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Frequency of Antiseptic Resistance Among *Staphylococcus aureus* and Coagulase-Negative Staphylococci Isolated From a University Hospital in Central Iran

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ABSTRACT

Objectives: Reduced biocide susceptibility in Staphylococci is associated with various antiseptic resistance genes encoding efflux systems. Our aim was to determine the susceptibility to three disinfectant agents, including benzalkonium chloride (BAC), benzethonium chloride (BZT), and chlorhexidine digluconate (CHDG) among clinical isolates of *Staphylococcus aureus* and coagulase-negative Staphylococci (CoNS). ***Methods***: The minimum inhibitory concentration (MIC) of 60 methicillin-resistant *S. aureus* (MRSA), 54 methicillin-sensitive *S. aureus* (MSSA) and 51 CoNS isolates from a single hospital to three biocidal agents (BAC, BZT, and CHDG) was determined. Biocide resistance genes (*qacA/B, smr, qacG, qacH, qacJ, and norA*) were analyzed by the polymerase chain reaction assay. ***Results:*** All isolates had MICs for BAC and BZT from 0.25 to 8 µg/mL, and for CHDG from 0.5 to 64 µg/mL. *qacA/B* was the most common biocide resistance gene among all 165 Staphylococcus isolates (76; 46%), which comprised 38 (63.3%) MRSA, 14 (25.9%) MSSA, and 24 (47%) CoNS. Eleven (6.7%) and 24 (14.5%) isolates among the 165 Staphylococci carried *smr* and *norA* genes, respectively. In contrast, other resistance genes such as *qacG*, *qacH*, and *qacJ* were absent in all Staphylococci studied. The *qacA/B* and *smr* genes were detected concomitantly in 3% of isolates, and 23.6% strains of the total 165 Staphylococcus isolates were negative for each studied gene. ***Conclusions:*** The carriage of several biocide resistance genes, including *qacA/B*, *smr*, and *norA*, alone or concurrently, is associated with reduced susceptibility. Use of antiseptics may select for antibiotic-resistant strains and assist their survival in the healthcare environment.

Methicillin-resistant *Staphylococcus aureus* (MRSA) is an important nosocomial pathogen infecting defenseless individuals in hospitals throughout the world.[1–3] There are growing numbers of studies implying the etiologic role of coagulase-negative Staphylococci (CoNS) in disease in immunocompromised patients, and the increased prevalence of multidrug-resistant strains.[4–6] These species have the ability to survive in medical facilities for months.[7] The emergence and rise of antibiotic resistance among Staphylococci is a burden in health care facilities and communities.[8]

Biocides (antiseptics, disinfectants, and preservatives) are chemical compounds applied to inactivate or destroy microorganisms in various settings (e.g., the health care sector, agriculture and the food industry).[9–11] By the mid-1900s, many biocidal compounds were in common use as industrial preservatives and in the medical field.[10,11] Today, biocides have become an integral part of the industrialized world and are invaluable compounds in the control of human and animal pathogens.[12] Large amounts of biocides are therefore consumed within the different settings, including the medical environment where they are used for disinfection, antisepsis, and cleaning.[9]

A wide variety of biocidal agents, including quaternary ammonium compounds (QACs), such as benzalkonium chloride (BAC) and benzethonium chloride (BZT) and divalent cations like chlorhexidine digluconate (CHDG)

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are commonly used in hospitals and healthcare facilities to decontaminate surfaces, disinfect the hands of hospital personnel, and treat hospital-acquired infections.[13,14]

The worldwide increase in antimicrobial resistance in bacterial pathogens leading to increased mortality and morbidity in humans highlights the importance of infection control practices and the key role of biocides in healthcare. However, the widespread use of biocides in hospitals has led to concerns about the emergence of disinfectant-resistant bacteria. Excessive use may also result in the appearance of cross-resistance between widely used biocides and antibiotics.[7,15] Epidemiological data on antiseptic susceptibility and the distribution of resistance genes are useful for nosocomial infection control. Multidrug efflux systems are possibly the most described resistance mechanism with regard to acquired resistance to biocides.

Antiseptic resistance genes, *qacA/B*, *qacC/D*, *qacE* to *qacJ* (chlorhexidine-resistant genes) and *norA* (fluoroquinolone efflux transporter protein) have been identified in *Staphylococcus* species.[2,9,16,17] The *qacA/B*, *smr* (Staphylococcal multidrug resistance, also known as *qacC/D*), and *norA* genes are found mainly in *S. aureus* clinical isolates and could be responsible for reduced susceptibility to certain antiseptic agents.[2,7–9,18,19] The *qacA/B* and *smr* genes are mostly found on plasmids, while *norA* is located on the *S. aureus* chromosome.[9,19] The *qacA/B*, detected in both *S. aureus* and CoNS isolates, confers reduced susceptibility to a wide range of antimicrobial organic cations, including QACs and biguanides. The *smr* gene encodes a protein that belongs to a small multidrug resistance family and confers reduced susceptibility to QACs and ethidium bromide.[2,9,14] Additionally, the chromosomal *norA* gene confers low-level resistance to hydrophilic fluoroquinolones, such as norfloxacin and levofloxacin, as well as antiseptic agents including QACs.[19,20] Other plasmid-borne *qac* genes, *qacG*, *qacH,* and *qacJ*, have been identified in *S. aureus* and CoNS isolates and their prevalence rates remain low in human carriage isolates.[13,17]

The incidence rate of MRSA in our hospital has increased to more than 80% of clinical isolates and the prevalence of methicillin -resistant coagulase-negative staphylococci also rising.[21] This study was designed to evaluate the efficacy of three different antiseptic agents, BAC, BZT and, CHDG, which are currently used against *S. aureus* and CoNS clinical isolates. We also sought to determine the prevalence of the antiseptic resistance genes *qac*, *smr*, and *norA* among these bacteria.

METHODS

Valiasr Hospital is a 320-bed university-affiliated therapy center located in Arak, Iran. In a 12-month period, from April 2013 to March 2014, various clinical specimens were collected from admitted patients and transported to the laboratory by brain heart infusion (BHI) broth and cultured. Institutional ethical approval was obtained before study commencement.

The isolates were identified using the API-Staph system (API System; bioMérieux, Paris, France). Standard reference species (*S. aureus* ATCC 25923, *S. epidermidis* ATCC 12228, and *S. saprophyticus* ATCC 15305) were used for quality control. All *S. aureus* isolates were also assessed for the presence of species-specific 442 bp genomic DNA fragment.[22,23]

MRSA strains were identified by disk diffusion testing on Muller-Hinton agar plate with a cefoxitin disk (30 μg) and an oxacillin disk (10 μg) (Mast, Merseyside, UK) according to Clinical & Laboratory Standards Institute (CLSI) recommendations.[22] The presence of the *mecA* gene was demonstrated using the polymerase chain reaction (PCR) only for phenotypically oxacillin-resistant isolates.[3,23] The reference strains *S. aureus* ATCC 43300 and *S. aureus* ATCC 25923 were used as positive and negative controls, respectively.

The minimum inhibitory concentration (MIC) of three BAC, BZT, and CHDG biocides (Sigma-Aldrich, St Louis, USA) were determined in duplicate using the microbroth dilution method in accordance with CLSI guidelines.[24] Briefly, MICs were determined by twofold serial dilution of each biocide solution (ranging from 0.25 to 128 μg/mL) in Mueller-Hinton broth (MHB). Each dilution was inoculated with 1/10 dilution of 5×10^6 CFU/mL from fresh culture in MHB set up in a microdilution plate. The plate was then incubated at 37 °C for 24 hours. MICs were determined by observing the presence or absence of growth in each dilution. Isolates were considered susceptible to BAC and BZT with a MIC ≤ 3 mg/L and CHDG with an MIC ≤ 1.0 mg/L and to have reduced susceptibility with an MIC between 1.5 and 3.0 mg/L.[14]

All Staphylococcus isolates were subjected to genomic DNA purification by a DNA extraction kit (Bioflux, Bioneer, Republic of Korea), as described by the manufacturer. PCR was carried out for detection of *qacA/B*, *qacC/D* (*smr*), *qacG*, *qacH*, *qacJ*, and *norA* genes using the primer sequences listed in Table 1. The volume of each reaction in the PCR was 25 µl. Each reaction mixture contained 12.5 µl of 2× MasterMix (SinaClon, Iran), including 1× PCR buffer, 1.5 mmol/L $MgCl_2$, 0.15 mmol/L dNTP, and 1.25 IU *Taq* DNA polymerase, 1 µl of each primer (1 µM), 1 µl of template DNA (0.5 µg), and 9.5 µl of sterile distilled water. PCR was performed in the peqSRAR 96X Universal Thermocycler (PEQLAB Ltd, Southampton, UK) for 30 cycles for all studied genes and based on conditions shown in Table 1. PCR products were analyzed by electrophoresis on a 2% (w/v) agarose gel. Three PCR products from each amplified gene were sent for sequencing (Gene-Fanavaran Co., Tehran, Iran) and confirmed through the basic local alignment search tool (BLAST) before the final analysis.

Pearson's chi-squared test was performed using SPSS Statistics (SPSS Statistics Inc., Chicago, US) version 18.0 to determine association of categorical variables. Statistical significance was set at *p*-values < 0.050.

RESULTS

In total, 165 Staphylococcus isolates were collected from the clinical specimens, including wounds (n = 39; 23.6%), blood (n=20; 12.1%), sputum (n = 18; 10.9%), urine (n = 7; 4.2%), abscess (n = 20; 12.1%), synovial fluid 18 (10.9%), tracheal aspirates (n = 32; 19.3%), and ascetic fluid (n = 11; 6.6%) of which 60 (36.3%) were MRSA, 54 (32.7%) were methicillin-sensitive *S. aureus* (MSSA), and 51 (31%) were CoNS strains. All isolates were sa442 positive [Figure 1a].

The 165 Staphylococcus isolates were screened for BAC, BZT and, CHDG resistance. Distribution of MICs of three biocides against all Staphylococci is shown in Table 2. MICs for BAC and BZT ranged from 0.5 to 64 µg/mL, and for CHDG from 0.25 to 8 µg/mL. MRSA isolates showed less susceptibility towards the three biocides tested.

The MIC inhibiting 90% of isolates (MIC90S) tested for BAC, BZT and CHDG in these bacteria were 64 µg/mL, 16 µg/mL, and 2 µg/mL, respectively. CHDG was the most effective biocide with the MIC50 = 1 µg/mL, and MIC90 = 2 µg/mL against MRSA, and MIC50 = 0.5 µg/mL to MIC90 = 1 µg/mL against both MSSA and CoNS.

The *mecA* gene was detected in all 60 *S. aureus* isolates, verifying them as MRSA [Figure 1b]. *qacA/B* was the most common biocide resistance gene among all 165 Staphylococcus isolates (n = 76; 46%) [Table 2]; which comprised of 38 (63.3%) MRSA, 14 (25.9%) MSSA, and 24 (47%) CoNS [Figure 1c]. Eleven (6.7%) and 24 (14.5%) isolates carried *smr* and *norA* genes, respectively [Figure 1d and 1e]. In contrast, other resistance genes

Table 1: The primer sequences and cycling parameters for detection of genes tested in this study.

Gene	Primer sequence (5'-3')	Caycling conditions	Amplicon size (bp)	Reference
sa442	F: AATCTTTGTCGGTACACGATATTCTTCACG R:CGTAATGAGATTTCAGTAGATAATAACAACA	95 °C, 30 s; 55 °C, 30 s; 72 °C, 30 s	108	21
mecA	F:TCCAGATTACAACTTCACCAGG R:CCACTTCATATCTTGTAACG	94 °C, 30 s; 53 °C, 30 s; 72 °C, 1 min	162	23
qacA/B	F: TGGCTTATACCTATTACCTATGG R: TGTAGAATATGATAATGGATGAC	94 °C, 1 min; 52 °C, 1 min; 72 °C, 1 min	169	Study own
qacC/D (smr)	F: GCCATAAGTACTGAAGTTATTGGA R: GACTACGGTTGTTAAGACTAAACCT	95 °C, 35 s; 52 °C, 30 s; 72 °C, 1.5 min	195	2
qacG	F: CAACAGAAATAATCGGAACT R: TACATTTAAGAGCACTACA	95 °C, 1 min; 48 °C, 45 s; 72 °C, 1 min	670	26
qacH	F: ATAGTCAGTGAAGTAATAG R: AGTGTGATGATCCGAATGT	95 °C, 1 min; 48 °C, 45 s; 72 °C, 1 min	550	26
qacJ	F: CTTATATTTAGTAATAGCG R: GATCCAAAAACGTTAAGA	95 °C, 1 min; 48 °C, 45 s; 72 °C, 1 min	667	26
norA	F: GTAATACCAGTCTTGCCTGT R: GTAATGGCTGGTCGTATCAT	95 °C, 30 s; 54 °C, 45 s; 72 °C, 1 min	878	1

Table 2: The MIC results of 165 Staphylococci isolates against three antiseptics.

Antiseptic agent	n (%) of isolates with related MIC, µg/mL										MIC50 µg/mL	MIC90 µg/mL
	0.25	0.5	1	2	4	8	16	32	64	128		
BAC												
MRSA (n = 60)	0 (0)	0 (0)	6 (10.0)	14 (23.3)	9 (15.)	14 (23.3)	5 (8.3)	5 (8.3)	7 (11.7)	0 (0)	8	64
MSSA (n = 54)	0 (0)	26 (48.1)	16 (29.6)	5 (9.2)	2 (3.7)	1 (1.8)	2 (3.7)	1 (1.8)	1 (1.8)	0 (0)	1	4
CoNS (n = 51)	0 (0)	15 (29.4)	0 (0)	8 (15.7)	11 (11.7)	4 (7.8)	6 (11.7)	5 (9.8)	2 (3.9)	0 (0)	4	32
BZT												
MRSA (n = 60)	0 (0)	4 (6.7)	10 (16.7)	14 (23.3)	9 (15.0)	9 (15.0)	10 (16.7)	1 (1.7)	3 (5)	0 (0)	4	16
MSSA (n = 54)	0 (0)	19 (35.2)	27 (50.0)	5 (9.2)	0 (0)	1 (18)	2 (3.7)	0 (0)	0 (0)	0 (0)	1	2
CoNS (n = 51)	0 (0)	15 (29.4)	0 (0)	10 (19.6)	4 (7.8)	9 (17.6)	13 (25.5)	0 (0)	0 (0)	0 (0)	4	16
CHDG												
MRSA (n = 60)	0 (0)	24 (40)	9 (15.0)	24 (40.0)	1 (1.7)	2 (2.3)	0 (0)	0 (0)	0 (0)	0 (0)	1	2
MSSA (n = 54)	10 (18.5)	34 (63.0)	7 (13.0)	1 (1.8)	2 (3.7)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0.5	1
CoNS (n = 51)	2 (3.9)	26 (51)	21 (41.1)	2 (3.9)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0.5	1

BAC: benzalkonium chloride; BZT: benzethonium chloride; CHDG: chlorhexidine digluconate; MIC: minimum inhibitory concentration; MRSA: methicillin-resistant S. aureus; MSSA: methicillin-susceptible S. aureus; CoNS: coagulase-negative Staphylococci.

such as *qacG*, *qacH*, and *qacJ* were absent in all Staphylococci studied.

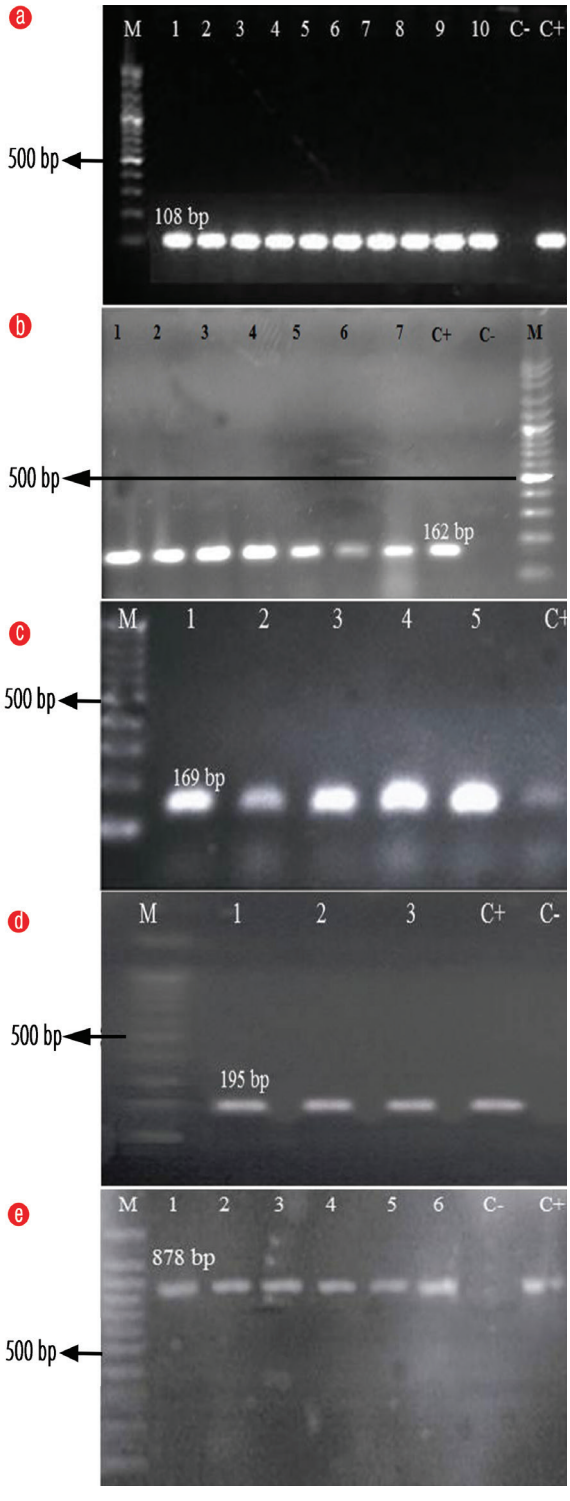
The *qacA/B* and *smr* genes were detected concomitantly in three (1.8%) isolates, of which one (1.7%), one (1.8%) and one (2.0%) were MRSA, MSSA, and CoNS, respectively. Coexistence of three *qacA/B*, *smr*, and *norA* genes was seen among three (5.0%) MRSA, two (3.7%) MSSA, and two (3.9%) CoNS. In addition, 39 (23.6%) strains of the total 165 Staphylococcus isolates were negative for each studied gene [Table 3].

The association of biocide resistance genes and MIC value in bacteria is shown in Table 4. Overall, isolates that harbored more resistance genes had reduced susceptibility to biocides.

Table 3: Distribution of the biocides resistance genes in 165 Staphylococcus isolates.

Resistance genes	n (%)			
	MRSA	MSSA	CoNS	Total
qacA/B	37 (61.7)	15 (27.8)	24 (47.1)	76 (46.1)
smr	6 (10.0)	2 (3.7)	3 (5.9)	11 (6.7)
norA	10 (16.7)	12 (22.2)	2 (3.9)	24 (14.5)
qacA/B + smr	1 (1.7)	1 (1.8)	1 (2.0)	3 (1.8)
qacA/B + norA	2 (3.3)	0 (0.0)	1 (2.0)	3 (1.8)
smr + norA	1 (1.7)	0 (0.0)	1 (2.0)	2 (1.2)
qacA/B + smr + norA	3 (5.0)	2 (3.7)	2 (3.9)	7 (4.2)
Negative	0 (0.0)	22 (40.7)	17 (33.3)	39 (23.6)
Total	**60 (100)**	**54 (100)**	**51 (100)**	**165 (100)**

MRSA: methicillin-resistant S. aureus; MSSA: methicillin-susceptible S. aureus; CoNS: coagulase-negative Staphylococci.



M: 100 bp Plus DNA Ladder; C-: Negative control; C+: Positive control.

Figure 1: Agarose gel electrophoresis of the polymerase chain reaction (PCR) amplification of the **(a)** *sa442*, **(b)** *mecA*, **(c)** *qacA/B*, **(d)** *smr*, and **(e)** *norA* genes. PCR product of isolates in numbered lanes.

DISCUSSION

The treatment and control of infections associated with Staphylococci, particularly MRSA and also CoNS, has become more difficult due to their ability to acquire resistance to many antimicrobials. This partly resulted from the ability of such bacteria to persist in healthcare environments and transmit among patients. Vigorous infection control measures, including standard hygiene and disinfection with efficient biocides, could lead to a decrease in the circulation of bacterial resistant clones in hospital environments.

In Iran, there are many reports about the increased antimicrobial resistance in Staphylococcal isolates,[3,25,26] but relatively few studies have investigated antiseptic susceptibility. Thus, assessments on the increase or decrease of antiseptic susceptibility in such problematic organisms could provide valuable information to support infection control and prevent healthcare-associated infections.

Our study can be considered the first comprehensive study in Iran evaluating the susceptibility to antiseptic agents and distribution of antiseptic resistance genes of 165 MRSA, MSSA, and CoNS strains collected from a single therapy center in Arak. CLSI guidelines (M100 document, 2015) and related published papers have been used as interpretive criteria for susceptibility testing.[5,14] The MICs of three test disinfectants against our Staphylococci isolates were ≤ 64 µg/mL, which is lower than concentrations recommended for use (2000, 1000, and 5000 µg/mL for BAC, BZT, and CHDG, respectively). This would suggest that if these biocides are used in the hospital environment in accordance with the manufacturers' instructions, 100% of bacteria should be inhibited or killed. However, it is noteworthy that in clinical practice, concentrations that might remain on surfaces after cleaning might provide a selective pressure on microorganisms. In the hospital environment, bacteria grow in biofilms on surfaces that have been shown to provide cells a 10–1000 fold higher tolerance of antimicrobial agents, and may be a contributor to disinfection failure.[27]

The *qacA/B* genes alone were found in 46.1% of tested bacteria: 61.7% of MRSA, 27.8% of MSSA, and 47.1% of CoNS. The high prevalence of these genes is likely to be due to selective pressure imposed by the various disinfection agents used in hospitals. Moreover, it is of note that significantly more MRSA than MSSA isolates were positive for the *qacA/B* gene. This may be the location of the *qacA/B* genes on widespread multi-resistance plasmids like the pSK1

Table 4: Correlation between biocide resistance genes and MIC value in Staphylococcal isolates.

Resistance genes	MIC range, µg/mL									Total
	BAC			BZT			CHDG			
	MRSA	MSSA	CoNS	MRSA	MSSA	CoNS	MRSA	MSSA	CoNS	
qacA/B	1–64	2–32	2–32	2–16	1–16	2–16	0.5–2	0.5–1	0.5–1	76
smr	2–4	2–4	2	1	1	2	0.5	1	0.5	11
norA	1–2	2	0.5	0.5–2	1–2	0.5	0.5	0.5–1	0.5	24
qacA/B+ smr	4- 32	2	4	2	2	4	2	2	0.5	3
qacA/B+ norA	64	-	16	64	-	8	2	-	1	3
smr+ norA	4	-	8	8	-	4	1	-	1	2
qacA/B+smr+norA	64	16–64	64	32–64	8–16	4–16	4–8	4	2	7
Negative	1	0.5–1	0.5	0.5–1	0.5–1	0.5	0.5	0.25–0.5	0.25–0.5	39
Total	**60**	**54**	**51**	**60**	**54**	**51**	**60**	**54**	**51**	**165**

MIC: minimum inhibitory concentration; BAC: benzalkonium chloride; BZT: benzethonium chloride; CHDG: chlorhexidinedigluconate; MRSA: methicillin-resistant S. aureus; MSSA: methicillin-susceptible S. aureus; CoNS: coagulase-negative staphylococci.

(*qacA*) and the β-lactamase/heavy metal resistance families, such as pSK23. These plasmids frequently harbor a number of determinants responsible for resistance to a range of antimicrobial agents.[9,28] Since MRSA multi-resistance is also, in part, due to such multi-resistance plasmids, the high incidence of *qacA/B* in MRSA is apprehensible.

The relatively low rate of carriage of *smr* (*qacC/D*) and *norA* genes found in the present study is in contrast to others. For example, *smr* was identified in 44.2% and *norA* in 36.7% of MRSA isolates in a study from the UK.[1] This may be due to the larger number of isolates screened and/or variation in the type of biocides used regularly, applying different selective pressures on Staphylococci isolates in each region. In addition, *qacG*, *qacH*, and *qacJ* genes were not detected in our study. These findings support the fact that among Gram-positive bacteria, the *qac* genes clearly predominate in Staphylococci with *qacA/B* genes most frequently reported followed by *qacC/D* genes.[15,29] Other *qac* genes, such as *qacG*, *qacH*, and *qacJ* have been less frequently observed.[17] On the other hand, compared to *qacA/B*, the role of other genes like *norA* as a contributing factor in the resistance of cationic antiseptic agents is very low.In another study conducted in neonatal intensive care units of a hospital in France from 51 CoNS isolated from catheter-associated bloodstream infections, 21 (41.2%) were resistance to at least one biocide and 30 (59%) isolates were positive for *qacA/B* resistance genes. This finding also emphasizes the important role of *qacA/B* genes in biocide resistance.[30]

We found a significant difference in MIC value between the isolates with and without biocide resistance genes. Furthermore, a considerable association was observed between the coexistence of several genes and the degree of susceptibility to all biocides tested. For example, isolates that carried *qacA/B*, *smr*, and *norA* genes, had MIC values higher than those positive for one or two resistance genes. This means that isolates carrying more biocide resistance genes may be able to persist on human skin and clinical environments where residual concentrations of biocides after cleaning may be lower than in-use concentrations.

CONCLUSION

At this time, the *qacG*, *qacH,* and *qacJ* resistance genes do not seem to pose a problem among Staphylococcus isolates collected from our hospital. However, the presence of other genes for biocide resistance, especially *qacA/B*, in the clinical Staphylococcal population and their ability to develop reduced susceptibility highlights the importance of effective infection control strategies, the use of biocides at concentrations recommended by the manufacturer, and the continued surveillance of resistance gene carriage.

Disclosure
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