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Prevalence, Antimicrobial Resistance, and Molecular Characteristics of Coagulase-Negative Staphylococci Isolated from Children's Blood Cultures in Northeastern Iran Within 2013 - 2019

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Abstract

Background: Coagulase-negative staphylococci (CoNS) are rolled in severe infections in animals and nosocomial infections in humans. Given that staphylococci other than *Staphylococcus aureus* are often reported only as CoNS in medical diagnosis laboratories, this study aimed to determine the exact species of this type of staphylococci in clinical samples.
Objectives: This study also aimed to evaluate antibiotic resistance, the ability to carry *cfr*, *qacA/B*, *mecA*, and *vanA* genes, and the diversity of staphylococcal cassette chromosome *mec* (SCC*mec*) elements in *mecA*-carrying isolates.
Methods: *Staphylococcus* spp. strains were isolated from the blood samples of children admitted to Imam Reza Hospital in Bojnurd, Northeastern Iran, between 2013 - 2019. All CoNS isolates were evaluated for resistance to vancomycin and oxacillin using agar screening and other routine anti-CoNS antibiotics using the Kirby-Bauer disk diffusion method, based on the latest Clinical and Laboratory Standards Institute guidelines. The CoNS strains were isolated based on conventional methods and polymerase chain reaction (PCR)-restriction fragment length polymorphism. The PCR was applied to determine the diversity of SCC*mec* elements in the CoNS isolates.
Results: In this study, 203 isolates were confirmed as CoNS belonging to nine staphylococci spp. *S. capitis* and *S. epidermidis* were the top two common CoNS. Type III was the dominant SCC*mec* type in *mecA*$^{+}$ isolates.
Conclusions: The findings of this study showed that CoNS isolated from blood cultures have a relatively high diversity and antibiotic resistance. Therefore, further attention should be paid to the isolation of these strains in laboratories, and they should not be easily considered as contamination.

Keywords: *Staphylococcus*, Coagulase, Drug Resistance, Polymerase Chain Reaction

1. Background

Coagulase-negative staphylococci (CoNS) are among the most prevalent bacteria isolated from clinical samples in medical microbiology laboratories. These bacteria commonly belong to normal flora and are regarded as contaminants in positive blood cultures. In some cases, such as immunocompromised patients and patients using indwelling devices, they can be opportunistic pathogens causing hospital-acquired infections (1-3). The CoNS are ranked among the first five common pathogens related to hospital-acquired infections, and a variable number of CoNS infections led to hospitalization (4). The CoNS can be a reservoir of antibiotic resistance genes in plasmid or other mobile genetic elements. The primary concern is the ability to exchange resistance-related elements between CoNS and *Staphylococcus aureus*. The most resistant CoNS are *S. hominis*, *S. haemolyticus*, and *S. epidermidis* (5, 6). Due to the ability of CoNS to acquire conjugative plasmids, to date, these bacteria are highly resistant to penicillin, oxacillin, clindamycin, ciprofloxacin, gentamycin, and erythromycin (6, 7).

Methicillin resistance is usually related to the *mecA* gene that encodes PBP2a with a low binding affinity to the methicillin family. The *mecA* gene is located on staphylococcal cassette chromosome *mec* (SCC*mec*) (8). These genetic elements are highly variable, and to date, 13 SCC*mec* types have been introduced in *Staphylococcus* spp. isolates from animals and humans (9, 10). Other important resistance mechanisms in staphylococci are the enzymatic change of antibiotics, removal of antibiotics from

the cell, and reduction of the binding affinity of antibiotic molecules to targets (2). In recent years, a homolog of the *mecA* gene called *mecC* was discovered in *S. aureus* and some other staphylococci spp. This gene produces a protein similar to PBP2a but, in the nucleotide sequence, differs from *mecA* and shows about 68% sequence similarity. This gene is located on the SCC*mec* XI element (11).

Staphylococci strains containing the *mecC* gene are usually isolated from animals and sometimes humans in Western Europe (12).

2. Objectives

Due to the preliminary diagnosis of non-aureus staphylococci under the general title of CoNS in most medical diagnostic laboratories of Iran and the importance of these bacteria as human pathogens and their ability to act as a reservoir of antimicrobial resistance genes for other virulent bacteria, this study investigated these bacteria at the species level, their antibiotic resistance, and their ability to carry *mecA/vanA* and *cfr-qacA/B* genes and the diversity of SCC*mec* elements in CoNS isolates, isolated from the blood cultures of patients admitted to a 96-bed teaching hospital in Bojnurd, Northeastern Iran.

3. Methods

3.1. Sample Collection

This study evaluated 203 CoNS spp. strains isolated from the blood samples of children admitted to Imam Reza Hospital in Bojnurd, Northeastern Iran. The CoNS ruled out contaminants and were selected as pathogens based on a laboratory-based algorithm and clinical examinations by related physicians (13). The clinical criteria included the presence of one or more of the following factors based on the patient's history:

(1) persistent fever, (2) hypotension, (3) leucopenia or leukocytosis, (4) invasive devices, (5) immunodeficiency, (6) sepsis, (7) long-term stay in the intensive care unit, (8) use of an intravenous catheter, and (9) surgery or procedures, such as dialysis.

The laboratory criteria included the pure growth of CoNS in cultures and identical isolates from two or more culture media.

3.2. Antimicrobial Susceptibility Testing

The antibiogram test was performed using tetracycline (TET), cefoxitin (CEF), moxifloxacin (MOX), levofloxacin (LEV), erythromycin (ERY), rifampicin (RIF), clindamycin (CLN), penicillin (PEN), gentamicin (GEN), minocycline (MIN), trimethoprim/sulfamethoxazole (COT), vancomycin (VAN), and linezolid (LIN) antimicrobial disks using the Kirby-Bauer disk diffusion method (MASTDISCS™, UK) based on Clinical and Laboratory Standards Institute guidelines (14).

3.3. CoNS Genus Identification

The CoNS strains were isolated based on conventional microbiology laboratory tests containing colony morphology, microscopic evaluation using gram staining, and biochemical tests, including catalase, acetoin production, coagulase, susceptibility to novobiocin and polymyxin B, pyrrolidonyl arylamidase test, ornithine decarboxylase test, and fermentation of glucose, mannitol, mannose, maltose, trehalose, and sucrose (15-18). Polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) test was performed to verify phenotypic test results (19).

3.4. Genomic DNA Extraction

The genomic DNAs of CoNS isolates were extracted using the Addbio Genomic DNA Extraction Kit (KR-2000, Addbio, South Korea). Lysostaphin was used in addition to lysis buffer at a final concentration of 20 μg/mL.

3.5. PCR-RFLP for CoNS Species Identification

This study used degenerate primers (forward: 5-GCCAAAAGAGACTATTATGA-3 and reverse: 5-ATTGYTTACCYGTTTGTGTACC-3) to amplify the fragment of the *dnaJ* gene with a variable length (20). The PCR amplification was performed using TaKaRa Gradient PCR TP600 thermal cycler in a volume of 50 μL using TAKARA Emerald Amp Max PCR pre-mixed Master Mix (TaKaRa, Japan). The thermal setting included the incubation of the PCR mixture for 180 seconds at 94°C and then short cycling by five cycles at 94°C for 30 seconds, 45°C for 30 seconds, and 72°C for 60 seconds. Then, the mixture was amplified in 40 cycles of denaturation at 94°C for 30 seconds, an annealing step at 50°C for 30 seconds, an extension step at 72°C for 60 seconds, and a final extension step at 72°C for 180 seconds (20). The PCR products were evaluated using 1.5% agarose gel electrophoresis containing SYBR™ Safe Stain (Sinaclone, Iran). As previously described, this study used the *XapI* restriction enzyme that provides specific enzymatic digestion profiles for the staphylococcal subspecies (19). Digestions were performed using 5 μL of the PCR products in a total volume of 15 μL with 1 μL reaction buffer in addition to 10 U of the *XapI* endonuclease enzyme (Fermentas, Germany) for 3 hours at 37°C. Then, agarose gel electrophoresis was performed on the digestion product using 2% agarose gel (Fermentas), and the produced fragments were evaluated using DNA safe stain and an ultra violet gel documentation system (19).

3.6. PCR Detection of Antimicrobial and Disinfectant Resistance Genes

The presence of the *vanA*, *mecA*, *cfr*, and *qacA/B* genes was evaluated by PCR using the primers in Table 1, as previously described.

3.7. SCCmec Typing

Staphylococcal cassette chromosome *mec* typing was performed as previously described (25).

3.8. Statistical Analysis

The results were transferred to a Microsoft Excel spreadsheet for analysis. Statistical analysis was performed using SPSS software (version 16.0). Similarities or differences were evaluated using an analysis of variance test. P-values $\leq$ 0.05 were considered statistically significant.

4. Results

A total of 203 infectious isolates belonged to nine species of staphylococci. Among the aforementioned isolates, *S. capitis* (n = 44) was the most common species, followed by *S. epidermidis* (n = 32), *S. warneri* (n = 29), *S. haemolyticus* (n = 25), *S. hominis* (n = 21), *S. simulans* (n = 17), *S. caprae* (n = 17), *S. cohnii* (n = 14), and *S. hyicus* (n = 4) (Figure 1). Antimicrobial susceptibility testing showed that penicillin resistance (94.1%) had the highest resistance rate in CoNS spp. A low resistance rate was detected for linezolid (3.9%), and vancomycin resistance was not observed in any of them (Table 2).

These results were also certified using the PCR-RFLP method. The mean of *mecA*-positive isolates was 22% by the PCR method. All the species had the *qacA/B* genes ranging from 25% to 58% (except *S. hyicus*), and the highest percentage was related to *S. caprae*. The *cfr* gene was found in *S. epidermidis* (15.6%) and *S. hominis* (14.3%) (Figure 2). In this study, the minimum inhibitory concentration (MIC) of $\leq$ 4 μg/mL was observed in 90% of the CoNS spp., and none of them had a vancomycin MIC of $\geq$ 32 μg/mL. SCC*mec* type III had the highest percentage of nine species of CoNS (Table 3). Other SCC*mec* types were not observed in *S. cohnii* and *S. hyicus* (except SCC*mec* type III).

5. Discussion

The CoNS are rolled in severe infections in animals and nosocomial infections in humans, particularly in immunocompromised hosts, and show a high rate of multiple antimicrobial resistance (26). In this study, 203 patients were confirmed as CoNS belonging to nine staphylococci spp. Moreover, *S. capitis* and *S. epidermidis* were the top two common CoNS spp. Furthermore, 13 antibiotics were tested for antimicrobial susceptibility. The results showed that penicillin, rifampicin, and erythromycin had the highest resistance rates in CoNS spp. A low resistance rate was detected for linezolid (3.9%), minocycline (30%), and moxifloxacin (36%). Therefore, these antibiotics should be cautiously selected for CoNS treatment. Consistent with the results of most previous studies, vancomycin resistance was not observed in CoNS spp. (27, 28). The results are in line with the results of previous studies wherein *Staphylococcus epidermidis* was the most common species of CoNS (28, 29). Additionally, Giormezis's study in Greece (30) showed that *S. epidermidis* and *S. haemolyticus* are responsible for 71.1% and 29.1% of CoNS infections, respectively.

A recent survey by Cui et al. about CoNS isolated from hospitalized patients in China showed that from 157 patients, the most prevalent species were *S. hominis* and *S. epidermidis*, respectively. All CoNS had a high resistance to penicillin, erythromycin, and oxacillin. Resistance to rifampicin and gentamicin was low, and none of the CoNS was resistant to linezolid or vancomycin (31). A study by Pedroso et al. about CoNS isolated from patients with bloodstream infections acquired in Brazil reported that the highest resistance belonged to benzylpenicillin (100%) and oxacillin (93.1%). The resistance of vancomycin (1.7%) had a low rate, and there was no resistance to tigecycline (32). The results of Paiva et al.'s study on CoNS isolates from blood samples obtained at a hospital in Porto, Portugal, showed that all 130 CoNS isolates were *mecA*-positive and identified as *S. epidermidis* (66.9%), *S. haemolyticus* (10.0%), *S. hominis* (9.2%), and *S. capitis* (8.5%), and the MICs of vancomycin ranged from 0.38 to 3 and 0.25 to 2 g/mL by E-test and broth microdilution method, respectively (33).

A study by Mittal et al. reported that all isolates were susceptible to vancomycin (MIC range: 1 - 4 μg/mL) (34). In this study, MIC of $\leq$ 4 μg/mL was also observed in about 90% of CoNS spp., and none of them had a vancomycin MIC of $\geq$ 32 μg/mL. Intermediate resistance to vancomycin (MIC range: 8 - 16 μg/mL) was not observed in *S. capitis*, *S. cohnii*, and *S. hyicus*. The results obtained by Nahaei et al. showed that the agar screening oxacillin method had a good relationship with PCR results (35). In the current study, the number of *mecA*-positive isolates was higher than agar-screening oxacillin-positive in nine species of staphylococci. In the agar screening oxacillin method, the highest growth rate was observed in *S. cohnii* (64.3%) and *S. hominis* (61.9%). The highest resistance on vancomycin agar screening media was observed in *S. haemolyticus* (12%) and *S. caprae* (11.8%).

In addition, the present study showed a difference between PCR and disk diffusion results. Methicillin resis-

Table 1. Primer Sequences Used in This Study

Gene	Primer Sequence	Product Size (bp)	Reference
mecA	5′-ATGTATGTGCGATTGTATTGC-3′	584	(21)
	5′-AGAAGATGGTATGTGGAAGTTAG-3′		
vanA	5′-ATCAAGCGGTCAATCAGTTC-3′	713	(22)
	5′-GGCAAGTCAGGTGAAGATG-3′		
cfr	5′-ACCATATAATTGACCACAAGCAGC-3′	746	(23)
	5′-TGAAGTATAAAGCAGGTTGGGAGTCA-3′		
qacA/B	5′-CCACTACAGATTCTTCAGCTACATG -3′	417	(24)
	5′-CTATGGCAATAGGAGATATGGTGT -3′		

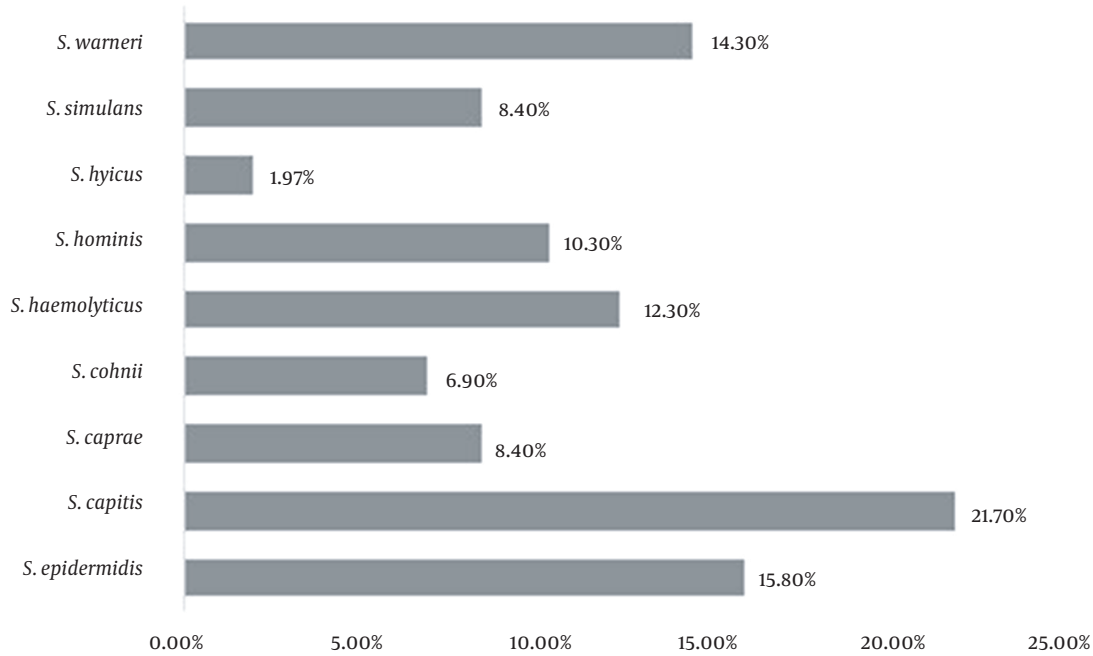


Figure 1. Prevalence of coagulase-negative staphylococci in blood cultures

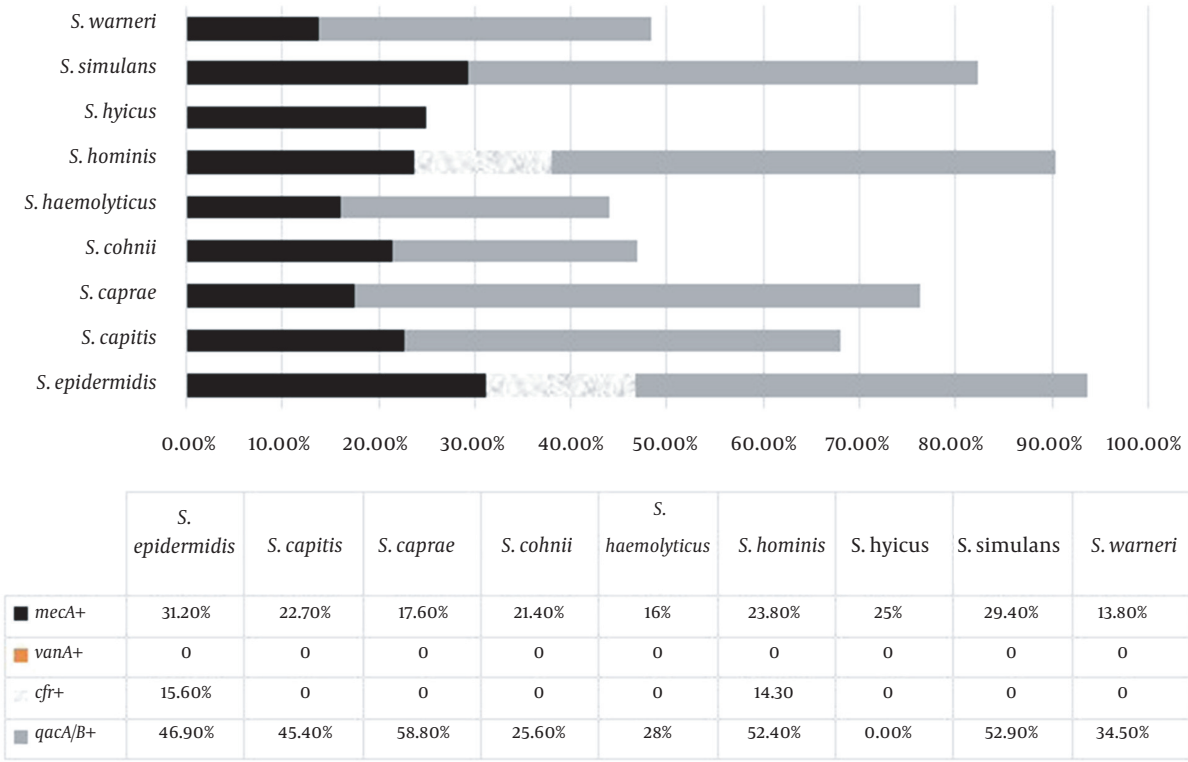
tance was 22% and 49.3% in PCR and disk diffusion methods, respectively. The CoNS isolates that showed phenotypical resistance to oxacillin but did not have the *mecA* gene might possess other mechanisms for resistance. Nahaei et al. reported that 10 CoNS isolates not containing the *mecA* gene were resistant to oxacillin using the disk diffusion method, and nine CoNS containing the *mecA* gene were susceptible to oxacillin disk screening. These differences might be related to the presence and the absence of the *mecA* gene expression and heteroresistance to oxacillin (35).

In other previous studies, the *mecA* gene was detected in 79% of CoNS (36). Genotypic analyses of Pedroso et al. showed that 40% of CoNS isolates were positive for *mecA*, and the *vanA* gene was not observed in any of them. Moreover, the highest percentage of SCCmec was observed in type IIIB (32.2%) (32). In this study, SCC*mec* type III was dominant. Other SCC*mec* types were not observed in *S. cohnii* and *S. hyicus* (except SCC*mec* type III). Staphylococcal cassette chromosome *mec* III could have been obtained from the hospital setting, patients, or healthcare workers, and it can be classified as a hospital-acquired infection. Another recent study by Taha et al. reported that two *mecA*-positive *S. lugdunensis* belonged to SCC*mec IVa*; however, it was previously thought that SCC*mec* exists only in *Staphylococcus aureus* (37).

Table 2. Results of Antimicrobial Susceptibility Testing [a]

	Staphylococcus epidermidis	*S. capitis*	*S. caprae*	*S. cohnii*	*S. haemolyticus*	*S. hominis*	*S. hyicus*	*S. simulans*	*S. warneri*	Total
PEN	28 (87.5)	39 (88.6)	16 (94.1)	12 (85.7)	22 (88)	20 (95.2)	4 (100)	25 (94.1)	25 (86.2)	191 (94.1)
ERY	15 (46.9)	27 (61.4)	10 (58.8)	8 (57.1)	15 (60)	12 (57.1)	2 (50)	10 (58.8)	15 (51.7)	114 (56.2)
CEF	17 (53.1)	20 (45.4)	9 (52.9)	9 (64.3)	10 (40)	13 (61.9)	1 (25)	8 (47.1)	13 (44.8)	100 (49.3)
TET	14 (43.75)	27 (61.4)	7 (41.2)	7 (50)	12 (48)	13 (61.9)	3 (75)	10 (58.8)	17 (58.6)	110 (54.2)
CLN	15 (46.9)	24 (54.5)	10 (58.8)	6 (42.9)	15 (60)	8 (38.1)	3 (75)	7 (41.2)	15 (51.7)	103 (50.7)
LEV	18 (56.2)	28 (63.6)	8 (47.1)	6 (42.9)	7 (28)	10 (47.6)	2 (50)	8 (47.1)	11 (37.9)	98 (48.3)
MOX	12 (37.5)	12 (27.3)	7 (41.2)	5 (35.7)	6 (24)	10 (47.6)	1 (25)	6 (35.3)	14 (48.3)	73 (36)
COT	13 (40.6)	19 (43.2)	7 (41.2)	8 (57.1)	8 (32)	12 (57.1)	0 (0)	7 (41.2)	14 (48.3)	39 (43.3)
GEN	18 (56.2)	20 (45.4)	6 (35.3)	7 (50)	15 (60)	14 (66.7)	2 (50)	9 (52.9)	15 (51.7)	106 (52.2)
RIF	20 (62.5)	24 (54.5)	8 (47.1)	8 (57.1)	16 (64)	11 (52.4)	3 (75)	9 (52.9)	16 (55.2)	115 (56.6)
MIN	8 (25)	12 (27.3)	6 (35.3)	5 (35.7)	6 (24)	7 (33.3)	1 (25)	6 (35.3)	10 (34.5)	61 (30)
VAN	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)
LIN	5 (15.6)	0 (0)	0 (0)	0 (0)	0 (0)	3 (14.3)	0 (0)	0 (0)	0 (0)	8 (3.9)

Abbreviations: CEF, cefoxitin; ERY, erythromycin; PEN, penicillin; CLN, clindamycin; TET, tetracycline; MOX, moxifloxacin; LEV, levofloxacin; GEN, gentamicin; COT, trimethoprim/sulfamethoxazole; MIN, minocycline; RIF, rifampicin; LIN, linezolid; VAN, vancomycin.
[a] Values are expressed as No. (%).



	S. epidermidis	*S. capitis*	*S. caprae*	*S. cohnii*	*S. haemolyticus*	*S. hominis*	S. hyicus	S. simulans	*S. warneri*
mecA+	31.20%	22.70%	17.60%	21.40%	16%	23.80%	25%	29.40%	13.80%
vanA+	0	0	0	0	0	0	0	0	0
cfr+	15.60%	0	0	0	0	14.30	0	0	0
qacA/B+	46.90%	45.40%	58.80%	25.60%	28%	52.40%	0.00%	52.90%	34.50%

Figure 2. Prevalence of antimicrobial resistance genes in coagulase-negative staphylococci

The *cfr* gene is associated with linezolid resistance. The resistance created by the *cfr* gene is usually plasmid-borne and can encode resistance to pleuromutilins, phenols, lincosamides, and streptogramin (38, 39). In a study by Mittal et al., linezolid resistance was observed in *S. haemolyticus*, *S. cohnii*, and *S. arlettae*. The aforementioned study suggested that *S. arlettae* could be an emerging pathogen (34). Dinakaran et et al. reported the isolation of *S. arlettae* from the blood culture in cardiovascular diseases (40). In the present study, linezolid resistance was observed in *S. epidermidis* (15.6%) and *S. hominis* (14.3%). These findings are consistent with the results of the presence of the *cfr* gene

Table 3. Results of Agar Screening, Minimum Inhibitory Concentration, and Staphylococcal Cassette Chromosome *mec* Typing Tests of Coagulase-Negative Staphylococci Species [a]

	Growth on Oxacillin Agar Screening Media	Growth on Vancomycin Agar Screening Media	Oxacillin MIC		Vancomycin MIC			SCC*mec* Type				
			≤ 0.25	≥ 0.5	≤ 4	8 - 16	≥ 32	I	II	III	IV	V
Staphylococcus epidermidis	17 (53.1)	2 (6.2)	15 (46.9)	17 (53.1)	31 (96.9)	2 (6.2)	0 (0)	2 (11.8)	2 (11.8)	10 (58.8)	2 (11.8)	1 (10)
S. capitis	20 (45.4)	0 (0)	24 (54.5)	20 (45.4)	44 (100)	0 (0)	0 (0)	0 (0)	0 (0)	16 (80)	4 (20)	0 (0)
S. caprae	9 (52.9)	2 (11.8)	8 (47.1)	9 (52.9)	15 (88.2)	2 (11.8)	0 (0)	3 (33.3)	0 (0)	5 (55.5)	1 (11.1)	0 (0)
S. cohnii	9 (64.3)	0 (0)	5 (35.7)	9 (64.3)	14 (100)	0 (0)	0 (0)	0 (0)	0 (0)	9 (100)	0 (0)	0 (0)
S. haemolyticus	10 (40)	3 (12)	15 (60)	10 (40)	22 (88)	3 (12)	0 (0)	0 (0)	0 (0)	7 (70)	3 (30)	0 (0)
S. hominis	13 (61.9)	1 (4.8)	8 (38.1)	13 (61.9)	20 (95.2)	1 (4.8)	0 (0)	2 (15.4)	0 (0)	8 (61.5)	3 (23.1)	0 (0)
S. hyicus	1 (25)	0 (0)	3 (75)	1 (25)	4 (100)	0 (0)	0 (0)	0 (0)	0 (0)	1 (100)	0 (0)	0 (0)
S. simulans	8 (47.1)	1 (5.9)	9 (52.9)	8 (47.1)	16 (94.1)	1 (5.9)	0 (0)	0 (0)	0 (0)	4 (50)	3 (37.5)	1 (12.5)
S. warneri	13 (44.8)	3 (10.3)	16 (55.2)	13 (44.8)	26 (89.6)	3 (10.3)	0 (0)	1 (7.7)	2 (15.4)	7 (53.8)	3 (23.1)	0 (0)

Abbreviations: MIC, minimum inhibitory concentration; SCC*mec*, staphylococcal cassette chromosome *mec*.
[a] Values are expressed as No. (%).

in both bacteria. Linezolid resistance might be created due to the horizontal transfer of resistance mediated by the *cfr* gene between patients.

The *qacA/B* genes caused reduced susceptibility to a wide range of antimicrobial organic cations. The current study's findings showed that all species had the *qacA/B* genes ranging from 25% to 58% (except *S. hyicus*), and the highest percentage was related to *S. caprae*. Taheri et al. reported that the *qacA/B* genes were detected in 3% of CoNS isolates (41). In another study, the *qacA/B* genes were detected in susceptible and resistant samples (53%) to chlorhexidine (42). The evidence showed a strong relationship between reduced susceptibility to chlorhexidine and the presence of *qacA/B* genes (43, 44). Different studies reported the detection of the qacA/B genes and the susceptibility of chlorhexidine depending on the region studied (45, 46).

5.1. Conclusions

This study evaluated antibiotic-resistant staphylococci, the ability to carry *mecA/vanA* and *cfr-qacA/B* genes, and the diversity of SCC*mec* elements in CoNS isolates. *S. capitis* and *S. epidermidis* were the two common species of CoNS. Moreover, rifampicin was the target of the highest resistance, and multidrug resistance was commonly observed in staphylococcal spp. Vancomycin resistance was not observed in any of the methods used. This antibiotic can be selected for CoNS treatment. In the PCR method, the mean of *mecA*-positive isolates was 22%; nevertheless, 49.3% of CoNS isolates were resistant to cefoxitin using the disk diffusion method. The difference in phenotypic and genotypic results might return to the presence of another resistance mechanism. Staphylococcal cassette chromosome *mec* type III as a hospital-acquired infection had the highest percentage of nine species of CoNS. The *cfr* gene was observed in *S. epidermidis* and *S. hominis*, which is related to linezolid resistance. Additionally, in this study, all species had the *qacA/B* genes (except *S. hyicus*), and the highest percentage was related to *S. caprae*. Due to the abundance of *mecA* genes and results of phenotypic methods between CoNS isolates, the prevalence of antibiotic-resistant strains is increasing. Furthermore, due to the difference between phenotypic and genotypic results, it is better to use phenotypic and genotypic methods simultaneously. Finally, due to the high prevalence of these bacteria and the high antibiotic resistance of these strains, further attention should be paid to these bacteria in infection control processes in hospitals.

Footnotes

Authors' Contribution: Roya Sadidi collected the data, carried out the conventional and PCR tests, and wrote the initial draft of the manuscript. Amir Azimian conceptualized and designed the study, carried out the conventional and PCR tests, performed the analyses, and conducted the final revision of the manuscript.

Conflict of Interests: The authors declare that there is no conflict of interest.

Data Reproducibility: The dataset presented in the study is available on request from the corresponding author during submission or after publication.

Ethical Approval: This study received the approval of the North Khorasan University of Medical Sciences Ethics Committee (approval code: IR.nkums.REC.1394.51) before beginning the project.

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