA gene in one isolate (1.7 %), while the vanB gene was not detected in any of the isolates. The nucleotide sequence of *vanA* was submitted to the GenBank database and assigned the accession number OR 947324.

PCR results for antiseptic resistance-encoding genes revealed frequencies of 5% for qacA/B, 20% for qacC, 18.3% for qacD, 26.4% for psmA, 1.2% for sasX, and 31.7% for smr. Analysis of agr gene types revealed that agr type I was the most prevalent (45%), followed by type II (20%), type IV (13.3%), and type III (8.3%). Notably, 8 isolates (13.3%) were nontypeable.

MLVA of 29 resistant isolates demonstrated high genetic diversity, revealing 5 clusters and 29 sequence types (STs) (Figs. 1 and 2). Among the markers, VNTR21_01 showed the highest genetic diversity, with a Simpson diversity index (D=0.926) among the 60 bacterial isolates.

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Published Online: March 31, 2025

Table 1. Sequences of primers used for tracking antibiotic resistance and antiseptic genes in *Staphylococcus aureus* isolates from cockroaches

Gene name	Primer name	Primer sequences (5′–3′)	Size (bp)	Source
vancomycin-resistant gene A	vanAf	GGCAAGTCAGGTGAAGATG	713	(20)
	vanAr	ATCAAGCGGTCAATCAGTTC		
vancomycin-resistant gene B	VanB F	GTGACAAACCGGAGGCGAGGA	433	(21)
	VanB R	CCGCCATCCTCCTGCAAAAAA		
aminoglycoside nucleoti-dyltransferase ANT (6)-Ia	ant(6)-Ia F	CGGGAGAATGGGAGACTTTG	563	(22)
	ant(6)-Ia R	CTGTGGCTCCACAATCTGAT		
aminoglycoside O-phos-photransferase APH (3')-IIIa	aph(3')-IIIa F	CTGATCGAAAAATACCGCT	354	(23)
	aph(3')-IIIa R	ACAATCCGATATGTCGATGGAG		
Aminoglycoside 4′-O-nucleotidyltransferase ANT (4′)-Ia	ant(4’)-Ia F	AATCGGTAGAAGCCCAA	135	(24)
	ant(4’)-Ia R	GCACCTGCCATTGCTA		
Aminoglycoside N-acetyltransferase [AAC(6′)] – Aminoglycoside O-phosphotransferase [APH(2″)	aac(6)-aph(2) F	GAGCAATAAGGGCATACCAAAAATC	505	(25)
	aac(6)-aph(2) R	CCGTGCAATTGTCTTAAAAAACTGG		
Quaternary Ammonium Compound Resistance genes A and B	qacA/B F	CTATGGCAATAGGAGATATGGTGT	416	(26)
	qacA/B R	CCACTACAGATTCTTCAGCTACATG		
Quaternary Ammonium Compound Resistance gene C	qacCF	AAACAATGCAACACCTACCACT	157	(27)
	qacCR	AACGAAACTACGCCGACTATG		
Quaternary Ammonium Compound Resistance gene D	qacD F	GCCATAAGTACTGAAGTTATTGGA	195	(28)
	qacD R	GACTACGGTTGTTAA-GACTAAAAACCT		
staphylococcal multidrug resistance	smr F	ATAAGTACTGAAGTTATTGGAAGT	286	(29)
	smr R	TTCCGAAAATGTTTAACGAAACTA		
Staphylococcus aureus surface pro-tein X	sasX F	ATTGAAGCTCAGACTCCTAG	123	(30)
	sasX R	GTTATCAGTTGTAGCAGTAGT		
Phenol-Soluble Modulin alpha	psmαF	TATCAAAAGCTTAATCGAACAATTC	176	(31)
	psmαR	CCCCTTCAAATAAGATGTTCATATC		

Table 2. PCR thermal cycling conditions used for amplification of antibiotic and antiseptic resistance genes

Resistance Gene	Initial Denaturation °C(min)[1cycle]	Denaturation °C (second)	Annealing °C (second)	Extension °C (second)	Final Extension °C (second) [1 cycle]
			[35 cycles]		
qacA/B	95 (5)	94 (30)	54 (30)	72 (30)	72 (30)
qacC	95 (5)	94 (30)	54 (30)	72 (30)	72 (30)
ant (6)	95 (5)	94 (30)	54(30)	72 (30)	72 (30)
psmα	95 (5)	94 (30)	51 (30)	72 (30)	72 (30)
Sasx	95 (5)	94 (30)	51(30)	72 (30)	72 (30)
VanA	95 (5)	94 (30)	52(45)	72 (30)	72 (30)
VanB	95 (5)	94 (30)	52 (45)	72 (30)	72 (30)
QacD	95 (5)	94 (30)	54 (30)	72 (30)	72 (30)
Smr	95 (5)	94 (30)	54(30)	72 (30)	72 (30)
aac6-aph2	95 (5)	94 (30)	55 (30)	72 (30)	72 (30)
aph3	95 (5)	94 (30)	60 (45)	72 (30)	72 (30)
ant4	95 (5)	94 (30)	53(45)	72 (30)	72 (30)

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Table 3. Primer sequences for multiple-locus variable number tandem-repeat analysis (MLVA) markers associated with *Staphylococcus aureus* (32)

VNTR name	primer sequence (5'–3')	PCR product size (bp)	Average repeat size (bp)	Range no. repeats
VNTR61_01	F-AATGCACATGAAACACTAATT R-GGTCAAGAATATTTAAAATCAATT	362	60	0-6
VNTR61_02	F-CTGTGAAGTTAGATAGATGAGTTT R- GCAATTAACGATTTCTTCAC	267	66	0-5
VNTR67_01	F-CGTGAATCTCTTTTATAAGAGTGT R-CCCTCCTATTAATATATATACCGT	345	67	0-10
VNTR21_01	F-GTCGATAAAGCATAAAGCTTT R-AGCAATGAATCAATAATTTTCA	149	21	0-13
VNTR63_01	F-TGAAGATGTAGTAGGAATGTTAGT R-AGAAAAAGCTAAAGAAGTTGAA	646	64	0-11

Table 4. Prevalence of aminoglycoside resistance genes in *Staphylococcus aureus* isolated from the internal and external surfaces of hospital cockroaches, Babol, 2024

Genes	Number of Isolates	Strain Percentage	P value*
ant*(6)-*Ia	13	46.4	0.01
aph*(3')-*IIIa	5	17.8	0.02
ant*(4')-*Ia	12	42.8	0.05
***aac*(6')-*Ie*+*aph*(2″)**	18	64.3	0.04
ant*(6)-*Ia*/*aph*(3')-*III a	1	3.6	0.78
ant*(6)-*Ia*/*ant*(4')-*Ia	5	17.8	0.23
***ant*(6)-*Ia*/*aac*(6')-*Ie*+*aph*(2″)**	7	0.25	0.01
aph*(3')-*IIIa*/*ant*(4')-*Ia	0	0.0	-
***aph*(3')-*IIIa*/*aac*(6')-*Ie*+*aph*(2″)**	2	7.4	0.45
***ant*(4')-*Ia*/*aac*(6')-*Ie*+*aph*(2″)**	8	28.6	0.01

*Because both variables are categorical, the Chi-square (χ^2) test was used to compare proportions. When the expected frequency in any cell was < 5, Fisher's exact test was applied instead

Table 5. Characteristics of cockroach isolates, including source, species, sex, life stage, resistance genes, and VNTR profiles

Isolate_ID	Source	Species	Sex	Life_Stage	Gene_Profile	VNTR61_01	VNTR61_02	VNTR67_01	VNTR21_01	VNTR63_01
57	External	*Blattella germanica*	Male	Immature	ant(6)-Ia	7	6	6	8	8
51	External	*Blattella germanica*	Male	Immature	ant(6)-Ia	10	1	1	8	8
40	Internal	*Blattella germanica*	Male	Adult	aac(6')-Ie+aph(2″)	3	7	3	8	3
38	Internal	*Blattella germanica*	Male	Adult	aac(6')-Ie+aph(2″)	4	8	5	5	9
30	Internal	*Periplaneta americana*	Female	Adult	ant(4')-Ia	4	1	3	4	3
56	External	*Blattella germanica*	Male	Immature	ant(6)-Ia	10	3	7	5	4
37	Internal	*Blattella germanica*	Female	Adult	aac(6')-Ie+aph(2″)	5	10	3	7	3
31	Internal	*Periplaneta americana*	Female	Adult	aac(6')-Ie+aph(2″)	9	5	4	4	7
34	Internal	*Periplaneta americana*	Female	Adult	aac(6')-Ie+aph(2″)	6	6	5	9	5

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Table 5. Continued ...

13	Internal	*Periplaneta americana*	Female	Adult	ant(6)-Ia	3	8	5	6	1
24	Internal	*Periplaneta americana*	Female	Adult	ant(4')-Ia	5	1	5	4	3
5	Internal	*Periplaneta americana*	Female	Adult	ant(6)-Ia	3	8	1	9	10
47	Internal	*Blattella germanica*	Male	Immature	aac(6')-Ie+aph(2″)	5	8	3	7	9
19	Internal	*Periplaneta americana*	Female	Adult	ant(4')-Ia	2	10	2	8	6
8	Internal	*Periplaneta americana*	Female	Adult	ant(6)-Ia	10	9	6	7	5
16	Internal	*Periplaneta americana*	Female	Adult	aph(3')-IIIa	8	8	3	3	8
17	Internal	*Periplaneta americana*	Female	Adult	aph(3')-IIIa	9	3	1	9	9
27	Internal	*Periplaneta americana*	Female	Adult	ant(4')-Ia	1	8	1	7	5
6	Internal	*Periplaneta americana*	Female	Adult	ant(6)-Ia	1	10	9	9	3
35	Internal	*Periplaneta americana*	Female	Adult	aac(6')-Ie+aph(2″)	10	10	8	10	8
1	Internal	*Periplaneta americana*	Female	Adult	ant(6)-Ia	10	7	4	9	4
26	Internal	*Periplaneta americana*	Female	Adult	ant(4')-Ia	4	6	2	5	2
25	Internal	*Periplaneta americana*	Female	Adult	ant(4')-Ia	5	7	3	2	6
58	External	*Blattella germanica*	Male	Immature	ant(6)-Ia	8	1	4	4	4
29	Internal	*Periplaneta americana*	Female	Adult	ant(4')-Ia	4	4	7	8	8
49	External	*Blattella germanica*	Male	Immature	ant(6)-Ia	4	8	10	2	9
46	Internal	*Blattella germanica*	Male	Immature	aac(6')-Ie+aph(2″)	7	4	1	4	7
22	Internal	*Periplaneta americana*	Female	Adult	ant(4')-Ia	7	5	9	1	10
52	External	*Blattella germanica*	Male	Immature	ant(6)-Ia	10	4	2	3	6
15	Internal	*Periplaneta americana*	Female	Adult	aph(3')-IIIa	8	10	10	8	6
48	External	*Blattella germanica*	Male	Immature	aac(6')-Ie+aph(2″)	2	10	8	4	7
21	Internal	*Periplaneta americana*	Female	Adult	ant(4')-Ia	5	2	1	4	1
42	Internal	*Blattella germanica*	Male	Adult	aac(6')-Ie+aph(2″)	4	5	4	8	6
23	Internal	*Periplaneta americana*	Female	Adult	ant(4')-Ia	6	5	4	8	5
11	Internal	*Periplaneta americana*	Female	Adult	ant(6)-Ia	6	6	1	10	5
55	External	*Blattella germanica*	Male	Immature	ant(6)-Ia	9	4	7	8	4
2	Internal	*Periplaneta americana*	Female	Adult	ant(6)-Ia	3	6	4	8	6
18	Internal	*Periplaneta americana*	Female	Adult	aph(3')-IIIa	5	4	4	4	3
39	Internal	*Blattella germanica*	Male	Adult	aac(6')-Ie+aph(2″)	6	8	1	2	10
4	Internal	*Periplaneta americana*	Female	Adult	ant(6)-Ia	6	9	8	7	5
12	Internal	*Periplaneta americana*	Female	Adult	ant(6)-Ia	8	3	6	5	9
10	Internal	*Periplaneta americana*	Female	Adult	ant(6)-Ia	8	2	1	10	1
60	External	*Blattella germanica*	Male	Immature	ant(6)-Ia	2	8	4	1	8
50	External	*Blattella germanica*	Male	Immature	ant(6)-Ia	5	5	10	6	6
44	Internal	*Blattella germanica*	Male	Adult	aac(6')-Ie+aph(2″)	3	5	7	3	6
59	External	*Blattella germanica*	Male	Immature	ant(6)-Ia	3	3	3	10	7
53	External	*Blattella germanica*	Male	Immature	ant(6)-Ia	4	1	1	8	7
14	Internal	*Periplaneta americana*	Female	Adult	aph(3')-IIIa	1	3	6	8	5
3	Internal	*Periplaneta americana*	Female	Adult	ant(6)-Ia	6	8	6	3	1
36	Internal	*Blattella germanica*	Female	Adult	aac(6')-Ie+aph(2″)	7	8	4	3	8
32	Internal	*Periplaneta americana*	Female	Adult	aac(6')-Ie+aph(2″)	2	2	5	10	5
41	Internal	*Blattella germanica*	Male	Adult	aac(6')-Ie+aph(2″)	7	3	5	10	9
54	External	*Blattella germanica*	Male	Immature	ant(6)-Ia	7	7	3	6	9
7	Internal	*Periplaneta americana*	Female	Adult	ant(6)-Ia	3	1	7	10	10
28	Internal	*Periplaneta americana*	Female	Adult	ant(4')-Ia	9	10	8	6	2
45	Internal	*Blattella germanica*	Male	Immature	aac(6')-Ie+aph(2″)	6	4	10	7	10
9	Internal	*Periplaneta americana*	Female	Adult	ant(6)-Ia	9	9	2	1	1
20	Internal	*Periplaneta americana*	Female	Adult	ant(4')-Ia	7	4	3	4	9
43	Internal	*Blattella germanica*	Male	Adult	aac(6')-Ie+aph(2″)	6	8	4	1	8
33	Internal	*Periplaneta americana*	Female	Adult	aac(6')-Ie+aph(2″)	7	8	6	9	2

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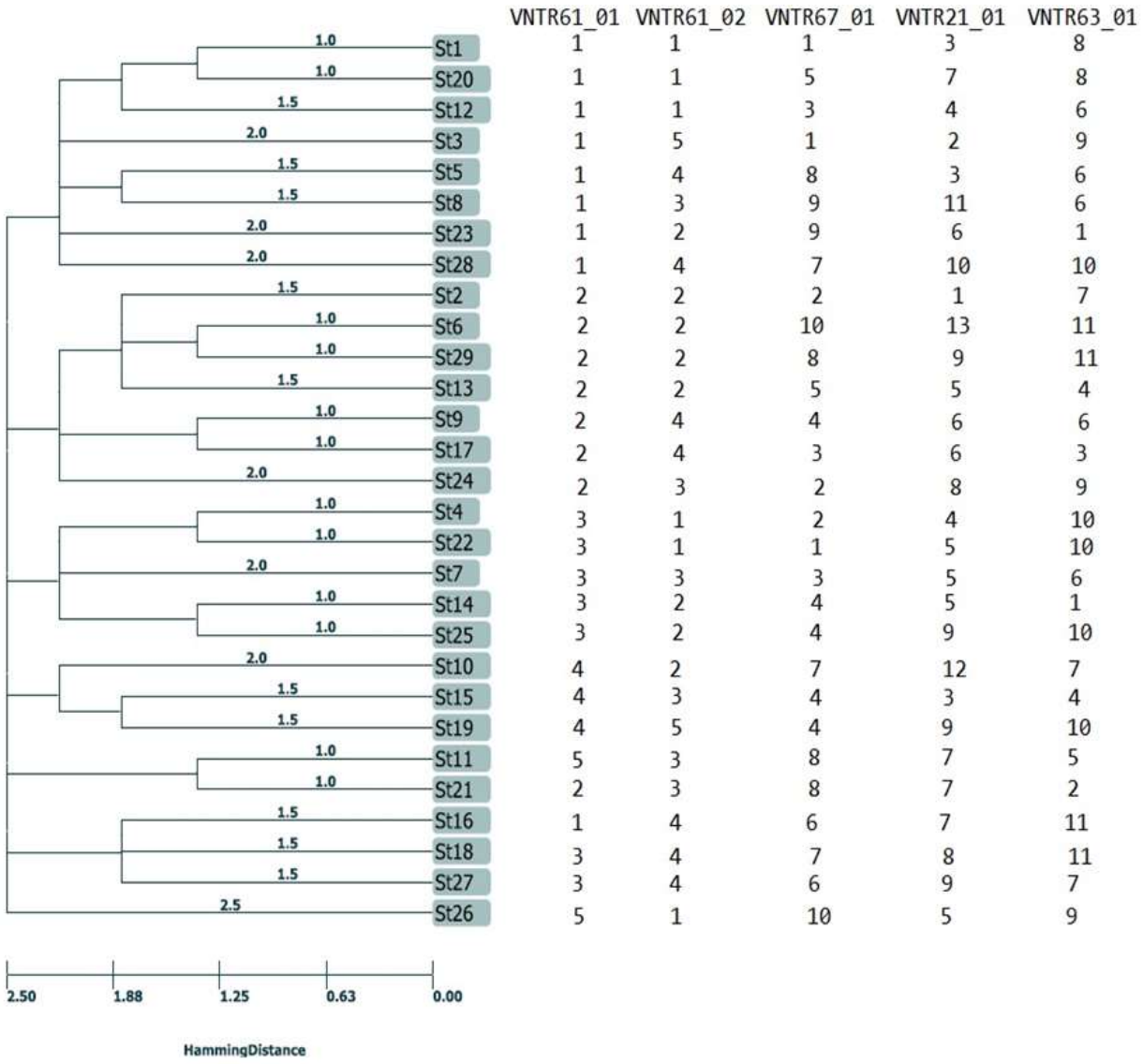

Fig. 1. Phylogenetic relationship of 29 *Staphylococcus aureus* isolates from hospital cockroaches in Babol, 2024. The tree was built using [PhyloViz 2 Software] based on VNTR profiles (VNTR61_01, VNTR61_02, VNTR67_01, VNTR21_01, VNTR63_01). The scale bar represents Hamming distance. The numbers in the columns indicate the number of repeat units (alleles) detected at each VNTR locus for each isolate

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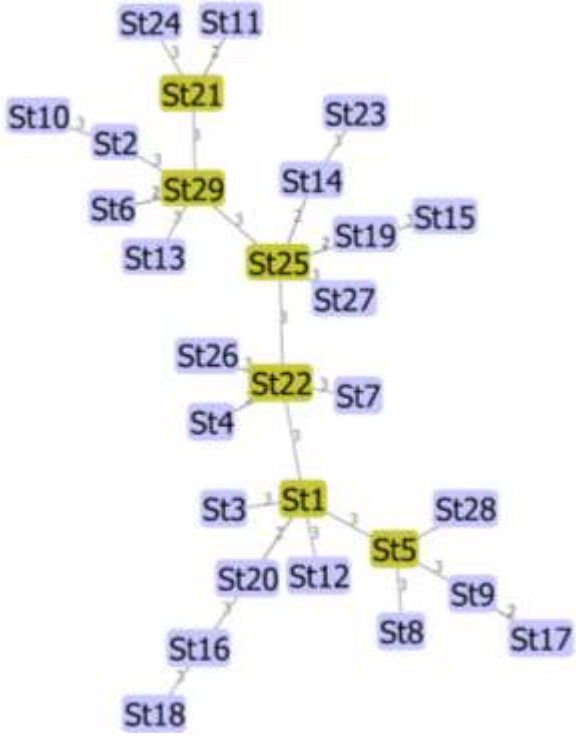

Fig. 2. Genetic relatedness based on MLVA of 29 *Staphylococcus aureus* isolates from hospital cockroaches, Babol, 2024. The tree was constructed using the [PhyloViz] method based on a Multiple-Locus Variable-number tandem repeat Analysis (MLVA) scheme targeting the following VNTR loci: [VNTR61_01], [VNTR61_02], [VNTR67_01], [VNTR21_01], and [VNTR63_01]. The numbers on the branches represent the number of allele differences between isolates. The proximity of lines indicates genetic similarity, with closer distances signifying greater similarity between isolates

Discussion

Cockroaches, notorious for their nocturnal habits and unsanitary behaviors, are recognized as significant pests due to their potential role in spreading pathogens, including *S. aureus.* Cockroaches indiscriminately deposit fecal material, contaminating food and the environment, thereby posing risks of food poisoning and acting as vectors for various bacteria and microorganisms (9). Particularly prevalent in human settlements, cockroaches are commonly found in areas associated with food storage, processing, and preparation, as well as in critical hospital environments such as wards, operating rooms, and intensive care units (34, 35).

The present investigation revealed that 12.5% of samples derived from external washings and 3.45% of intestinal samples procured from hospital cockroaches were positive for *S. aureus* strains. Conversely, a study conducted by Abdolmaleki et al. in 2019 demonstrated positivity rates of 12.26% and 98.6% in external washing and intestinal samples, respectively, among hospital cockroaches. The prevalence of *S. aureus* strains in *P. americana* and *B. germanica* hospital cockroaches was 11.61% (72 out of 620) and 6.81% (30 out of 440), respectively (11). Globally, prevalence rates of *S. aureus* in cockroach samples from countries such as Bangladesh, Iran, Nigeria, Ethiopia, Algeria, Brazil, and China have ranged from 7.7% to 69.2%(17, 36-41).

Among the 376 cockroaches collected across three hospital centers, 60 isolates of *S. aureus* (15.95%) were identified. Among these, 28 isolates (46.7%) demonstrated resistance to gentamicin, and 2 isolates exhibited resistance to vancomycin. Islam et al. (2016) reported a 23% prevalence of kanamycin resistance in *S. aureus* strains isolated from cockroach samples (17). In addition, Fowoyo and Ogunbanwo (41) reported a substantial prevalence of resistance to gentamicin among *S. aureus* strains, reaching 11.40%.

Rong et al. (42) reported the prevalence of antibiotic resistance in *S. aureus* strains, with rates of 4.20%, 6.70%, and 0% observed for amikacin, gentamicin, and vancomycin, respectively. Heidari et al. (43) conducted a study on *S. aureus* isolated from cockroaches in Chaharmahal and Bakhtiari hospitals and reported that 44% of 100 samples were infected, with methicillin-resistant *Staphylococcus aureus* (MRSA) accounting for 18.18% of those cases. MRSA strains exhibited significant resistance to methicillin (100%), cefixime (87.5%), and vancomycin (75%). Menasria et al. (38) reported from Algeria that 13 out of 21 isolates of the genus *Staphylococcus* were identified

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as *S. aureus* from cockroaches, with most of these isolates demonstrating sensitivity to various antibiotics, including chloramphenicol, gentamicin, pristinamycin, ofloxacin, clindamycin, and vancomycin.

However, in contrast to the findings of the present study, nearly half of the isolates in the mentioned study exhibited resistance to gentamicin. Nevertheless, the results regarding vancomycin sensitivity were notably similar to those observed in our study. Notably, no study on aminoglycoside resistance genes in cockroaches has been conducted. However, in an in vivo investigation involving the cockroach species *Pycnoscelus surinamensis*, researchers explored the transfer of antimicrobial resistance. The study revealed that treated microbiomes could lead to the transmission and emergence of antimicrobial resistance in populations not initially exposed to antimicrobial agents (39).

The current study is the first to investigate aminoglycoside resistance genes (AMEs) in hospital cockroaches. The prevalence of AMEs–*aac*(6')-*Ie+aph*(2″), *ant*(4')-*Ia*, *aph*(3')-*IIIa* and *ant*(6)-*Ia*–was 64.3%, 42.8%, 17.8% and 46.4%, respectively. The high frequency of *aac*(6')-*Ie+aph*(2″), conferring resistance to a broad range of aminoglycosides, is clinically significant, as it critically limits therapeutic options for potential staphylococcal infections. Sabzehali et al. (44) reported slightly higher frequencies of *ant*(4')-*Ia* (89%), *aac*(6')/*aph*(2'') (84.5%), and *aph*(3')-*IIIa* (66%), which may reflect demographic and geographic variations.

Regarding quaternary ammonium compound (QAC) resistance genes, the frequencies of the *qacA/B*, *qacC*, *qacD* and *smr* genes in the *S. aureus* isolates from the present study were 5%, 20%, 18.3%, and 31.7%, respectively. In a study by Zaki et al. (6), quaternary ammonium compound (QAC) resistance genes were identified in 13 *S. aureus* isolates, with *qacA/B* (22.2%), *qacJ* (22.2%), *smr* (17.8%), *qacG* (17.8 %) and *qacH* (6.7%) being the most prevalent. Prevalence of antiseptic resistance genes varies globally, ranging from 10% to 80% (42, 43).

In the present study, the prevalent agr types in *S. aureus* isolated from cockroaches were distributed as agr type I (45%), type II (20%), type IV (13.3%), and type III (8.3%).

In contrast, Bibalan and colleagues (47) observed different proportions of agr group genes in *S. aureus* isolates from healthy individuals, patients, and food, with the agr group I gene being the most abundant (43.3%), followed by agr group III (28.87%), agr group II (22.68 %), and agr group IV (5.15%).

Furthermore, for epidemiological investigations, variations in the number of short tandem repeat sequences in MLVA are utilized to generate DNA profiles (44).

Different MLVA schemes have been devised for typing *S. aureus* in various studies (45). Analysis of 29 antibiotic-resistant isolates in the current study using MLVA revealed 5 clusters and 29 STs. Schouls et al. (2009) conducted MLVA on 1681 *S. aureus* isolates from Dutch patients and 100 isolates from pigs and identified 511 typing sequences distributed across 11 clusters. Discrepancies in results may arise due to factors such as geographical location, sample size, marker quantity, and isolate origin (46).

To further explore potential associations, the 29 antibiotic-resistant *S. aureus* isolates were analyzed for correlations between MLVA clusters and resistance profiles, QAC resistance genes, and agr types. Statistical analysis revealed that certain MLVA clusters were predominantly associated with specific antibiotic resistance patterns and agr types. For instance, isolates in Cluster 1 showed a higher frequency of gentamicin resistance and harbored the *aac* (6')-*Ie+aph*(2'') gene, whereas Cluster 3 included isolates with elevated prevalence of QAC resistance genes (*qacC* and *smr*). However, no statistically significant correlation was observed between MLVA clusters and vancomycin resistance. Similarly, agr type I was most frequent in Cluster 2, while agr type IV appeared exclusively in Cluster 5. These findings suggest that while some genetic clusters may

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preferentially harbor certain resistance determinants, resistance traits, and agr types are not strictly confined to specific clusters, highlighting the genetic diversity and potential for horizontal gene transfer among *S. aureus* populations associated with hospital cockroaches.

Research on *S. aureus* carried by cockroaches in hospital environments is critical for controlling nosocomial infections, which contribute to higher morbidity, mortality, prolonged hospital stays, and increased healthcare costs. Cockroaches' ability to traverse diverse hospital areas facilitates pathogen dissemination, and their persistence is promoted by environmental factors such as food availability and structural deficiencies. These findings underscore the urgent need for effective and integrated pest management systems in healthcare facilities to mitigate the risks posed by these resilient vectors.

Conclusion

In general, cockroaches, prevalent across diverse geographical regions and commonly found in hospital environments, act as mechanical vectors for the transmission of *S. aureus*. Research focusing on the resistance of *S. aureus* to gentamicin, vancomycin, and quaternary ammonium compound (QAC) antiseptics transported by cockroaches highlights their role in the dissemination of nosocomial pathogens, which contribute to increased morbidity, mortality, prolonged hospital stays, and higher healthcare costs. MLVA analysis revealed significant genetic diversity among antibiotic-resistant isolates, with certain clusters showing correlations with specific antibiotic resistance profiles, *QAC* resistance genes, and agr types. These findings underscore the urgent need for effective cockroach control and integrated pest management strategies in hospital settings to mitigate the spread of resistant *S. aureus* strains.

Acknowledgements

The authors would like to thank all their colleagues at the Infectious Diseases and Tropical Medicine Research Center, Health Research Institute, Babol University of Medical Sciences, Babol, Iran, for their cooperation in this study. The authors of this study are also grateful for the kind support of Shahid Beheshti, Ayatollah Rouhani, and Yahainejad hospitals, Babol University of Medical Sciences, Babol, Iran. This study was funded by the Infectious Diseases and Tropical Medicine Research Center, Health Research Institute, Babol University of Medical Sciences, Babol, Iran.

Ethical considerations

This study was approved by the ethical committee of the Research Center of Babol University of Medical Science (accepted Number, IR. MUBABOL.HRI.REC.1402.013).

Conflict of interest

The authors declare there is no conflict of interest.

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