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Detection of Integrons and Staphylococcal Cassette Chromosome *mec* Types in Clinical Methicillin-resistant Coagulase Negative Staphylococci Strains

Fahimeh Hajiahmadi[a], Elham Salimi Ghale[a], Mohammad Yousef Alikhani[a], Alireza Mordadi[a], Mohammad Reza Arabestani[a,b]

[a]Department of Microbiology, Hamadan University of Medical Sciences, Hamadan, Iran
[b]Brucellosis Research Center, Hamadan University of Medical Sciences, Hamadan, Iran

Objectives: Integrons are thought to play an important role in the spread of antibiotic resistance. This study investigates class 1 and 2 integron-positive methicillin-resistant coagulase-negative staphylococci strains isolated in Iran and characterizes their patterns of antimicrobial resistance.
Methods: Hundred clinical isolates of coagulase-negative staphylococci were characterized for integron content and staphylococcal cassette chromosome *mec* (SCCmec) type.
Results: Sixteen isolates carried class 1 (*intI1*) integrons and four isolates carried class 2 (*intI2*) integrons. One resistance gene array was identified among the class 1 integrons (*aadA1* cassette). The distribution of SCCmec types in 50 methicillin-resistant coagulase-negative staphylococci strains showed that SCCmec types III and V dominated among the tested strains.
Conclusion: This is the first report of methicillin-resistant coagulase-negative staphylococci strains that carry two mobile genetic elements, including class 1 and 2 integrons and SCCmec, in Iran.

Key Words: coagulase-negative staphylococci, integrons, staphylococcal cassette chromosome *mec*

Corresponding author:
Mohammad Reza Arabestani
E-mail: mohammad.arabestani@gmail.com

INTRODUCTION

Coagulase-negative staphylococci (CoNS) are a frequent cause of nosocomial and bacterial infections, especially in patients with medical devices and immunocompromised patients [1]. CoNS are also the most frequently isolated pathogens in intravascular catheter-related infections and infections from other implanted devices [2]. Many isolates of CoNS are becoming resistant to most beta lactams due to the *mecA* gene. The *mecA* gene encoding methicillin resistance is located on a mobile genetic element, designated as staphylococcal cassette chromosome *mec* (SCCmec). These elements are widely disseminated among *Staphylococcus* species, which might be due to horizontal transmissions [3]. In addition to the *mec* gene complex (the *mecA* gene and its regulators), SCCmec is also contained in the *ccr* gene complex, where it encodes site-specific recombinases that are responsible for the integration and excision of the cassette in the bacterial genome [4,5].

SCCmec displays a more polymorphous structure in methicillin-resistant coagulase-negative staphylococci (MR-CoNS), which are more frequent carriers of SCCmec than *Staphylococcus aureus*. The *ccr* and *mec* genes may be combined together in CoNS from an unknown source,

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although frequent *ccr-mec* combinations have not been described in methicillin-resistant *S. aureus* (MRSA) [6–8]. Therefore, SCCmec transfers from MR-CoNS to methicillin-sensitive *S. aureus* may occur [9–11]. However, this mechanism is not known. Eleven different types and several subtypes of SCCmec have been identified. SCCmec types I, II, and III mainly cause nosocomial infections and are significantly larger than the other types. Community-associated MRSA mainly carries SCCmec types IV and V [12].

In recent years, the role of integrons and gene cassettes in the dissemination of antibiotic resistance among pathogenic bacteria has been well documented [13]. Several classes of integrons, based on the homology of their respective integrase genes, have been described from pathogenic gram-negative bacteria. Of these, the class 1 integrons are the most commonly found and widely distributed. These integrons form a gene capture and expression system that is known to be responsible for multidrug resistance. They consist of an integrase gene (*intI*), which encodes a site-specific recombinase enzyme; a primary recombination site called *attI*, which is recognized by the integrase; and a promoter region (promoter cassette), which directs transcription of the integrated genes [14]. These integrons can carry single or multiple gene cassettes, which become a part of the integron when integrated; these gene cassettes can be coded for resistance to different antibiotics [15].

Although class 1 integrons are widely distributed among clinically isolated gram-negative bacteria, they have also been observed in gram-positive bacteria, including *Corynebacterium*, *Staphylococcus*, *Streptococcus*, *Enterococcus*, *Brevibacterium*, and *Aerococcus* (mostly in *Staphylococcus*) [16]. Class 1 integron-positive, gram-negative bacteria include Enterobacteriaceae, *Acinetobacter*, *Aeromonas*, *Alacaligenes*, *Burkholderia*, *Campylobacter*, *Pseudomonas*, *Stenotrophomonas*, and *Vibrio* [17]. The presence of class 1 integrons in Enterobacteriaceae is higher than in nonfermenting bacteria.

A small number of studies about integrons have focused on clinical staphylococcal isolates. The presence of class 1 integrons in clinical staphylococcal isolates was shown in China and other countries at high rates in MR-CoNS strains [18–22]. Therefore, increasing antibiotic resistance mediated by integrons in gram-positive bacteria has become a serious public health concern worldwide. Integrons and SCCmec are the reservoirs of many types of genes and can be transferred from one strain to another; therefore, their simultaneous existence would likely speed up the genetic exchange and effectiveness of genome evolution in staphylococci. In this article, we aim to determine the occurrence and prevalence of MR-CoNS-associated integrons, as well as the SCCmec types, in a hospital setting during 2014–2015.

MATERIALS AND METHODS

1. Bacterial strains

During 2014–2015, 100 CoNS isolates of three staphylococcal species from human sources (*Staphylococcus epidermidis*, *Staphylococcus haemolyticus*, and *Staphylococcus saprophyticus*) were isolated from various clinical specimens in the microbiology laboratories of three major hospitals of Hamedan, Iran. The majority of the samples were blood (44%); the others were isolated from urine specimens (38%), wounds (4%), and catheters (14%). Specimens were cultured on blood agar and chocolate agar (Merck, Darmstadt, Germany) and isolates were identified by standard biochemical methods. Verified isolates were preserved at −70°C in Trypticase soy broth (Merck) containing 20% (v/v) glycerol for further analysis.

2. Antimicrobial susceptibility testing

Antimicrobial susceptibility testing was performed by the standard disk diffusion method according to the Clinical and Laboratory Standards Institute (CLSI) guidelines [23] for the following: chloramphenicol (30 μg), cefoxitin (30 μg), clindamycin (2 μg), doxycycline (30 μg), erythromycin (15 μg), gentamicin (10 μg), levofloxacin (5 μg), novobiocin (5 μg), rifampicin (5 μg), trimethoprim-sulfamethoxazole (25 μg), and vancomycin (30 μg) (MAST, Merseyside, UK). Isolates that were shown to be resistant to at least three different classes of antimicrobial agents were classified as multi-drug resistant. *S. aureus* ATCC33591 strain was used for controlling the test.

3. Template DNA preparation

Template DNA was prepared using a GeneMATRIX Quick Blood DNA Purification Kit (EURx Ltd., Gdansk, Poland) according to the manufacturer's instructions.

4. Detection of the *mecA* gene and SCCmec typing

The presence of the *mecA* gene was detected in all isolates by polymerase chain reaction (PCR) using specific primers (Table 1 [5,24–27]). PCR primer sets (Table 1) were selected from published papers based on their specificity, compatibility, and the ability to target fragments of SCCmec types I–V. The 25-mL PCR mixtures consisted of 5 μL template DNA, 0.2 mM of each deoxynucleoside triphosphate, 10 pmol of each primer, 10 mM Tris–HCl, 1.5 mM $MgCl_2$; 50 mM KCl, and 1.5 U of Taq DNA polymerase.

Amplification involved an initial denaturation at 94°C for 15 minutes, followed by 30 cycles of denaturation (94°C for 30 seconds), annealing (57°C for 1.5 minutes), and extension (72°C for 1.5 minutes), with a final extension step (72°C for 10 minutes).

The amplified DNA was separated by gel electrophoresis on 1% agarose and visualized under ultraviolet transillumination. For quality control, we used *S. aureus* ATCC29247 strains (for *mecA* gene), *S. aureus* NCTC 10442 (for Type I), *S. aureus* N315 (for Type II), *S. aureus* 85/2082(for Type III), *S. aureus* JCSC 4744 (for Type IV), and *S. aureus* WIS (for Type V).

5. Integron characterization and sequencing of resistance-encoding gene cassettes

The presence of integron genes and resistance-encoding gene cassettes associated with class 1 was investigated by PCR using specific primers (Table 1). PCR was performed in a reaction mixture with total volume of 25 μL, containing 2 μL template DNA, 0.2 mM of each deoxynucleoside triphosphate, 10 pmol of each primer, 10 mM Tris-HCl, 1.5 mM $MgCl_2$, 50 mM KCl, and 1.5 U of Taq DNA polymerase. PCR was performed with the Eppendorf and Biorad thermocycler (ASTEC Co., Hamadan, Iran).

Amplification was performed as follows: initial denaturation step at 94°C for 5 minutes, followed by 35 cycles consisting of denaturation (94°C for 30 seconds), annealing (55°C, 30 seconds for *intI1*, *intI2*, and 5′CS and 3′CS), and extension (72°C for 1 minute), followed by a final extension step. The PCR products of variable regions were cut out from the agarose gel and purified using a QIAquick gel extraction kit (Qiagen, Valencia, CA, USA). The sequencing of gene cassettes of class 1 integrons was performed using the ABI 3730X capillary sequencer (Genfanavaran and Macrogen, Seoul, Korea). Finally, sequence data were analyzed with Chromas software and aligned with the GenBank databases using the BLAST algorithm, which is available through the National Center for Biotechnology Information website (http://www.ncbi.nlm.nih.gov).

RESULTS

1. Antimicrobial susceptibility testing

The antimicrobial resistance of 100 CoNS isolates, including 56 isolates of *S. epidermidis*, 40 isolates of *S. haemolyticus*, and 4 isolates of *S. saprophyticus*, are presented in Table 2. In all, 64% (32 isolates) of MR-CoNS isolates and 18% (9 isolates) of methicillin-susceptible CoNS isolates were multi-drug resistant. All 20 integron-bearing MR-CoNS strains were resistant to cefoxitin (100%, n = 20), followed by trimethoprim/sulfamethoxazole (60%, n = 12) and doxycycline/erythromycin (25%, n = 5). The percentage of resistance to levofloxacin, clindamycin, gentamicin, tetracycline, and chloramphenicol ranged from 15 to 20% (Table 2). None of the tested isolates showed resistance to vancomycin, rifampicin, or novobiocin.

2. Integron analysis

Of the 100 CoNS isolates, 16 isolates carried class 1(*intI1*) integrons and 4 isolates carried class 2 (*intI2*) integrons. Class 1 integrons were more frequent among CoNS isolates compared with class 2 integrons. All isolates that carried integrons had the *mecA*

Table 1. Primers used in this study

Gene target	Primer sequence (5′ to 3′)	Amplicon/product size (bp)	Reference
intI1	F: CAGTGGACATAAGCCTGTTC R: CCCGACGCATAGACTGTA	160	[33]
intI2	F: TTGCGAGTATCCATAACCTG R: TTACCTGCACTGGATTAAGC	288	[33]
3CS,5CS	F: GGCATCCAAGCAGCAAG R: AAG CAG ACT TGA CCT GA	Variable	[34]
mecA	F: TCCAGATTACAACTTCACCAGG R: CCACTTCATATCTTGTAACG	162	[35]
Type I	F: GCTTTAAAGAGTGTCGTTACAGG R: GTTCTCTCATAGTATGACGTCC	613	[5]
Type II	F: GATTACTTCAGAACCAGGTCAT R: TAAACTGTGTCACACGATCCAT	287	[36]
Type III	F: CATTTGTGAAACACAGTACG R: GTTATTGAGACTCCTAAAGC	243	[35]
Type IV	F: GCCTACTCTTCTGAAAAGCGTCG R: CTTATTCGAAGAAACCG	776	[5]
Type V	F: GAACATTGTTACTTAAATGAGCG R: TGAAAGTTGTACCCTTGACACC	325	[5]

Table 2. Prevalence of antibiotic-resistant strains among 50 MR-CoNS and 50 MS-CoNS

Antimicrobial agent	MR-CoNS (n = 50)			MS-CoNS (n = 50)		
	S. saprophyticus (n = 2)	*S. haemolyticus* (n = 23)	*S. epidermidis* (n = 25)	*S. saprophyticus* (n = 2)	*S. haemolyticus* (n = 17)	*S. epidermidis* (n = 31)
Clindamycin	0	5	4	0	4	6
Chloramphenicol	0	7	8	0	2	6
Doxycycline	0	2	7	0	6	10
Erythromycin	0	9	4	1	4	12
Cefoxitin	2	23	25	0	0	0
Gentamicin	0	6	8	1	2	3
Levoflxacin	0	3	1	1	2	5
Novobiocin	2	0	0	2	1	0
Rifampicin	1	0	0	0	1	2
Trimethoprim-sulfamethoxazole	1	13	14	1	5	14
Vancomycin	0	0	0	0	0	0

MR, methicillin-resistant; CoNS, coagulase-negative staphylococci; MS, methicillin-sensitive; *S.*, *Staphylococcus*.

Table 3. Genotypic characteristics of 20 integron-bearing MR-CoNS strains

Species	Source	Integron class	Gene cassette	SCCmec typing
S. epidermidis	Urine	1	–	NT
	Blood	1	–	V
	Wound	1	–	III
	Urine	1	–	NT
	Blood	1	–	III
	Blood	1	aadA1	V
	Catheter	1	aadA1	I
	Blood	1	aadA1	NT
	Blood	1	–	NT
	Urine	2	–	III
	Blood	2	–	I
S. haemolyticus	Blood	1	–	NT
	Urine	1	–	II
	Blood	1	–	NT
	Blood	1	–	III
	Urine	1	–	NT
	Urine	1	aadA1	V
	Urine	1	aadA1	III
	Blood	2	–	II
	Urine	2	–	V

MR-CoNS, methicillin-resistant coagulase-negative staphylococci; SCCmec, staphylococcal cassette chromosome *mec*; *S.*, *Staphylococcus*; NT, not typeable; –, not carrying gene cassettes.

Table 4. Distribution of SCCmec types in 30 non-integron-bearing MR-CoNS strains

Species	SCCmec typing (No. of isolates)					
	I	II	III	IV	V	NT
S. epidermidis (n = 14)	0	1	8	3	0	2
S. haemolyticus (n = 14)	1	3	2	1	7	0
S. saprophyticus (n = 2)	0	0	0	0	0	2

SCCmec, staphylococcal cassette chromosome *mec*; MR-CoNS, methicillin-resistant coagulase-negative staphylococci; NT, not typeable; *S.*, *Staphylococcus*.

gene (100%). One type of gene cassette array was found in class 1 integrons. Sequence analysis of the integron variable regions indicated the presence of aminoglycoside 3′-adenyltransferase (*aadA1*; 1,000 bp). A total of 37.5% of MR-CoNS isolates (6 of 16 isolates) harbored *aadA1* gene cassettes.

3. SCCmec typing

Fifty isolates of CoNS were positive for *mecA* and a certain type of SCCmec. The distribution of SCCmec types in the 50 MR-CoNS strains showed that SCCmec types III and V dominated among the tested strains (Tables 2 and 3). Of the 20 integron-bearing MR-CoNS strains, five strains had type III SCCmec, four strains had type V SCCmec, two strains had type II SCCmec, two strains had type I SCCmec, and seven strains were untypeable (Table 3). None of the tested strains had type IV. Of the 30 non-integron-bearing MR-CoNS strains, ten strains had type III SCCmec, eight strains had type V SCCmec, five strains had type II

SCCmec, four strains had type IV SCCmec, and two strains had type I SCCmec, with five strains being untypeable (Table 4).

DISCUSSION

Despite the remarkable advances in therapeutic options and novel drug discovery efforts, the development of antimicrobial resistance in microorganism's remains as a serious public health concern in the treatment of infectious diseases. The horizontal transmission of antimicrobial resistance genes via mobile elements, such as plasmids and transposons, plays an important role in the evolution and dissemination of multi-drug resistance [22,28].

In this study, we isolated three species of CoNS strains—*S. epidermidis*, *S. saprophyticus*, and *S. haemolyticus*—which were simultaneously carrying gene cassettes within a class 1 integron and SCCmec. We identified one type of gene cassette array and five different types of SCCmec. To our knowledge, this is the first report of CoNS strains that carry two mobile genetic elements, including class 1 and class 2 integrons and SCCmec, in Iran.

Integrons are now well known as a primary source of resistance genes, and they are suspected to facilitate the spread of antimicrobial resistance genes and the rapid evolution of resistance within microbial populations [16]. Integrons can harbor more than 100 different antibiotic resistance gene cassettes, which encode adaptations that extend beyond antibiotic resistance and pathogenicity. Other types of mobile genetic elements, such as plasmids, transposons, and even entire chromosomes, have been identified as major sources of integrons, which are able to facilitate the spread of genetic material between species or genera of bacteria [29].

In the present study of 100 CoNS isolates, 16 (16%) and 4 (4%) clinical isolates were found to carry class 1 integrons and class 2 integrons, respectively. These results indicate that class 1 integrons are more prevalent than class 2 integrons in clinical isolates. This is similar to the findings of a study of China from 2001 to 2006. In this study, 209 MRSA and 53 MR-CoNS strains were isolated from various clinical samples. Class 1 integrons were detected in 122 MRSA strains. None of the isolates contained class 2 or class 3 integrons [30]. In another study by Veise et al [31], 200 *Staphylococcus* isolates were isolated from nasal and throat swabs at Sanandaj Hospital in Iran. Class 1 integrons were detected in 81 isolates (40.1%), including 37 (40.1%) *S. aureus*, 35 (23.5%) *S. epidermidis*, and 9 (36.0%) *S. saprophyticus* strains. The results of this study indicate a high prevalence of integrons in *Staphylococcus* isolates. The differences in the prevalence of integron genes can be due to the differing geographic regions, the bacteria strains, or the indiscriminate use of antibiotics. The low prevalence of integrons in our study may be carried on other genetic elements [32].

We also found one gene cassette in *intI1*-positive isolates, including *aadA1*—an encoded protein that may contribute to the bacterial isolates' resistance to aminoglycosides (streptomycin/spectinomycin). In our study, the class 1 integron containing the *aadA1* gene was similar to that found in gram-negative bacteria [33]. Therefore, the transfer of resistance genes that may occur between ram-positive and gram-negative organisms could lead to the construction of diverse resistance to the usual antibiotics [34]. All integron-positive isolates had more resistance to antibiotics than integron-negative isolates, and resistant phenotypes usually correlated with the presence of integrons. Furthermore, a considerably large number of the MR-CoNS isolates (20 out of 50; 40%) carried class 1 integrons. Our results suggest that the identification of and screening for integrons may assist in guiding treatment regimens and could complement existing antibiotic resistance surveillance programs by providing information about antibiotic resistance and widespread dissemination among bacterial populations.

Our study also addressed the role of SCCmec in the transmission of MR-CoNS strains. The SCCmec element is a mobile genetic element that carries the *mecA* gene and is widely distributed among MR-CoNS species. Among 50 MR-CoNS strains, SCCmec types III and V dominated. Similar results were previously described in a study performed by Murugesan et al [35] in South India, in which types I and IV SCCmec dominated among the tested strains. Xu et al [36] found types I, II, and V SCCmec to be the most common in China.

Integrons and SCCmec can serve as reservoirs for different resistance genes. Therefore, they might act as a vehicle for the exchange of useful genes and resistance genes to improve the survival for staphylococci in various environments. The transfer of these genes is dependent on the host species, the environment, and the geographical location. As is generally accepted, the indiscriminate use of antibiotics can proliferate the emergence and selection of antibiotic-associated mobile genetic elements, which to some extent is reflected by the domination of nosocomial SCCmec and the prevalence of integrons. In fact, these elements act as a reservoir for many kinds of genes and are able to exchange genes between species; for this reason, bacteria can adapt to environmental and stressful conditions [22].

In Iran, antibiotic resistance is increasing due to the indiscriminate use of antibiotics and poor performance of regulations on the clinical use of antibiotics. The widespread occurrence of integrons and SCCmec will be a major challenge in the treatment and control of infectious diseases.

CONFLICTS OF INTEREST

No potential conflict of interest relevant to this article was reported.

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