Annals of R.S.C.B., ISSN: 1583-6258, Vol. 25, Issue 6, 2021, Pages. 1329-1347
Received 25 April 2021; Accepted 08 May 2021.

"A molecular study on Healthcare-associated *Staphylococcus aureus* MRSA (HA-MRSA) strains isolated from hospitalized patients with skin lesions in Iraq: 16S RNA sequencing and PCR detection of *mecA* and *tst-1*"

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Abstract

Unveiling the molecular identity of the clinical strains implicated in human skin pathogenesis is being a mandatory task from epidemiological point of view and combating the emergence of multidrug resistance phenomenon. The present work underlines a molecular study on eighteen *Staphylococcus aureus* MRSA clinical strains (HA-associated MRSA) isolated from human skin lesions, consulted by dermatologist at Al-Qasim General Hospital, Imam Al-Sadiq Hospital, Babil Governorate, Iraq, using 16S RNA gene sequence analysis and PCR detection of the virulence genes *mecA* and *tst-1*. The suspect clinical strains, isolated from the human skin lesions on blood agar, showed typical *Staphylococcus aureus* features as follow: catalase positive, coagulase positive, golden yellow colonies on mannitol salt agar, and characteristic *S.aureus* profile on APISTAPH kit and VITEK2 system. All *S.aureus* clinical strains under study assigned as MRSA strains, deduced from their pattern of methicillin resistance. The 16S RNA sequence analysis of six MRSA strains, randomly selected, confirmed their affiliation to *S.aureus*. The occurrence of the two virulence genes *mecA* and *tst-1* in all *S.aureus* MRSA

Annals of R.S.C.B., ISSN: 1583-6258, Vol. 25, Issue 6, 2021, Pages. 1329-1347
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clinical strains under study was 100 and 100 %, respectively as inferred from the banding pattern of PCR amplification of partial fragments of 855 and 326 bp, respectively using gene specific primers. The present finding would greatly underpin the predominance of *S.aureus* MRSA clinical strains among hospitalized patients with skin lesions infection whilst, increasing sample size is recommended in prospective studies for further exploring the epidemiology of HA-MRSA strains in Iraq.

Keywords: *Staphylocccus aureus*; Skin infection; *mecA*; *tst-1*; 16S RNA sequencing

Introduction

Staphylococcus aureus (*S. aureus*) is classified as a Gram-positive bacterium that is being able to inhabit the skin, nose, and throat of human as a commensal bacterium. Healthy people (nearly 30%) could be colonized by *S.aureus* asymptomatically [Chambers 2001; Lowy 1998]. Obviously, this colonization is a mandatory risk factor in order to initiate the infectivity [Graham et al., 2006; Kuehnert et al., 2006; Perl et al., 2002; Shopsin et al., 2000; Von Eiff et al., 2001]. *S. aureus* is the causative agent of numerous infections as bacteremia, joints, bones, pleuropulmonary, heart, and skin infections (Wertheim et al. 2005; Gorwitz et al. 2008; Tong et al. 2015; Lee et al. 2018; Peacock and Paterson 2015). Additionally, *S. aureus* is one of the leading causes of food poising and nosocomial infections [Lowy 1998] where the immunocompromised patients could easily acquire the infection from the healthy carrier individuals.

The first report highlighting the emergence of methicillin resistance among *S.aureus* clinical strains was in 1961 [Jevons 1961; Barber 1961]. Currently, methicillin-resistant *S. aureus* (MRSA) is a major cause of healthcare-correlated infections. The emergence of MRSA strains is attributed to the gain of *mecA* gene localized on a mobile genomic island namely

staphylococcal chromosome cassette *mec* (SCC*mec*) by the aid of methicillin-sensitive *S. aureus* [Baba et al., 2002; Okuma et al., 2002].

According to the source of the etiological agent MRSA, it can be divided into two categories: healthcare-associated MRSA (HA-MRSA) and community-associated MRSA (CA-MRSA). The HA-MRSA have emerged among hospitalized inpatients exposed to nosocomial infections. Whilst, the CA-MRSA have emerged among individuals without any history for hospital admission. The methicillin resistance among HA-MRSA strains is attributed to the acquisition of staphylococcal chromosome cassette *mec* (SCC*mec I, II,* and *III*) [Ma et al., 2002; Ito et al., 2004].

The secretion of the pyrogenic toxins like tsst-1 is one the characteristic features of *S.aureus* is (Deurenberg et al. 2005). This toxin is well-known as shock syndrome toxin 1 that does encode the protein tst-1 (21.9kDa), the etiologic agent of toxic shock syndrome (Fraser et al. 2000).

From the insight of epidemiology, it is mandatory to explore the occurrence of the two virulence genes *tst-1* and *mecA* among *S.aureus* clinical strains (Healthcare-associated *S.aureus* MRSA); isolated from hospitalized patients with skin lesions. Consequently, the goal of the current work is molecular typing of HA-associated *S.aureus* MRSA clinical strains isolated from patients with skin lesions regarding to the two virulence genes *tst-1* and *mecA* in Iraq. The aim of the study is extended to employ the 16S rRNA gene sequence analysis to confirm the affiliation of the HA-associated *S.aureus* MRSA strains under study on both genus and species levels.

Patients and methods

Patients (Cohort study)

A total of eighteen patients with skin lesions, consulted a dermatologist at Al-Qasim General Hospital, Imam Al-Sadiq Hospital, Babil Governorate, Iraq ,from 7/10/ 2020 to 1/3/ 2021, was enrolled in this work. The cohorts study did include the two genders (males and females), whereas their ages spanned from 1-60 years. Moreover, a sheet for each participant was prepared encompassing the age, name, gender, and signs of the disease.

Molecular biology chemicals, reagents, and kits

MyTaq 2X Master Mix, agarose, and DNA ladder were purchased from Bioline, USA. All primers used in this study were synthesized by Integrated DNA Technology (IDT Co., USA). Genomic Bacterial DNA isolation kit was purchased from Anatolia Co., Turkey. GeneJET PCR purification kit was purchased from Thermo Fisher Scientific Co., USA.

Samples collection

A touch from the skin lesions from participant using sterilized cotton swab was taken. All gathered cotton swabs carrying the suspect pathogens were immediately transferred to the transport Amies' medium [Amies 1967].

Bacteriological media

Amies' medium was utilized for transport purposes of the cotton swab holding the suspect pathogens taken from the skin lesions [Amies 1967]. Human Blood agar was utilized for the purposes of enrichment [MacFaddin 1985]. Mannitol salt agar (MSA) was the selective differential medium [MacFaddin 1985] to differentiate between the *Stapylococcus aureus* and *Staphylococcus epidermidis*. Müller–Hinton broth was utilized for the test of antibiotic

sensitivity. Nutrient broth and nutrient agar were utilized for activation and growing of the isolated pathogens for long-term preservation purposes at -20 ^{o}C using in presence of 20% (v/v) glycerol [MacFaddin 1985]. All media were obtained from Oxoid-Thermofisher Scientific Co., USA.

S.aureus clinical strains from skin lesions: presumptive and confirmatory identification on a phenotypic level

The cotton swab holding the suspect pathogen from the skin lesions were cultured on Human blood agar medium. Then all cultured blood agar plates were incubated for 24 hrs at 37 ^{o}C in a static incubator. Next day, the raised colonies on the surface of blood agar plates were subjected to Gram-staining procedure. The colonies exhibiting Gram-positive cocci appearance were tested for exploring their capabilities for catalase and coagulase production. Then colonies displaying the positive output for catalase and coagulase production were cultured on mannitol salt agar (MSA) plates at 37 ^{o}C for 24 hrs for the differentiation between the *S.aureus* and *S.epidermidis*. The colonies showing golden yellow appearance on MSA plates were subjected to a panel biochemical tests through API STAPH Kit (BIOMERIEUX, France) and VITEK2 system (Faculty of Medicine, Microbiology Department, Baghdad Province, Iraq) according to the instructions of the manufacturer.

Methicillinsensitivity test

All suspect clinical strains isolated from the skin lesions were tested for methicillinsusceptibility test using the disk diffusion method according to the guidelines of CLSI (Clinical & Laboratory Standards Institute) [CLSI 2018]. Briefly, each tested clinical strain was cultured on nutrient agar for overnight. Two colonies were picked and diluted in Müller-Hinton broth until reaching 0.5

McFarland standard. Then, a disc containing 30 µg of oxacillin was used in the disk diffusion method according to the Kirby-Bauer method [Bauer et al. 1966].

Genomic DNA isolation

Genomic DNA was isolated from all tested clinical strains using the Genomic Bacterial DNA isolation kit (Anatolia, Turkey) according to the manufacturer's instruction. DNA quality was checked by running the isolated DNA on 1% agarose gel electrophoresis followed by visualization under Ultraviolet (UV-illumination) using UV-Transilluminator (Cleaver Scientific., UK). Whereas the quantitation of the isolated DNA was performed by the aid of Nano-drop Spectrophotometer (Applied Biosystem, USA).

16 S RNA gene sequence analysis and phylogeny

The 16S RNA gene sequence analysis from all clinical strains under study was performed to identify these strains on a molecular level and determine the genetic relatedness among them as well. The full length of 16S RNA from each clinical strain was PCR amplified using the universal primer set F8- 27 (5' -AGAGTTTGATCCTGGCTCAG-3') and R1510-1492 (5' -GGTTACCTTGTTACGACTT-3') of *Escherichia coli* 16S RNA gene [Eden et al., 1991]. The PCR was conducted according to a procedure reported previously [Embaby et al., 2014]. Briefly, the PCR reaction mixture included 30 ng genomic DNA, 25 μL MyTaq Master Mix 2X (Bioline, USA), 0.3 μM of each forward and reverse primer, and the final volume was completed to 50 μL with nuclease-free water. The PCR was performed in The PCR thermocycler (Biometra Co., Germany). The PCR conditions were set as follow: an initial denaturation at 96◦C for 4 min, 30 cycles: each cycle composed of denaturation at 94◦C for 45 sec, annealing at 55◦C for 30 sec, extension at 72◦ C for 1.5 min, and a final extension for 10 min at 72◦C. The amplified PCR

products were visualized under UV-illumination after running in 1% agarose gel electrophoresis. After that, the PCR products were purified using GeneJET PCR purification kit (Thermo Fisher Co., USA). Then, the purified PCR products were directly sequenced as sequencing templates using the above-mentioned primers for 16s RNA (27F and 1492R). DNA Sequencing was performed at Integrated DNA Technology (IDT) Co., USA. All nucleotide DNA sequences were subjected to editing and assembly by the aid of CLC Sequence Viewer 7.0 software. The edited and assembled 16S RNA sequences for all tested clinical strains were subjected to analysis by BLASTN (Basic Local Alignment Search Tool for nucleotides) online program of NCBI (National Center for Biotechnology Information), located at the webserverhttps://blast.ncbi.nlm.nih.gov/Blast.cgi?PROGRAM=blastn&PAGE_TYPE=BlastSearch&LINK_LOC=blasthome. BLASTN analysis does determine the genetic affiliation of each 16S RNA query sequence in relation to other closely related 16S RNA sequences deposited in the International Nucleotide Sequence Database Collaboration (INSDC). After that, multiple sequence alignment was conducted, using CLC Sequence Viewer 7.0, among the 16S RNA nucleotide sequences of the tested clinical strains under study and some hits (16S r DNA sequences) with the closest match, inferred from BLASTN analysis. Moreover, a phylogenetic tree was constructed using CLC Sequence Viewer 8.0 to delimit the genetic relatedness among the tested clinical strains. All 16S RNA nucleotide sequences obtained in this study were deposited in the GenBank.

PCR detection of *mecA* and *tst-1*

The distribution of the two virulence genes *mecA* and *tst-1* among the clinical strains isolated from human skin lesions were investigated by PCR amplification of a partial fragment of each gene using two gene specific primers sets. The next two gene specific primer sets F-*mecA*: 5'-

TGAGTTGAACCTGGTGAAGTT-3'/ R-*mecA*: 5'- TGGTATGTGGAAGTTAGATTGG -3' [Koosha et al. 2016] and F-*tst-1*: 5'- ACCCCTGTTCCCTTATCATC -3'/R-*tst-1*:5'- TTTTCAGTATTTGTATCGCC-3' [Blomster-Hautamaa et al. 1986] were used to detect the existence of *mecA* and *tst-1* in each clinical strain, respectively. For each clinical strain under study, two PCR were performed separately using the aforementioned mentioned two primer sets. Each PCR reaction mixture included 30 ng genomic DNA, 0.25 µM of each forward and reverse primer, 25 µL of PCR Master mix (2X) (MyTaq, Bioline Co., USA), and the final volume was completely by nuclease free water to 50 µL. The programming of the PCR thermocycler (Biometra Co., Germany) was set to be: 95 ^{o}C, 5 min for initial denaturation, 35 cycles each cycle: 94 ^{o}C, 45 sec for denaturation, 57 ^{o}C for *mecA* and 58 ^{o}C for *tst-1*, 50 ^{o}C, 45 sec for annealing, 72 ^{o}C, 45 sec (for *mecA*), 25 sec (for *tst-1)* for elongation, and final elongation for 72 ^{o}C, 10 min. Post PCR, 5 µL of each PCR product was loaded onto 1% agarose gel electrophoresis to verify the existence of the expected band at the expected length. A DNA ladder (100 bp DNA) was run in agarose gels parallel with the PCR products. Post electrophoresis, the agarose gels were exposed to UV-illumination. The expected PCR products lengths were 855 and 326 bp resulting from PCR amplification of a partial fragment of *mecA* and *tst-1*, respectively.

Results

Phenotypic affiliation of clinical strains

All suspect clinical strains were Gram-positive cocci, catalase positive, coagulase positive, and mannitol fermenter with golden yellow colonies on MSA plates. Results of VITEK2 system revealed that all tested strains are affiliated to *Staphylococcus aureus*. Moreover, the profile of all tested clinical strains on APISTAPH kit did verify that all strains were affiliated to *S.aureus*

with a percent of confirmation ranging from 90 and 97.4% covering the tested strains, in accordance with the identification scheme settled in the software (bioMérieux). Form the phenotypic characterization, eighteen *S.aureus* clinical strains were obtained and further subjected to molecular investigations. These strains were nominated on the strain level as AEMQ1Iraq to AEMQ18Iraq.

Phenotypic typing of *S.aureus* strains regarding oxacillin susceptibility

All tested *S.aureus* clinical strains were oxacillin resistant; deduced from the full capability of all strains to grow on Müller-Hinton agar plates overlaid with discs containing 30 µg of oxacillin each. This in turn evidenced that all tested *S.aureus* strains should be assign the MRSA strains.

Molecular typing of *S.aureus* MRSA based on 16S RNA sequence analysis

The 16S RNA sequence analysis of six MRSA strains, randomly selected, by BLASTN algorithm did confirm their molecular affiliation to *S.aureus* with a percent of identity of 99%. A phylogenic tree showing the degree of relatedness among the six tested strains was depicted in Figure 1. Moreover, the phylogenetic tree could group the tested strains into four clades (groups). AEMQ2Iraq and AEMQ3Iraq in one clade with a very close degree of relatedness and the AEMQ5Iraq and AEMQ6Iraq in another clade with a very close degree of relatedness. However, the AEMQ1Iraq and AEMQ4Iraq were very distally related and each could be assigned to a different clade. However, AEMQ1Iraq is slightly near from AEMQ5Iraq and AEMQ6Iraq.

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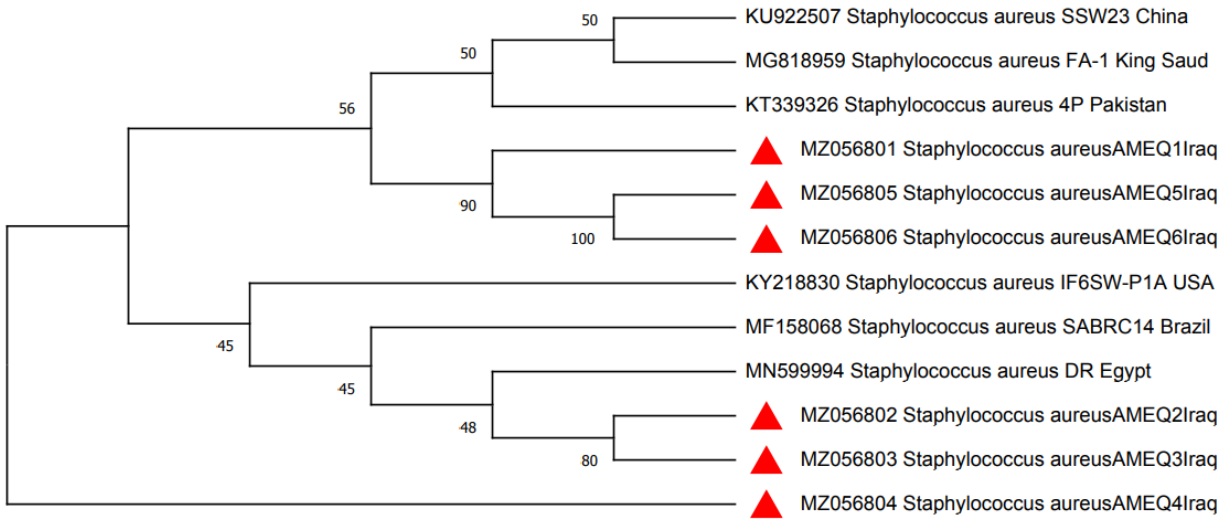

Figure 1: Phylogenic tree constructed by CLC Sequence Viewer 8.1 using the neighbor joining method depicting the degree of relatedness among six MRSA strains under study, randomly selected. The red triangles refer to the 16S r RNA sequence of the tested six *S.aureus* MRSA strains. The numbers refer to the bootstrapping values with 500 re-samplings.

Molecular profile of *S.aureus* MRSA strains: *mecA* and *tst-1*

The distribution of the two virulence genes *mecA* and *tst-1* among the eighteen skin lesions pathogens *S.aureus* MRSA strains were displayed in Figure 2 and Figure 3. The *mecA* gene was partially amplified by PCR from all tested *S.aureus* MRSA strains; giving the expected band size of 855 bp (Figure 2). Similarly, the *tst-1* gene was partially amplified by PCR from all tested *S.aureus* MRSA strains (Figure 3) giving the expected band size of 326 bp. Thus, the occurrence of *mecA* and *tst-1* was 100 and 100%, respectively.

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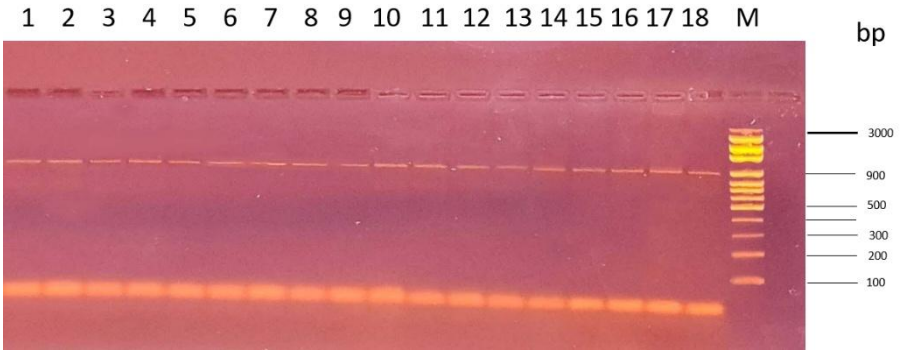

Figure 2: 1% agarose gel electrophoresis showing the PCR partial amplification of *mecA* gene (855 bp) from eighteen *S.aureus* MRSA clinical strains isolated through this study from patients suffering from skin lesions. M: DNA ladder. Lanes 1-18: PCR product of *mecA* partially amplified from eighteen *S.aureus MRSA* clinical strains nominated as AEMQ1Iraq to AEMQ18Iraq.

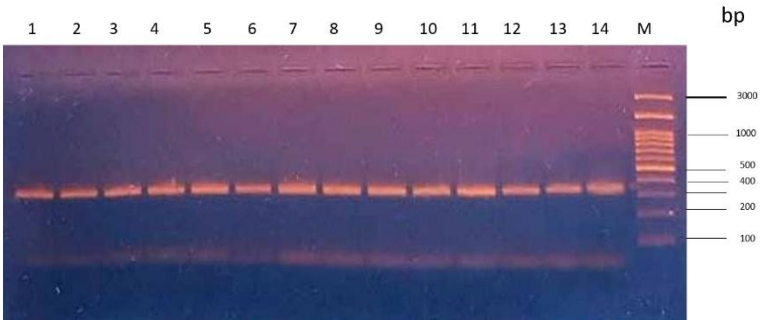

Figure 3: 1% agarose gel electrophoresis showing the PCR partial amplification of *tst-1* gene (326 bp) from eighteen *S.aureus* MRSA clinical strains isolated through this study from patients suffering from skin lesions. M: DNA ladder. Lanes 1-14: PCR product of *mecA* partially amplified from fourteen *S.aureus* MRSA clinical strains nominated AEMQ1Iraq to AEMQ14Iraq as representative of the total strains under study.

Discussion

S.aureus MRSA (HA-MRSA) clinical strains have considerable health implications worldwide especially those strains colonizing the skin patients due to their potential to cause life-threating infections. The identification of an MRSA clinical isolate from colonized skin hospitalized patients has crucial ramifications for patients on two levels: community level and hospital setting level. Moreover, the molecular typing of HA-associated MRSA strains is a very crucial task aiming to record the major molecular profiles predominating among these strains in a certain country to help eradicate or reduce their prevalence. In this context, this study aimed to trace the occurrence of the two virulence genes *mecA* and *tsst-1* among HA-associated MRSA strains; isolated from colonized skin hospitalized patients in Iraq. Additionally, the molecular identity of the HA-MRSA strains was verified using the 16S rRNA gene sequence analysis. In this study, the *S.aureus* clinical strains were phenotypically methicillin resistant whereas the genotypic profile of the methicillin resistance was unraveld. Our finding stated that all HA-MRSA strains under study carried the *mecA* gene in a frequency of occurrence 100% ((n=18/18). Unlike previous studies reported the methicillin resistance nevertheless the absence of the *mecA* gene (Elhassan et al. 2015; Bastidas et al. 2019) whereas 90.2% (n=111/123) and 6.1% (n=9/148) of the MRSA strains were found to harbor the *mecA* gene in Sudan and Ecuador, respectively. In addition, a previous study addressed the frequency of occurrence of *mecA* gene among MRSA stains isolated from hospitalized patients with skin lesions was 86.66% (n=13/15) in Iraq (unpublished data). These previous findings regarding the absence of *mecA* despite the methicillin resistance in MRSA strains phenotypically might be attributed to the presence of other genetic elements other than *mecA* like *mecC* assuming to be responsible for the methicillin resistance (Patterson et al 2014; Bastidas et al. 2019). In contrast, our finding regarding the presence of *mecA* was in a good agreement with previous finding stating 100% (n=76/76) and

100% (n=49/49) occurrence of *mecA* among MRSA strains isolated from Iraqi and Syrian communities, respectively [Rasheed and Hussein 2020]. A previous study reported the 100% occurrence of the *mecA* gene (n=10/10) among MRSA strains isolated from pasteurized milk camel [Yehia et al., 2020]. Another previous study addressed the 100% occurrence of *mecA* gene among MRSA strains colonized in nursing homes in Saudia Arabia [Albarrag et al., 2020].

Remarkably, the occurrence of *tst-1* gene among *S.aureus* MRSA clinical strains in this study was 100% (n=18/18). The investigations in preceding studies evidenced a narrow range of prevalence level for the *tst-1* gene among the *S.aureus* MRSA strains. For example, the level of the *tsst-1* gene among the *S.aureus* MRSA strains was < 20% [Tsen et al.1998; Becker et al. 2003; Fenner et al. 2008; Demir et al. 2011; Hoseini Alfatemi et al. 2014; Mairi et al., 2019]. Whereas, another previous studies did address 48 and 68% prevalence levels of *tst-1* gene among MRSA clinical strains (Kimura et al. 1992; Xie et al. 2011; Koosha et al. 2016).

Despite the discrepancy in the literature of review concerning the occurrence of tst-1 gene among MRSA clinical strains, the expression of the tst-1 toxin protein is not being confirmed. Unless, the detection of the tst-1 toxin is performed with positive output. This would underpin the obligation of unveiling the ability of MRSA clinical strains to secret the tst-1 toxin protein in our strains in prospective studies. At most, the divergence between our findings and other previous findings might be attributed to the geographic regions. Reportedly, *S.aureus* isolated from different geohraphic regions demonstrated a diverse virulence gene profiles.

The 16S ribosomal RNA (rRNA), synonymously called 16S RNA, is a highly conserved gene within strains belonging to the same species and among species belonging to the same genus. So far, 16S rRNA sequence analysis is considered the gold standard for speciation of bacteria

among different genera and within the same genus [Woo et al., 2000]. Despite the shortage in the 16S RNA gene sequence analysis approach for the discrimination among the strain, the six clinical MRSA strains AEMQ1Iraq to AEMQ6 Iraq were grouped into four different clades based on their 16S RNA gene sequence analysis. Moreover, the results of 16S RNA gene sequence analysis for six randomly selected strains AEMQ1Iraq to AEMQ6 were in a great agreement with those of APISTAPH kit and VITEK2 system. In our study, the APISTAPH kit and the VITEK2 system could successfully identify the 18 MRSA strains without obtaining false-negative results (Ruane et al., 1986; Piper et al., 1988). Present findings regarding the potential of APISTAPH kit and VITEK2 system for the speciation of the bacterial strains were in disagreement with previous findings addressing the shortage of these biochemical system in the identification of some strains on the species level.

The prospective studies would address the use of greater sample size where the profile of the virulence genes mecA and tst-1 would be investigated among the tested strains.

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