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ORIGINAL RESEARCH

Application of *tuf* gene sequence analysis for the identification of species of coagulase-negative staphylococci in clinical samples and evaluation of their antimicrobial resistance pattern

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Introduction: Coagulase-negative staphylococci (CoNS) are normal inhabitants of human skin and mucous membranes. However, CoNS represent one of the major nosocomial pathogens, especially in immunocompromised patients. The increasing incidence of CoNS and mainly methicillin-resistant strains underlines the need for an accurate identification of Staphylococcus isolates at the species level. Analysis of the *tuf* gene proved to be an accurate tool for the species identification of CoNS. The aims of this study were to identify the CoNS species by *tuf* gene-based polymerase chain reaction method and sequencing, and to determine the frequency of CoNS clinical isolates resistant to methicillin (MRCoNS) and other antibiotics.

Methods: A total of 200 staphylococci isolates were collected from various clinical samples. Phenotyping methods were used for initial identification followed by polymerase chain reaction amplification of *tuf* gene with subsequent sequencing. The phylogenetic relationships among species were analyzed using the neighbor-joining method based on the partial gene sequence of *tuf*. Microbroth dilution test was used for screening methicillin resistance, and disk diffusion susceptibility testing was performed for evaluation of antibiotic resistance among the isolates.

Results: In the present study, 125 isolates were identified as CoNS; among them, *Staphylococcus epidermidis* 54(43.2%) and *Staphylococcus haemolyticus* 50 (40.0%) were demonstrated as the most prevalent species. Resistance to methicillin was detected in 54.4% of the CoNS based on microbroth dilution method. In disk diffusion susceptibility testing, the greatest resistance of CoNS was demonstrated for cefoxitin (65.4%), cotrimethoxazole (54.4%), and clindamycin (49.6%), while daptomycin (87.2%) and linezolid (83.2%) showed the greatest effectiveness for CoNS isolates.

Conclusion: Our results confirmed the predominance of *S. epidermidis* and *S. haemolyticus* among CoNS isolates. The high prevalence of MRCoNS strains is a serious concern and strongly suggests the need for control program measures in our hospitals in order to reduce MRCoNS infections, especially in immunocompromised patients.

Keywords: coagulase-negative staphylococci, *tuf* gene, antimicrobial resistance, PCR, susceptibility testing

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Introduction

The pathogenicity of coagulase-negative staphylococci (CoNS) was not accepted until 1980, but later investigations demonstrated that CoNS are common cause of a vast range of important infections and diseases, and in general, newborns, individuals with neutropenia and artificial organs are at highest risk of infection.[1–4] The most

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commonly observed species of CoNS are *Staphylococcus epidermidis*, *Staphylococcus haemolyticus*, *Staphylococcus saprophyticus*, and *Staphylococcus lugdunensis*.[1,2] Among the CoNS, *Staphylococcus hominis* appears to be the most prevalent cause of septicemia, bacteremia, and endocarditis in immunocompromised patients and infants,[5] whereas *S. saprophyticus* and *S. lugdunensis* have been reported as the common causes of urinary tract infection in young females and infective endocarditis, respectively.[6] There are overwhelming evidences and reports of increasing methicillin resistance among CoNS, and this phenomenon creates new complications for infected patients such as patients with artificial heart valves.[7,8] Vancomycin is a drug of choice for the treatment of methicillin-resistant CoNS associated with neonatal infections.[9] However, in recent years, there are increasing reports of vancomycin resistance among staphylococci isolates, and the exact mechanism of resistance has not yet been completely understood.[10]

The *tuf* gene cluster, which is located in the short tandem repeat region on bacterial chromosome, shows a significant diversity among members of Staphylococci. The small size and its conserved location in bacterial chromosome insist on its superiority in DNA sequencing compared with 16SrRNA for construction of phylogenetic tree on species and genus level in Staphylococci, Enterococci, and Streptococci isolates.[11,12] Bergeron et al emphasized on the value of the partial *tuf* gene sequence for the identification of all staphylococcal species.[13] Analysis of the *tuf* gene proved to be more discriminative for CoNS clinical isolates, and provides a reference method with high accuracy for recognizing hospital infections related to *S. epidermidis* and *S. haemolyticus* in critical care units.[14,15]

Due to the increase in infections caused by CoNS in our settings, the present study was aimed to identify the CoNS species based on *tuf* gene by polymerase chain reaction (PCR) sequencing method in clinical isolates and to determine the frequency of CoNS resistance to methicillin (MRCoNS) and other antibiotics. We expect that our findings would help us have a better understanding of the frequency of CoNS clinical isolates and their antibiotic resistance pattern in Ahvaz, Southwestern Iran.

Methods

Bacterial isolates

A total of 200 staphylococci spp. isolates belonging to patients admitted to Golestan teaching hospital, Ahvaz, Iran, were collected during 1-year period, from January to December 2015. The study was approved by the joined institutional review board and ethics committee of the Ahvaz Jundishapur University of Medical Sciences, after submission of preliminary proposal and necessary permission for sample collection was granted. As part of the university's policy, referred patients were requested to provide written informed consent and were asked to sign the informed consent in case that their sample was used for research purposes apart from routine clinical investigation. The staphylococci were isolated from various clinical samples including blood, urine, deep wound, ear discharge, cerebrospinal fluid, and endotracheal secretion. The isolates were cultured on appropriate culture media of blood agar and mannitol salt agar (Merck, Darmstadt, Germany), and were identified as CoNS according to standard morphological criteria and biochemical tests including catalase, coagulase, and DNase.[16] CoNS isolates were inoculated in Trypticase soy broth with 20% glycerol and were kept at –70°C until use.

Antimicrobial susceptibility testing

The resistance patterns of CoNS isolates were evaluated by disk diffusion method according to Clinical and Laboratory Standards Institute (CLSI) guidelines,[17] using following antibiotic disks: linezolid 30 µg, quinupristin/dalfopristin15 µg, ticarcillin 75 µg, cotrimoxazole 25 µg, clindamycin 2 µg, daptomycin 30 µg, and cefoxitin 30 µg (MAST Co., Berkshire, UK).

Microbroth dilution method

The evaluation of minimal inhibitory concentration for oxacillin was performed by microbroth dilution according to CLSI guidelines.[17] *Staphylococcus aureus* ATCC29213 and ATCC43300 were used as sensitive and resistant controls for Oxacillin, respectively. The Fisher's exact test was used to evaluate the statistically significant relationship between methicillin-sensitive CoNS (MSCoNS) and MRCoNS isolates.

DNA extraction and PCR amplification

DNA was extracted from bacterial colonies by using simple boiling method as previously described.[18] The concentration of extracted DNA was measured by biophotometer (Eppendorf, Hamburg, Germany) at 260–280 nm and was stored at –20°C until use.

PCR amplification was performed on an Eppendorf thermocycler (Roche Co., Mannheim, Germany) by application of a set of primers of 5′-GCCAGTTGAGGACGTATTCT-3′ and 5′- CCATTTCAGTACCTTCTGGTAA-3′, which can amplify a 412 bp fragment of *tuf* gene,[19] as previously described.[20] The *S. epidermidis* ATCC 49134 was used as positive control in amplification reaction. The PCR products were loaded on a 1.5% (w/v) agarose gel with 0.5 µg/mL ethidium bromide and were analyzed using gel electrophoresis, and photographed

by using the gel documentation system (ProteinSimple, San Jose, CA, USA). The obtained sequences of *tuf* gene for each isolate were aligned separately By MEGA 5 (Molecular Evolutionary Genetics Analysis) software and compared with all existing sequences of CoNS annotated in GenBank database.

Phylogenetic trees were obtained from DNA sequences using the neighbor-joining method and Kimura's two-parameter distance correction model with 1,000 bootstrap replications supported by the MEGA 5 software.[21]

Results

Based on culture and standard biochemical criteria, 125 out of 200 total tested staphylococci isolates were identified as CoNS. These were originated from samples obtained from 60 (48%) male and 65 (52%) female patients, but there was no statistically significant difference in gender distribution.

The distribution of CoNS according to the hospital departments is presented in Table 1, and as it shows, nearly more strains were isolated from patients referred to outpatient department (OPD; n=64[51.2%]) in comparison with the rest of the strains that were isolated from hospitalized patients (n=61[48.8%]).

The majority of CoNS were isolated from urine samples (n=82[65.6%]). The rest of isolates were derived from wound, catheter, blood, sputum, and trachea with lower frequency (Table 2). The most prevalent species revealed by conventional methods were *S. epidermidis*, *S. haemolyticus*, and *S. hominis*, respectively.

The frequency of various CoNS species demonstrated by PCR amplification of *tuf* gene followed by DNA sequencing were *S. epidermidis*, 54(43.2%), *S. haemolyticus*, 50 (40.0%), *S. hominis*, 11 (8.8%), *S. saprophyticus*, 6 (4.8%),

Table 1 Distribution of different CoNS species among various departments of hospital

Total no.	*Staphylococcus petrasii* No. (%)	*Staphylococcus warneri* No. (%)	*Staphylococcus hominis* No. (%)	*Staphylococcus saprophyticus* No. (%)	*Staphylococcus haemolyticus* No. (%)	*Staphylococcus epidermidis* No. (%)	Department
64	0 (0)	1 (1.56)	4 (6.25)	4 (6.25)	27 (42.18)	28 (43.75)	**OPD**
15	0 (0)	0 (0)	1 (6.6)	1 (6.6)	7 (46.6)	6 (40.0)	**ICU**
6	0 (0)	0 (0)	1 (16.66)	0 (0)	4 (66.66)	1 (16.66)	**NICU**
2	0 (0)	0 (0)	0 (0)	0 (0)	1 (50.0)	1 (50.0)	**Pediatrics**
7	0 (0)	1 (14.28)	1 (14.28)	0 (0)	2 (28.57)	3 (42.85)	**Internal (M)**
12	1 (8.33)	0 (0)	1 (8.33)	0 (0)	5 (41.66)	5 (41.66)	**Internal (W)**
13	0 (0)	0 (0)	3 (23)	0 (0)	2 (15.38)	7 (53.84)	**Nephrology**
6	0 (0)	0 (0)	0 (0)	1 (16.66)	2 (33.33)	3 (50)	**Surgery**
125	1 (0.8)	3 (2.4)	11 (8.8)	6 (4.8)	50 (40.0)	54 (43.2)	**Total**

Abbreviations: ICU, intensive care unit; M, men; NICU, neonates intensive care unit; OPD, outpatient department; W, women.

Table 2 The frequency of MSCoNS and MRCoNS species among different clinical samples

CoNS (no.)	MRCoNS No. (%)	MSCoNS No. (%)	Wound No. (%)	Urine No. (%)	Blood No. (%)	Trachea No. (%)	Catheter No. (%)	Sputum No. (%)
***Staphylococcus epidermidis* (54)**	35 (64.8)	19 (35.2)	7 (53.8)	36 (43.9)	2 (28.6)	0 (0)	6 (50)	3 (37.5)
***Staphylococcus haemolyticus* (50)**	23 (46)	27 (54)	4 (30.8)	35 (42.7)	3 (42.9)	1 (33.3)	2 (16.7)	5 (62.5)
***Staphylococcus hominis* (11)**	7 (63.6)	4 (36.4)	0 (0)	6 (7.3)	0 (0)	2 (66.7)	3 (25)	0 (0)
***Staphylococcus saprophyticus* (6)**	2 (33.3)	4 (66.7)	1 (7.7)	4 (4.9)	1 (14.3)	0 (0)	0 (0)	0 (0)
***Staphylococcus warneri* (3)**	0 (0)	3 (100)	0 (0)	1 (1.2)	1 (14.3)	0 (0)	1 (8.3)	0 (0)
***Staphylococcus petrasii* (1)**	1 (100)	0 (0)	1 (7.7)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)
Total (125)	68 (54.4)	57 (45.6)	13 (100)	82 (100)	7 (100)	3 (100)	12 (100)	8 (100)

Abbreviations: MRCoNS, methicillin-resistant coagulase-negative staphylococci; MSCoNS; methicillin-sensitive coagulase-negative staphylococci.

Staphylococcus warneri, 3 (2.4%), and *Staphylococcus petrasii*, 1 (0.8%). *S. epidermidis* was the most frequent species isolated from OPD (n=28 [43.75%]) and hospitalized patients (n=26 [42.62%]), followed by *S. haemolyticus* with rates of 27 (42.18%) and 23 (37.70%) from OPD and hospitalized patients, respectively. *S. petrasii* comprised the lowest frequency in this study with only one strain isolated from wound sample of a patient in internal department.

Phylogenetic analysis confirmed the PCR amplification results analyzed by Blast. The CoNS type strains were grouped into separate clusters, with high bootstrap percentages, corresponding to distinct species (Figure 1). The reference strains and their corresponding accession numbers are presented in Table 3.

Methicillin (oxacillin) resistance was demonstrated in 68 (54.4%) of CoNS based on microbroth dilution method (Table 2). Among the MRCoNS isolates, 41 (60.30%) were isolated from hospitalized patients, which was statistically significant ($P<0.005$). *S. epidermidis*, *S. hominis*, and *S. haemolyticus* showed the highest methicillin resistance rates of 64.8%, 63.6%, and 46%, respectively.

According to the results of disk diffusion susceptibility testing, the greatest resistance of CoNS was demonstrated for cefoxitin (65.6%), cotrimethoxazole (54.4%), and clindamycin (49.6%). Thirty-five CoNS strains (54.6%) in OPD patients and 47 CoNS strains (77%) in hospitalized patients were resistant to cefoxitin, but we could not find any significant difference between them. *S. hominis*, *S. epidermidis*, and *S. saprophyticus* were again accounted as the most resistant strains for cefoxitin with resistance rates of 72.7%, 72.2%, and 66.7%, respectively. Daptomycin (87.2%) and linezolid (83.2%) showed the greatest effectiveness against CoNS

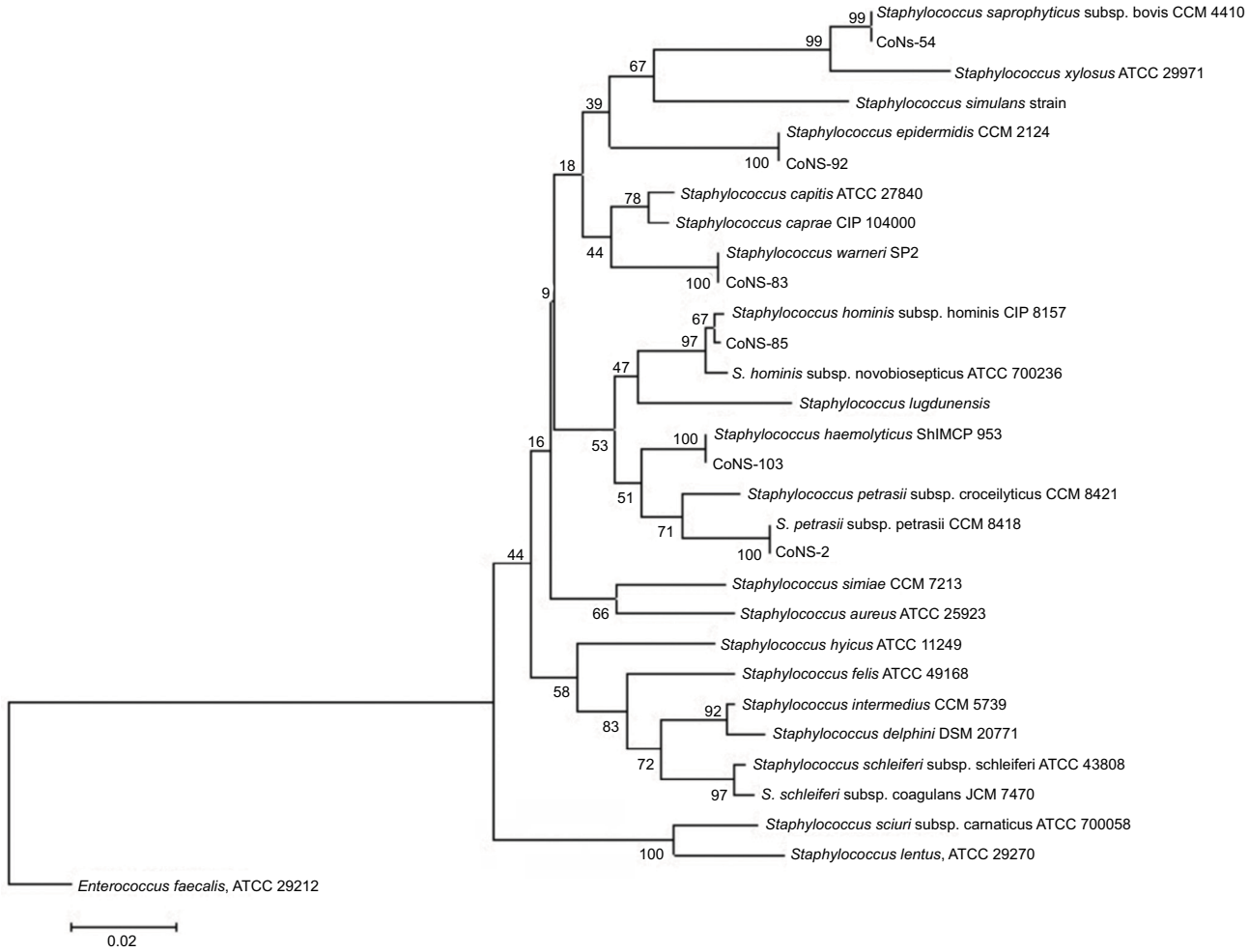

Figure 1 Phylogenetic tree inferred from the analysis of the partial gene sequence of *tuf* from CoNS isolates and reference strains (Table 3).
Notes: The tree was created using the neighbor-joining method with K2 model based on nucleotide alignment. The support of each branch, as determined from 1,000 bootstrap samples, is indicated by percentages at each node. Bar: 0.02 substitutions per nucleotide position.

Table 3 The reference strains used for construction of dendrogram

Reference strain	Accession number
Staphylococcus saprophyticus subsp. bovis **CCM 4410**	HM352934.1
Staphylococcus xylosus **ATCC 29971**	XAY763438.1
Staphylococcus simulans	HM352949.1
Staphylococcus epidermidis **CCM 2124**	HM352922.1
Staphylococcus capitis **ATCC 27840**	EU571071.1
Staphylococcus caprae **CIP 104000**	EU652787.1
Staphylococcus warneri **SP2**	KR336537.1
Staphylococcus hominis subsp. hominis **CIP8157**	EU652801.1
S. hominis subsp. ***novobiosepticus*** **ATCC700236**	HM352995.1
Staphylococcus lugdunensis	HM352926.1
Staphylococcus haemolyticus **ShIMCP 953**	HM071886.1
Staphylococcus petrasii subsp. croceilyticus **CCM 8421**	KM488624.1
S. petrasii subsp. petrasii **CCM 8418**	KM488623.1
Staphylococcus simiae **CCM 7213**	HM352931.1
Staphylococcus aureus **ATCC 25923**	CP020020.1
Staphylococcus hyicus **ATCC 11249**	CP008747.1
Staphylococcus felis **ATCC 49168**	CP027770.1
Staphylococcus intermedius **CCM 5739**	HM352933.1
Staphylococcus delphini **DCM 20771**	HM352940.1
Staphylococcus schleiferi subsp. schleiferi **ATCC 43808**	HM352937.1
S. schleiferi subsp. coagulans **JCM 7470**	HM352936.1
Staphylococcus sciuri subsp. carnaticus **ATCC 700058**	HM352946.1
Staphylococcus lentus **ATCC 29270**	HM352944.1
Enterococcus faecalis **ATCC 29212**	NC004668

isolates. The only isolate, *S. petrasii*, was the most sensitive strain in the present study and did not show any resistance to all tested antibiotics (Table 4).

There were statistically significant correlations between MSCoNS and MRCoNS with regard to antibiotic resistance to clindamycin, ticarcillin, cefoxitin, and quinupristin/dalfopristin ($P<0.001$; Table 5). However, the correlation was not significant for daptomycin, linezolid, and cotrimoxazole ($P>0.05$).

Discussion

CoNS can colonize on any open surface of body and they are considered as nonpathogenic or opportunistic agents, but they are among the most common causes of secondary infections.[22] These bacteria are capable of attaching themselves to prosthesis and catheter; they have the ability to form biofilms which are strong virulence factors.[1,23] Because of the increase in the clinical significance of CoNS, there is a need for a more accurate and sensitive method to identify CoNS species in clinical samples.[11] Correct species identification is essential to provide an accurate understanding of pathogenic capacity of diverse CoNS species and could help the clinicians for an appropriate treatment strategy.[22]

In the present study, the frequency of CoNS isolates from OPD and hospitalized patients were demonstrated as 51.2% and 48.8%, respectively. *S. epidermidis* and *S. haemolyticus* were the two most prevalent isolates representing skin flora, which under certain circumstances could act as a potential source of infection.[24,25]

Our findings also indicated that in outpatients, 92% of urinary tract colonization was due to CoNS; however, this study could not emphasize on the role of these bacteria in urinary infection. Moreover, *S. haemolyticus* and *S. epidermidis* were responsible for 42.9% and 28.6% of bacteremia, respectively. This finding was in concordant with a similar report from Turkey showing the involvement of *S. haemolyticus* (43%), and *S. epidermidis* (11.5%) in bacteremia cases.[25]

In a review of global studies conducted between 1983 and 2007 on the frequencies of isolated CoNS from patients, *S. epidermidis* was found to be the leading cause of infections followed by *S. haemolyticus* with a significant distance,[22,25] whereas, we found these prevalent isolates in closer numbers compared with other species of CoNS. In other studies also, *S. epidermidis* and *S. haemolyticus* were reported as the most common CoNS isolated from clinical specimens.[26–28] In a latter study conducted by Barros et al, however, compared

Table 4 The resistance pattern of different CoNS isolates against tested antibiotics

CoNS (no.)	OXA No. (%)	CD No. (%)	TC No. (%)	CFO No. (%)	TMP/SMX No. (%)	DAP No. (%)	LZD No. (%)	SYN No. (%)
***Staphylococcus epidermidis* (54)**	35 (64.8)	34 (63)	14 (25.9)	39 (72.2)	28 (51.9)	6 (11.1)	11 (21.1)	25 (48)
***Staphylococcus haemolyticus* (50)**	23 (46)	17 (34)	15 (30)	30 (60)	29 (58)	4 (8)	4 (7.8)	7 (13.7)
***Staphylococcus hominis* (11)**	7 (63.6)	7 (63.6)	6 (54.5)	8 (72.7)	8 (72.7)	3 (27.3)	2 (16.6)	4 (33.3)
***Staphylococcus saprophyticus* (6)**	2 (33.3)	3 (33.3)	2 (33.3)	4 (66.7)	2 (33.3)	2 (33.3)	3 (50)	2 (33.3)
***Staphylococcus warneri* (3)**	0 (0)	1 (33.3)	1 (33.3)	1 (33.3)	1 (33.3)	1 (33.3)	1 (33.3)	1 (33.3)
***Staphylococcus petrasii* (1)**	1 (100)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)
Total (125)	68 (54.4)	62 (49.6)	38 (30.4)	82 (65.6)	68 (54.4)	16 (12.8)	21 (16.8%)	39 (31.2%)

Abbreviations: CD, clindamycin; CFO, cefoxitin; DAP, daptomycin; LZD, linezolid; OXA, oxacillin; SYN, Synercid (quinupristin/dalfopristin); TC, ticarcillin; TMP/SMX, trimethoprim/sulfamethoxazole.

Table 5 Statistical analyses of antibiotic resistance patterns of CoNS isolates in present study in relation to their oxacillin resistance (statistical test with Fisher's exact test)

P-value	Chi-squared test	Oxacillin		Antibiotics	
		MSCoNS No.	MRCoNS No.		
0.24	1.5	7	14	R	Linezolid
		50	54	S	
0.001	15.2	8	31	R	Quinupristin/dalfopristin
		49	34	S	
0.001	13.4	8	30	R	Ticarcillin
		46	35	S	
0.029	4.3	25	43	R	Cotrimoxazole
		31	25	S	
0.001	28.3	14	48	R	Clindamycin
		40	16	S	
0.5950	0.5	6	10	R	Daptomycin
		51	58	S	
0.001	18.5	26	56	R	Cefoxitin
		31	12	S	

Abbreviations: MRCoNS, methicillin-resistant coagulase-negative staphylococci; R, resistant; S, sensitive.

with our findings, a lower prevalence for these species was reported.

The antibiotic resistance among CoNS has created a major problem for health care sectors worldwide.[22] The *mecA* gene is responsible for resistance against methicillin, which is located on staphylococcal chromosomal cassette elements.[29,30] It has been proven that this element has the ability of horizontal transfer among species of staphylococci especially *S. aureus*.

In the present study, the prevalence of MRCoNS was 54.4%, out of which 60.30% belonged to patients from OPD and 39.7% related to hospitalized patients and the statistical analysis demonstrated a significant difference between them. In a report from European countries on the prevalence of methicillin-resistant staphylococci, extremely high rates were demonstrated, which varied from 61.9% in Spain to 83.7% in Greece.[31] In a similar study from China, the prevalence of MRCoNS between 2004 and 2009 was reported to be 79.1%, which compared with our results was higher.[26] Furthermore, recent studies from Iran and Saudi Arabia reported rates of 81.1% and 63%, respectively.[32,33] This study demonstrated the highest prevalence of antibiotic resistance against cefoxitin (n=82 [65.6%]) among the tested CoNS. Fifty-six (68.3%) out of 82 cefoxitin-resistant strains also presented oxacillin resistance, and this relationship has proven to be statistically significant (*P*=0.0001). In a similar study from Brazil, only 32% of their CoNS strains were reported to be cefoxitin resistant, which was lower than our findings. Moreover, in their study, *S. epidermidis* and *Staphylococcus simolance* showed the highest resistance to cefoxitin (41.5%),[34] while our study presented *S. hominis* (72.7%) followed by *S. epidermidis* (72.2%) as the most resistant species of CoNS to cefoxitin.

To overcome the antibiotic resistance, currently new drugs including linezolid, daptomycin, tigecycline, and quinupristin/dalfopristin have been administered and shown to be useful.[31,35] Investigations are still emphasized on the continuous susceptibility of CoNS clinical strains to these antibiotics.[35,36]

Conclusion

Our results confirmed the predominance of *S. epidermidis* and *S. haemolyticus* among CoNS isolates. The accurate identification of CoNS to the species level in reference laboratories is important to establish the role of each staphylococcal species as an infectious agent and to conduct epidemiologic investigations.[18]

The high prevalence of MRCoNS strains is a serious concern and strongly suggests the need for control program measures in our hospitals in order to reduce MRCoNS infections especially in immunocompromised patients. In this regard, molecular epidemiology surveillance could be a useful way to investigate the transmission patterns of CoNS to control nosocomial infections due to these bacteria.

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Disclosure

The authors report no conflicts of interest in this work.

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