Iraqi Journal of Biotechnology, May 2019, Vol. 18, No. 2, 289-299

Characterization of Five Types of Staphylococcal Cassette Chromosomal *mec* Genes in Methicillin-Resistant *Staphylococcus aureus* (MRSA) Isolates from Iraqi Patients

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Received: March 5, 2019 / **Accepted:** April 10, 2019

Abstract: The distribution of Staphylococcal Cassette Chromosomal *mec* (SCCmec) types I, II, III, IV and V was assessed in 137 methicillin-resistant *S.aureus* (MRSA) isolates obtained from patients from different hospitals in Baghdad city. Each types responsible for certain virulence factors. It was found that 38 (27.73%) isolates of MRSA out of 137 contained SCC*mec*I, the lest percentage of SCC*mec* types in all MRSA isolates was type II, It was found that 8 (5.83%) isolates only were positive in this type. While 22 isolates (16.05%) contain SCC*mec* III. The number of isolates detected in SCC*mec* type IV were 53 (38.68%) isolates out of 137 while 86 (62.77%) for type V which represented the highest percentage with contrast with other types.

Key words: *S. aureus*, virulence factors, (SCCmec) types, MRSA.

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Introduction:

Staphylococcal cassette chromosome (SCC) elements are a complex mobile genetic elements that often carry antimicrobial resistance and in some cases virulence-associated genes. Nearly all MRSA strains contain the *SCCmec* element. They integrate into the Staphylococcal chromosome at a specific site (*attB* or the integration site sequence *ISS*) within the 3' end of the *orfX* gene encoding a ribosomal methyl transferase (1). The large *SCCmec* types I to III are present in HA-MRSA strains and were likely transferred to *S. aureus* from a commensal Staphylococcal species on a few occasions .The smaller SCC*mec* types IV and V which associated with CA_MRSA are believed to have been transferred to methicillin-susceptible backgrounds frequently, with the resultant emergence of novel, fit MRSA strains bearing the type IV or V elements (2, 3).

Materials and Methods:

1- Clinical Isolates:

Two hundred and ten different clinical samples were collected from different sources (wound , urine , burns , boils and abscesses) from three hospitals in Baghdad city included Al-kindy teachinq hospital, Al-Yarmouk hospital and Ibn Al-balady hospitals during the period January 2015 till the end of June 2015. The clinical samples

were collected from different patients attending the hospitals using sterile swabs, placed into a transport medium and transported to the laboratory immediately.

2-Isolation and identification of *S. aureus* by traditional methods:

- Culturing on selective media:

The collected specimens were inoculated on the blood, incubated at 37ºC for 24 hours. Isolates were examined for their shape, size, color, pigments, and hemolytic activity. Then they were transferred and streaked on mannitol salt agar (MSA) and incubated at 37°C for 24 hr. *Staphylococcus aureus* was identified depending on the morphological features on culture media and biochemical tests using API Staph.

3-Molecular identification of *S. aureus* :

- Bacterial Genomic DNA Extraction:

An overnight culture in brain heart infusion broth was collected by centrifugation and extraction of DNA from isolated bacteria, carried out by using genomic DNA kit (Gene aid). Preserved DNA with 50-100µl of TE solution in Eppendorf tubes at 20-Cº.

- Detection of *S. aureus* by polymerase chain reaction (PCR):

1- Detection of *S. aureus* by *nuc* gene specific primer:

PCR used for detection of the *nuc* gene for conformation the identification of the *S. aureus*, according to (4).These primers synthesized by Cinna Gen Company (Table 1).

Table (1): The sequence and concentration of forward and reverse primers of *nuc* gene.

Primers Type	Primers Sequence	Concentration in picomoles	Product size
nuc Forward	5-GCGATTGATGGTGATACGGTT--3	30262.27	300bp
nuc Reverse	5-AGCCAAGCCTTGACGAACTAAAGC-3	35265.50	300bp

PCR reaction was conducted in master mix tube containing lypholyzied master mix with 20µl of reaction mixture containing, 1 µl of each primer, 5 µl DNA template and 13 µl of deionized water (Table 2).

Table (2): The mixture of conventional PCR working solution for detection of, *nuc* gene in *S. aureus*.

Working solution	
Water	13 µl
Forward primer	1 µl
Reverse primer	1 µl
DNA	5 µl
Final volume	20 µl

Amplification was conducted using a master cycler Eppendorf programmed with 35 cycles for Initial denaturation 95°C for 3 min., Denaturation for 94°C 1min., Annealing 55°C for 30 sec., Extension 72°C for 1.5min and final Extension 72°C for 3.5min.

2- Detection of *Mec A* gene in *S. aureus* by specific primer:

PCR used for detection of the *MecA* gene for conformation the identification of the *S. aureus*, according to (5). These primers synthesized by Cinna Gen Company (Table 3).

Table (3): The sequence and concentration of forward and reverse primers of *MecA* gene.

Primers Type	Primers Sequence	Product size
mecA Forward	5-AACAGGTGAATTATTAGCACTTGTAAG-3	170 bp
mecA Reverse	5-ATTGCTGTTAATATTTTTTGAGTTGAA-3	170 bp

PCR reaction was conducted in 20µl of reaction mixture containing, 1 µl of each primer, 5 µl DNA template and 13 µl of deionized water.

Amplification was conducted using a master cycler Eppendorf programmed with 35 cycler of Initial denaturation 95°C for 4 min., Denaturation 94°C for 30 sec., Annealing 55°C for 1min., Extension 72°C for 1min and final Extension 72°Cfor 5min. (Table 4).

Table (4): PCR program for *mecA*gene amplification by the conventional method.

Thermocycler conditions	Temperature (°C)	Time (min)
Initial denaturation	95 °C	4 min
Cycles number : 35 cycle		
Denaturation **Primmer annealing** **Primmer extension**	95 °C 58 °C 72 °C	0.5 min 1 min 1 min
Final extend	72 °C	5 min

Table (5): The sequence of forward and reverse primers of each gene of *SCCmec* types.

Primers Type	Primers Sequence (5` – 3`)	Product size bp	References
SCC*mec* I Forward	TTCGAGTTGCTGATGAAGAAGG	495	(6)
SCC*mec* I Reverse	ATTTACCACAAGGACTACCAGC	495	(6)
SCC*mec* II Forward	AATCATCTGCCATTGGTGATGC	284	(6)
SCC*mec* II Reverse	CGAATGAAGTGAAAGAAAGTGG	284	(6)
SCC*mec* III Forward	TTCTTAAGTACACGCTGAATCG	414	(6)
SCC*mec* III Reverse	GTCACAGTAATTCCATCAATGC	414	(6)
SCC*mec* IV Forward	GCCTTATTCGAAGAAACCG	776	(7)
SCC*mec* IV Reverse	CTACTCTTCTGAAAAGCGTCG	776	(7)
SCC*mec* V Forward	GAACATTGTTACTTAAATGAGCG	325	(7)
SCC*mec* V Reverse	TGAAAGTTGTACCCTTGACACC	325	(7)

PCR reaction was conducted in 20µl of reaction mixture containing, 1 µl of each primer, 5 µl DNA template and 13 µl of deionized water (Table 6).

Table (6): The mixture of conventional PCR working solution for detection of, each gene of *sccmec* types in *S. aureus.*

Working solution	
Water	13 µl
Forward primer	1 µl
Reverse primer	1 µl
DNA	5 µl
Final volume	20 µl

Table (7): PCR program for *sccmec type I, II, III gene* amplification by the conventional methods.

Thermocycler conditions	Temperature (°C)	Time (min)
Initial denaturation	95 °C	10 min
Cycles number : 35 cycle		
Denaturation	94 °C	0.5 min
Primer annealing	53 °C	0.5 sec
Primer extension	72 °C	1 min
Final extend	72 °C	10 min

Table (8): PCR program for *sccmec type IV* gene amplification by the conventional methods.

Thermocycler conditions	Temperature (°C)	Time (min)
Initial denaturation	95 °C	5min
Cycles number : 35 cycle		
Denaturation	95 °C	0.5 min
Primer annealing	57 °C	0.5 sec
Primer extension	72 °C	0.5 min
Final extend	72 °C	4 min

Table (9): PCR program for *sccmec type V gene* amplification by the conventional methods.

Thermocycler conditions	Temperature (°C)	Time (min)
Initial denaturation	94 °C	5min
Cycles number : 35 cycle		
Denaturation	94 °C	1 min
Primer annealing	51 °C	1 sec
Primer extension	72 °C	1 min
Final extend	72 °C	10 min

Triplex PCR of *SCCmec* type I, II, III (Duarte and Hermínia 2002):

The triplex PCR used to detect three genes in one run of PCR, the mixture of reaction is shown in (Table 10) and the PCR programme for this mixture is shown in (Table 11).

Table (10): Triplex PCR Working Solution

Working solution with lyophilized master mix	
Water	9µl
SCC *mec*I primer F	1 µl
SCC *mec*I primer R	1µl
SCC *mec*II primer F	1µl
SCC *mec*II primer R	1µl
SCC *mec*III primer F	1µl
SCC *mec*III primer R	1µl
DNA	5 µl
Final volume	20 µl

Table (11): Triplex PCR Protocol.

Thermocycler conditions	Temperature (°C)	Time (min)
Initial denaturation	94 °C	4 min
Cycles number : 35 cycle		
Denaturation	94 °C	1 min
Primer annealing	57 °C	1 min
Primer extension	72°C	2 min
Final extend	72°C	5 min

Analysis of PCR Products:

The PCR products and the ladder marker (10µl) were resolved by electrophoresis. 10 µl of the product were loaded on 2% agarose gels containing 2µl ethidium bromide (5µg/ml) and run at 5volt /cm for 2 hours. Finally, bands were visualized on a UV transiluminator and photographed using a digital camera.

Result And Dissection:

1- Clinical Samples:

- Identification of *S. aureus* by traditional methods:

A total number of 210 clinical samples were collected from different three hospitals in Baghdad city Al-kindy technical hospital, Al-Yarmouk and Ibn Al-balady hospitals. The specimens included nasal swab, wound swab, burn swab, abscess and pus, sputum, ear swab, urine and blood culture. One hundred fifty isolates identified as *Staphylococci* on a mannitol salt agar depending on yellow color of the colonies, the media considered a selective and differential growth medium which is used for encouraging the growth of Staphylococci and inhibit others by containing high concentration of NaCl and phenol red as an indicator (Figure 1). 150 isolates gave a positive result and were identified as *S. aureus* due to the production of catalase enzyme which is distinguished them from Streptococcus spp. Finally, the API Staph. System was used for accurate identification of the isolates at generic and species level. The test was applied on all isolates, which previously identified by conventional biochemical tests the results gained from API Staph system were in covenant with those obtained from biochemical identification (8).

Figure (1): Appearance of *S. areas* isolates on mannitol salt agar.

Identification by Molecular Methods:

All isolates were submitted to conventional PCR for further identification on molecular level by using specific primers for detection *nuc* gene, from (150) S. *aureus* isolates recognizing depending on traditional methods,(143) isolates were positive for *nuc* gene with product size 300pb (Figure 2).

The *nuc* primers set was recognized all tested isolates belonged to *S. aureus*, but not other bacteria tested Published data indicate that treatment with antibiotics does not interfere with the detection of the *nuc* gene as long as minimum quantities of the target DNA sequences are still present in the clinical specimens.

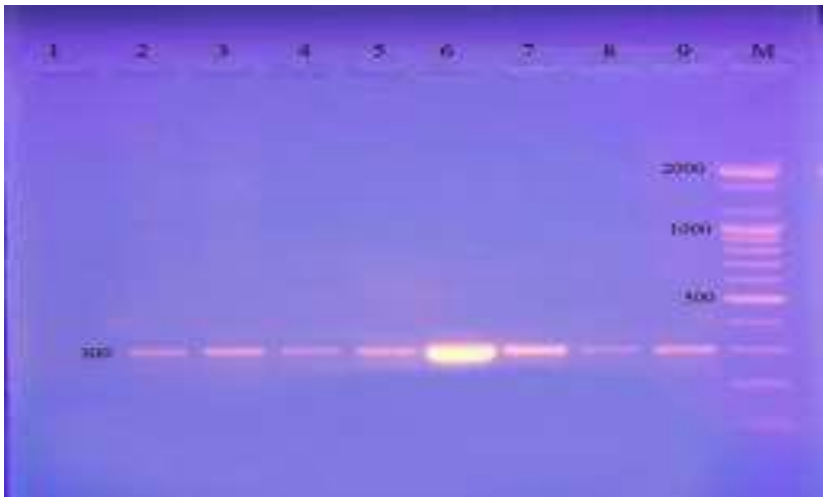

Figure (2): Agarose gel electrophoresis of PCR amplification products of *S.aureus, nuc* gene (2% agarose, , 5V/Cm, 2hr.). M: The DNA marker (100 bp ladder),Lanes (2-9) positive amplification of 300 bp for *nuc* gene.

The isolates that were previously identified by morphological, biochemical characteristics and molecular level by *nuc* gene primers as *S. aureus* were tested for antibiotic susceptibility using Methicillin antibiotic discs (5µg/disc) by applying the antibiotic disc diffusion method. The results of this study confirmed that out of (143) tested *S. aureus* isolates that were (137) isolated exhibited a high level of resistance to Methicillin, the target antibiotic, which is reflected MRSA. This result is agreed with the outcome obtained by (9) in Saudi Arabia. Rapid and accurate detection of methicillin resistance in *S. aureus* is essential for the use of appropriate antimicrobial therapy and for the control of nosocomial spread of MRSA strains. Thus evaluation the efficiency of the disk diffusion method is important. All the positive isolates (137) that were characterized as MRSA by the (methicillin disc test) were subjected to PCR to detect the presence of *mecA* gene, all of them gave positive results with 170pb PCR product (Figure 3). The acquisition of *mec A* gene is considered to be the first genetic requisite for methicillin resistance in *Staphylococci* (10)

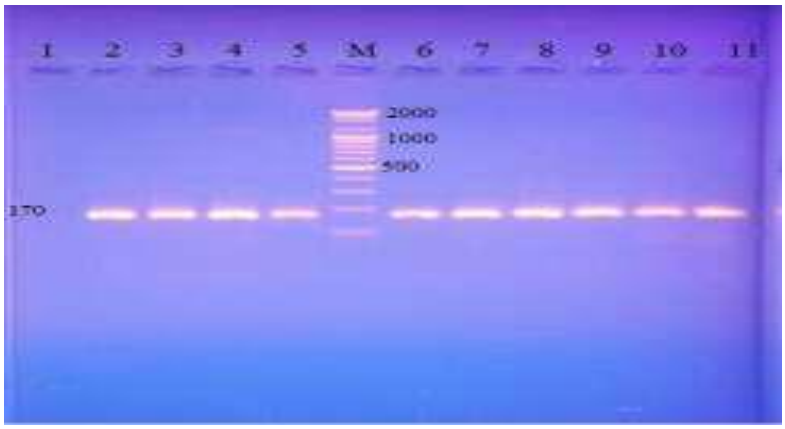

Figure (3): Agarose gel electrophoresis of PCR amplification products of *S.aureus*, *mecA* gene (2% agarose, 5V/Cm, 2hr.). M: The DNA molecular Wight marker (100 bp ladder).; Lanes (2-11) positive amplification of 170 bp for *mecA* gene.

MRSA developed resistance to β-lactam antibiotics through the acquisition of the *mecA* gene that encodes penicillin-binding protein 2a (PBP2a), which has a significantly reduced affinity for β-lactam antibiotics, thereby conferring β- lactam resistance (11). The detection of *mecA* by the Polymerase Chain Reaction (PCR) is considered a gold-standard technique for methicillin resistance detection (12). This is mainly because other methods such as the phenotypic methods may be difficult to interpret and some isolates do not express their *mecA* gene unless selective pressure via antibiotic treatment is applied (5).

Detection of *SCCmec* types I, II, III, IV, V:

In this study, the distribution of *SCCmec* types I, II, III, IV and V was evaluated in methicillin-resistant *S.aureus* obtained from patients in hospitals. In this study 38 (27.73%) isolates of MRSA out of 137 contained *SCCmec*I and it is showed a positive result in the gel electrophoresis when observed under U.V. light, the product size of its primer used in PCR was 495 bp (Figure 4).

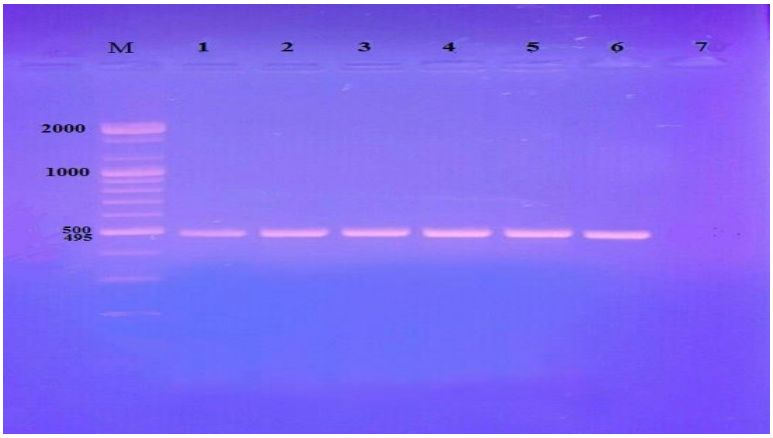

Figure (4): Agarose gel electrophoresis of PCR amplification products of *S.aureus, SCCmec* I gene (495bp) (2% agarose, , 5V/Cm, 2hr.). M: The DNA marker (100 bp ladder). ; Lanes (1-6) positive lane 7 Negative.

The lest percentage of *SCCmec* types in all MRSA isolates was type II. It was found that 8 (5.83%) isolates only were positive for this type (Figure 5), While 22 isolates (16.05%) contain *SCCmec* III (Figure 6).

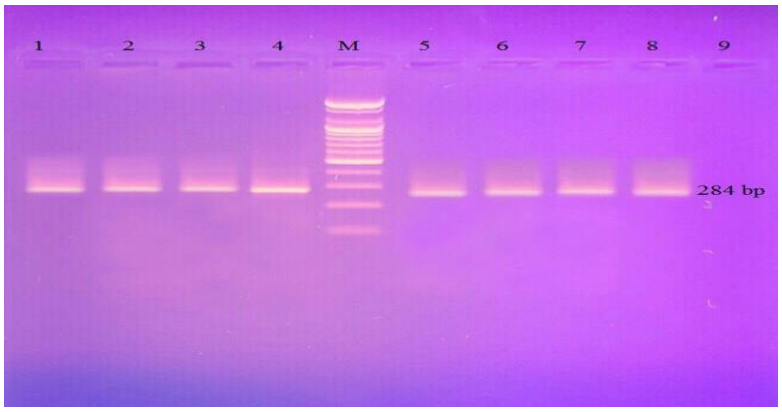

Figure (5): Agarose gel electrophoresis of PCR amplification products of *S.aureus, SCCmec* IIgene (284bp) (2% agarose, 5V/Cm, 2hr.). M: The DNA marker (100 bp ladder). ; Lanes (1-8) positive, lane 9 Negative.

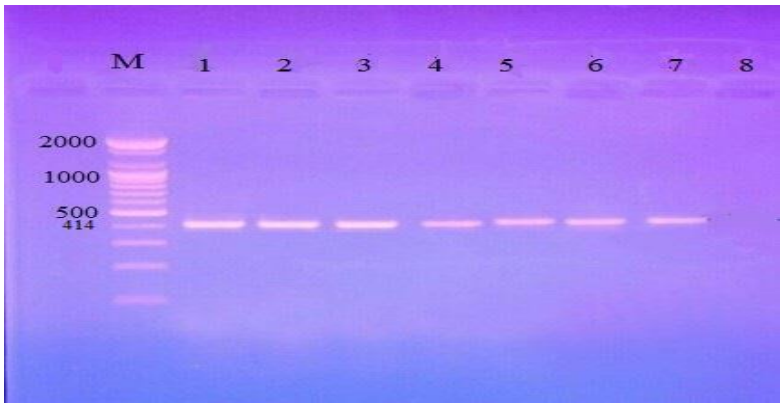

Figure (6): Agarose gel electrophoresis of PCR amplification products of *S.aureus, SCCmec* III gene (414bp) (2% agarose, 5V/Cm, 2hr.). M: The DNA marker (100 bp ladder). ; Lanes (1-7) positive, lane 8 Negative.

This percentage of the presence of SCC*mec*III is not close with some other studies in other countries ,the SCC*mec* type III found in the widely distributed MRSA in Brazil (Brazilian clone) may have originated from coagulase-negative staphylococci (CoNS) (13). The most relevant point is that SCC*mec* type III encodes the largest number of resistance genes, and this information is epidemiologically important for institutional infection control.

Several researcher have noted increased number of MRSA isolates that contained *SCCmec* type IV were due to increased hospitalization of patients with Community Acquired methicillin resistant *S. aureus* (CA-MRSA) infections or to an increased prevalence of isolates containing *SCCmec* type IV among Hospital Acquired methicillin resistant *S. aureus* (HA-MRSA) isolates (14,15). The number of isolates detected in staphylococcal cassette chromosome *mec* type IV (Figure 7) which were 53 (38.68%) isolates out of 137 and for V (Figure 8) it was 86 (62.77%) isolates out of 137 were found more than the other types, suggesting its greater

promiscuity and successful persistence. The distribution of *SCCmec* types and other gene in MRSA strains summarized in Figure (9).

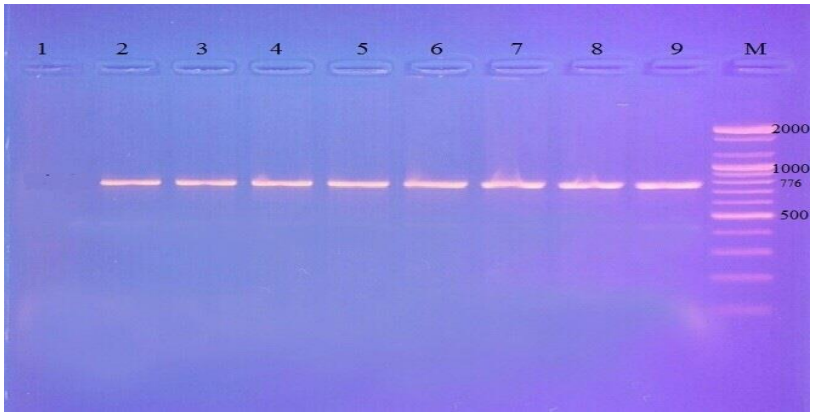

Figure (7): Agarose gel electrophoresis of PCR amplification products of *S.aureus, SCCmec* IV gene (776bp) (2% agarose, TBE buffer (1X), 5V/Cm, 2hr.). M: The DNA molecular Wight marker (100 bp ladder). ; Lanes (2-9) positive, lane 1 Negative.

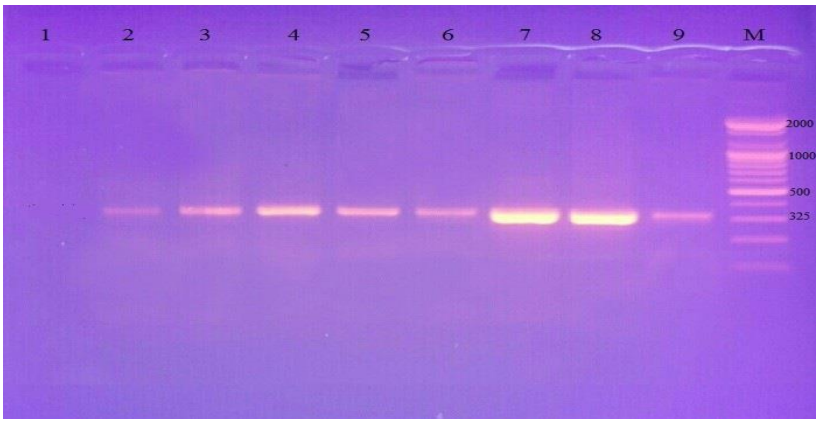

Figure (8): Agarose gel electrophoresis of PCR amplification products of *S.aureus, SCCmec V*gene (325bp) (2% agarose, 5V/Cm, 2hr.). M: The DNA marker (100 bp ladder). ; Lanes (2-9) positive, lane 1 Negative.

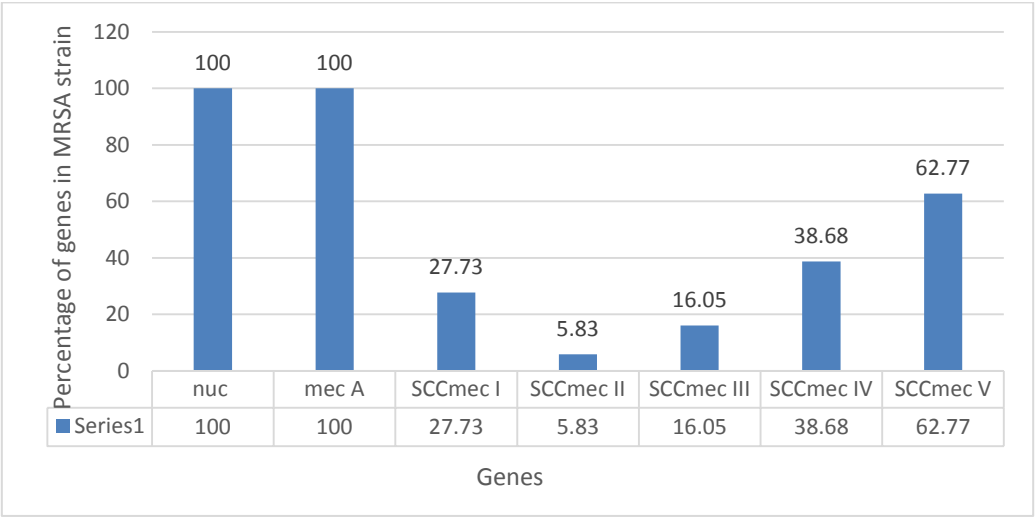

Figure (9): The distribution of *SCCmec* types and other gene in MRSA strains.

This may be the result of greater efficiency of transfer and/or a lesser fitness cost to the recipient clone, possibly because of its smaller size and lack of the excess baggage included in other *mec* types (16). The relatively greater fitness of CA-MRSA strains carrying the *mec* type IV and V gene may account for its remarkable success in displacing other MRSA strains in some hospitals after introduction from the community. These types of *mec* elements are small; appear to be highly mobile, allowing for efficient spread; and are strongly associated with community-acquired strains of MRSA at the present time. It is noteworthy that SCC*mec* type IV was first identified in *S. epidermidis* in 1970 and only described around 10 years later in *S. aureus* (17).

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