Annals of R.S.C.B., ISSN:1583-6258, Vol. 25, Issue 4, 2021, Pages. 3227 – 3238
Received 05 March 2021; Accepted 01 April 2021.

Molecular profile of *mecA*, *tst-1*, *Hla, Hlb, eta,etb,Erma,* and *Ermb* virulence genes in *Staphylococcus aureus* using RAPD - PCR .

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Abstract

The present work highlights the molecular typing of thirty *Staphylococcus aureus* clinical strains isolated from skin lesions of patients, consulted a dermatologist at Diwaniyah Teaching Hospital in Iraq, using RAPD – PCR to detect of eight virulence genes (*mecA*, *tst-1*, *Hla*, *Hlb*, *eta*,*etb*, *Erma*, and *Ermb).* Tested strains gave the profile of *St.aureus*, deduced from yellow colonies on mannitol salt agar , and oxacillin (methicillin) resistance(MRSA). The occurrence of virulence genes *eta*, *etb*, *Hlb*, *Erma*, and *Ermb* among the thirty pathogenic *Staph.aureus* were 16.66 , 50 ,60, 16.66 and 26.66% ,respectively. The RAPD-640 PCR could categorize the thirty clinical strains under study into 19 groups strains (19 clades), regarding the obtained DNA banding profile in each profile. The present data would underpin that *Staph.aureus* MRSA is the most predominate pathogen among patients with skin lesions .

Keywords: *Staphylocccus aureus*; Skin infection; *tst-1, Hla, Hlb, eta,etb, Erma, Ermb*,RAPD –PCR.

Introduction

Skin infections could be caused by many types of bacteria, however, the most prevalent skin pathogens are assigned to the bacterial genera *Staphylococcus* and *Streptococcus. Staphylococcus aureus(S.aureus)* is considered a widespread commensal and a persistence expedient pathogen. Usually, the individuals of a certain population could be stratified into two types infrequently or continuously colonized with *S.aureus*. Colonization of the human skin and nasal mucosa is frequently reported elsewhere worldwide .Upon damaging the cutaneous and mucosal barriers, *S .aureus* can cause numerous contagions such a skin infections, joints, bactere mia, heart, bones, pleura pulmonary, and soft tissues infections(1) .

In 1960S, methicillin-resistant *St.aureus* MRSA strains was detected. The contagions with *St.aureus* MRSA strains are usually associated with complications in treatment due the acquired multidrug resistance. Consequently, methicillin-resistant *St.aureus* MRSA strains contagions have become a global burden. (2) . Reportedly, there are several virulence genes carried by *St.aureus* clinical strains. These genes are namely *tst-1, Hla, Hlb, eta,etb,Erma,* and *Ermb.*

The ribosomal binding site modification like methylation or mutation in *23S rRNA* gene are encoded by erm genes (*ermA*, *ermB*, *ermC*, and *ermF*). The predominate genes *ermA* and *ermC* are responsible for resistance to macrolide, lincosamide, and streptogramin B (MLS_B) antibiotic in *St.aureus* (3).

The objective of the present work is to unravel the allocation of the eight virulence genes *mecA*, *tst-1*, *Hla*, *Hlb*, *eta*, *etb*, *Erma*, and *Ermb* among *St.aureus* clinical strains; isolated of patients suffered from skin lesions to obtain a molecular profile for these clinical strains.

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Patients and methods

Thirty patients suffering from skin lesions whom admitted to Diwaniyah Teaching Hospital, Iraq from December 2019 to April 2020, was included in this study.A total of thirty sterile cotton swabs were collected from patients. All swabs were transferred at once to the transport Amies medium(4). For enrichment, sheep blood agar was utilized,Mannitol salt agar (MSA)and Müller–Hinton broth were used in the course of selective differentiation and antibiotic susceptibility, respectively(5).

*Staphylococcus aureus:*isolation and identification

The clinical samples in the form of cotton swabs were streaked onto sheep blood agar medium. All plates were incubated at 37 ^{o}C for 24 hrs in a static incubator. Next day, Gram-staining was carried out for the raised colonies on sheep blood agar plates. The catalase test was done for all colonies showing Gram-positive cocci appearance.Then, the colonies with an output of catalase positive test were subjected for confirming their identification with API STAPH (BIOMERIEUX, France) according to the instructions of the manufacturing. The colonies showing positive API STAPH profile were further subcultured on MSA plates for the differentiation between *Staphylococcus aureus* and *Staphylococcus epidermidis*. The yellow colonies on MSA plates indicating the presence of *St.aureus*; capable of mannitol fermentation. The mannitol fermenter colonies with yellow appearance on MSA plates were subjected to coagulase test. Finally, all positive coagulase colonies were nominated as *St.aureus* and were preserved for long term at -80 ^{o}C after the addition of glycerol with a final concentration of 15% for overnight cultures in nutrient broth.

Detection of *S. aureus* MRSA strains

All *St.aureus* clinical strains were subjected to antibiotic susceptibility to pick up the *St.aureus* MRSA strains.The antibiotic susceptibility was performed using the disk diffusion method according to the guidelines of CLSI (Clinical &Laboratory Standards Institute)(6). Briefly, the tested strains were grown on nutrient agar plates for overnight and incubated statically at 37 ^{o}C. Two colonies from each tested strain were picked and diluted in Müller-Hinton broth to reach 0.5 McFarland standard. Then, a disc of methicillin (30 µg) (oxacillin) was used in the disk diffusion method according to the Kirby-Bauer method(7).

Genomic DNA isolation

The isolation of genomic DNA from all bacterial isolates was performed using the Genomic Bacterial DNA isolation kit (Anatolia, Turkey) according to themanufacturer's instructions.The quality of the isolated genomic DNA was checked by running aliquots of the DNA on 1% agarose gel electrophoresis. The agarose gels were stained with ethidium bromide and were visualizedunder ultra violet illumination using UV-Transilluminator (8). The DNA concentration was determined using Nano-drop Spectrophotometer (Applied Biosystem, USA).

PCR detection of *mecA, tst-1, Hla, Hlb, eta, etb, Erma,* and *Ermb* genes

The distribution of the eight virulence genes *mecA*, *tst-1*, *Hla*, *Hlb*, *eta*, *etb*, *Erma,* and *Ermb* were unraveled in the bacterial strains under investigation through PCRpartial amplification of each gene by the aid of eight gene specific primers sets. The eight gene specific primer sets F-*mecA*:5'-

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TGAGTTGAACCTGGTGAAGTT-3'/ R-*mecA*: 5'- TGGTATGTGGAAGTTAGATTGG -3'(9) ,F-*tst-1*: 5'- ACCCCTGTTCCCTTATCATC-3'/R-*tst-1*:5'-TTTTCAGTATTTGTATCGCC-3', F-eta:5'-TTTGCTTTCTTGATTTGGATTC-3'/R-eta:5'- GATGTGTTCGGTTTGATTGAC-3', F-*etb*: 5'-ACCGCAGGAAAACATATAGCCC-3'/R-*etb*: 5'-GCGACTATCTTCCGCATAATCA-3', F-Erm-b: GTTCAAGAACAATCAATACAGAG-3'/R-Erm-b: 5'-GGATCAGGAAAAGGACATTTTAC-3', F-Erm-a:5'-CCGTTTACGAAATTGGAACAGGTAAAGGGC-3'/R-Erm-a:5'-GAATCGAGACTTGAGTGTGC-3', F- Hla: 5'- GAAAACACGTATAGTCAGCTCAGTAAC -3'/R-Hla:5'- GTCATTTCTTCTTTTTCCCAATCG-3'/R-Hla:5'-GGTGAAAAAAACAAAATCCAATTCAC-3', and F- Hlb:5'-GGTGAAAAAAACAAAATCCAATTCAC-3'/R-Hlb:5'-GGTGAAAAAAACAAAATCCAATTCAC-3', for the partial amplification of the eight genes *mecA*, *tst-1*, *eta*, *etb*, *Ermb*, *Erma*, *Hla*, and *Hlb*,respectively.The synthesis of the eight primer sets was carried out in Integrated DNA Technology, USA.The PCR thermocycler (Biometra, Germany)was programmed as follow: 95 ^{o}C, 5 min for initial denaturation, 35 cycles each cycle: 94 ^{o}C, 45 sec for denaturation, 57^{o}C (for *mecA*), 50^{o}C (for *tst-1*), 54 ^{o}C (for *Hlb*,*etb*,and *Erma*), 58 ^{o}C (*Hla*,*eta*,and *Ermb*) 45 sec for annealing, 72^{o}C, 45 sec (for *mecA*), 25 sec (for *tst-1*),35 sec (for *Hlb*,*etb*,and *Erma*), 40 sec (for *Hla*,*eta*,and *Ermb*) for extension, and 72 ^{o}C, 10 min for final extension. After the PCR termination, the presence of the expected PCR products from each reaction was checked on 1% agarose gel electrophoresis alongside with 50 and 100 bpDNA ladders (abm, Canada). The visualization of agarose gels was performed under UV-Transillumiantor(Cleaver Scientific, UK). The expected lengths of PCR products were 985, 951,855, 464, 421, 360, 326, and 226 bp resulting from the partial amplification of *Hlb*,*Hla*, *mecA*, *eta*, *Ermb*, *Erma*, *tst-1*, and *etb* genes, respectively.

RAPD–PCR technique

RAPD640-PCR was conducted in this work for DNA fingerprinting of the bacterial isolates under study using the RAPD-640: 5'- CGTGGGGCCT-3'(10). The synthesis of the primers was done in Integrated DNA Technology, USA. Shortly, the PCR mixture for RAPD-PCR included 12.5 µL of PCR master mix (MyTaq, Bioline, USA), 3 µL of (0.3 µM) of primer, 9.5 µL of distilled water, and 2.0 µL (100 ng) of template DNA. All reaction mixtures were put in the thermocyclcer (Biometra, Germany). The PCR cycling conditions were set to be as follow: an initial denaturation step at 94 ºC for 2 min, 45 cycles each cycle 94 ºC for 1 min, 25 ºC for 1 min, 72 ºC for 5 min, and a final extension at 72 ºC for 8 min. The electrophoresis of RAPD640 DNA fragments was conducted on 1.5% agarose gel alongside with 50 and 100 bp DNA ladders(abm, Canada) at 100 Volt for 60 min using DNA submarine unit (Cleaver Scientific, UK). The resultant DNA bands were visualized by exposure to UV-Transilluminator (Cleaver Scientific, UK). The RAPD 640 DNA fragments were analyzed optically and their molecular weight were determined. DNA standard curves were established using50 and 100 bp DNA ladders (abm, Canada).

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Results

Phenotypic identification of clinical strains from the skin lesions

A thirty bacterial pathogens were isolated from the thirty patients suffered from skin lesions participated in the present study. These clinical strains were nominated as skin lesion strains (SKLS) and were assigned the numbers SKLS1 to SKLS30 in a serial order. The results of phenotypic identification revealed that all clinical strains under study were Gram-positive cocci, coagulase positive, catalase positive, and mannitol fermenter displaying yellow colorization on MSA. Additionally, all clinical strains under study showed positive Staphylococci profile on API STAPH. As a result, all clinical strains under study were assigned as *Staphyloccus aureus*.

The resistance profile of Oxacillin (methicillin)

The present data verified that that all tested strains showed resistance towards the oxacillin (methicillin) with a frequency of occurrence 100% (n=30/30). This in turn would indicate that all tested *St.aureus* clinical strains named as *St.aureus* MRSA.

Molecular profile for *Hlb,Hla, mecA, eta, Erm-b, Erm-a, tst-1,* and *etb*

The distribution of the eight virulence genes *Hlb,Hla, mecA, eta, Ermb, Erma, tst-1*, and *etb* of the thirty skin lesions pathogens *St.aureus* clinical strains was monitored by partial amplification of each gene by PCR. For *mecA*, all tested skin lesions pathogens *St.aureus* clinical strainsdisplayed positive PCR product band pattern with the expected length (855 bp) (Fig1 and Fig 2). Similarly, all tested *St.aureus* clinical strains showed positive PCR products banding pattern within the expected size 326 bp for *tst-1* gene (Fig 3).

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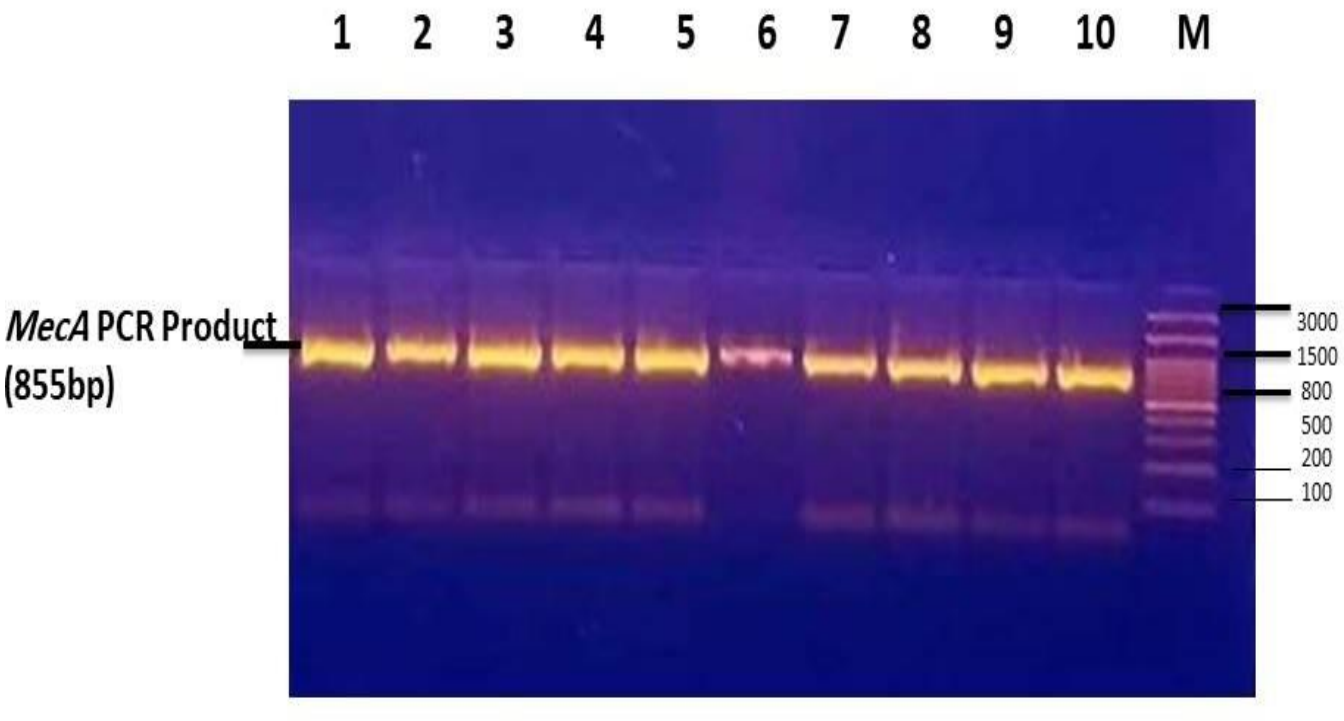

Fig 1: Agarose gel electrophoresis (1.5%) showing the PCR products of *mecA*gene after partial amplification using gene specific primer. M: 100 bp DNA ladder. Lanes (1-7): PCR products of *mecA* gene partial amplification (855 bp) from seven *S.aureus* skin lesionsclinical strains as representative examples.

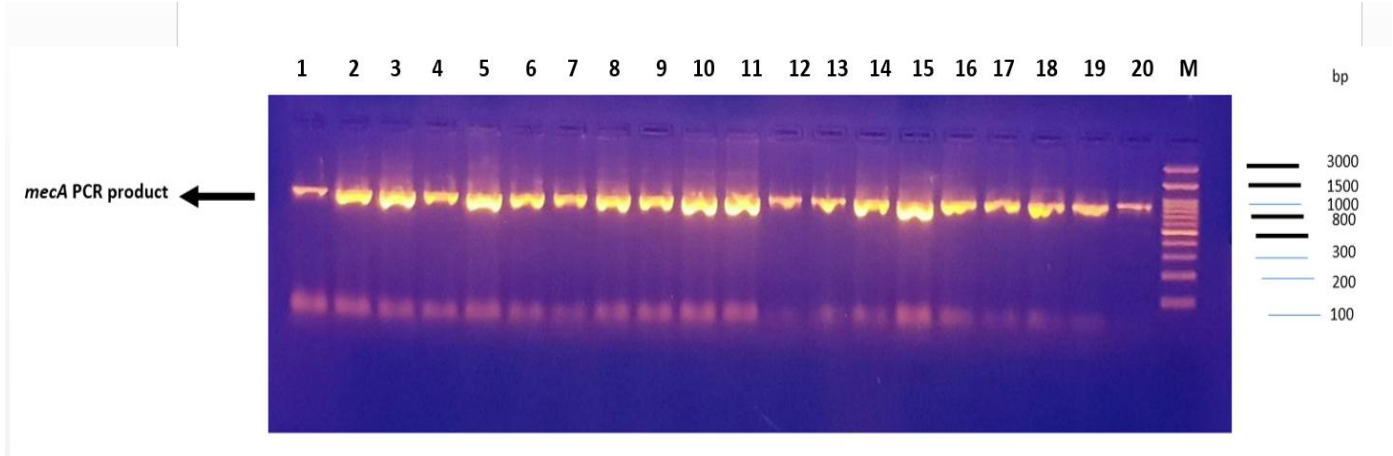

Fig 2: Agarose gel electrophoresis (1.5%) showing the PCR products of *mecA* gene after partial amplification using gene specific primer. M: 100 bp DNA ladder. Lanes (1-20): PCR products of *mecA* gene partial amplification (855 bp) from twenty*St.aureus* skin lesions clinical strains under study as representative examples.

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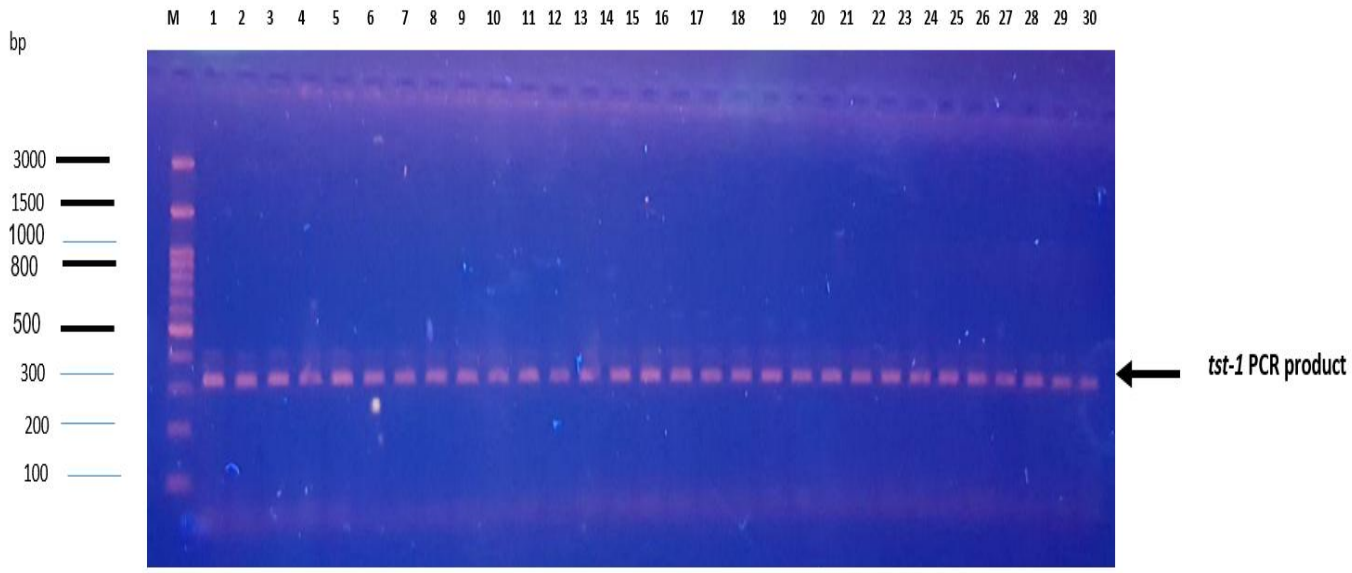

Fig 3: Agarose gel electrophoresis (1.5%) showing the PCR products of *tst-1* gene after partial amplification using gene specific primer. M: 100 bp DNA ladder. Lanes (1-30): PCR products of *tst-1* gene partial amplification (326 bp) from thirty *St.aureus* skin lesions clinical strains.

RAPD-640 profile for thirty *St.aureus* skin lesions clinical strains

The thirty skin lesions*St.aureus* MRSA clinical strains were discriminated based on RAPD640-PCR DNA banding pattern as shown in Fig 4,5,6. RAPD640-PCR did display 19 profile of DNA banding patterns (Table 1) among the thirty clinical *St.aureus* MRSA clinical strains . The highest and the lowest numbers of banding pattern were 15 and 2 DNA banding pattern of RAPD640-PCR. Whilst, the most frequent banding pattern occurred among the thirty *St.aureus* MRSA clinical strains was 10 DNA bands. The molecular weight of each DNA band displayed by RAPD640-PCR was estimated by the aid of DNA standard curve (Fig 7).

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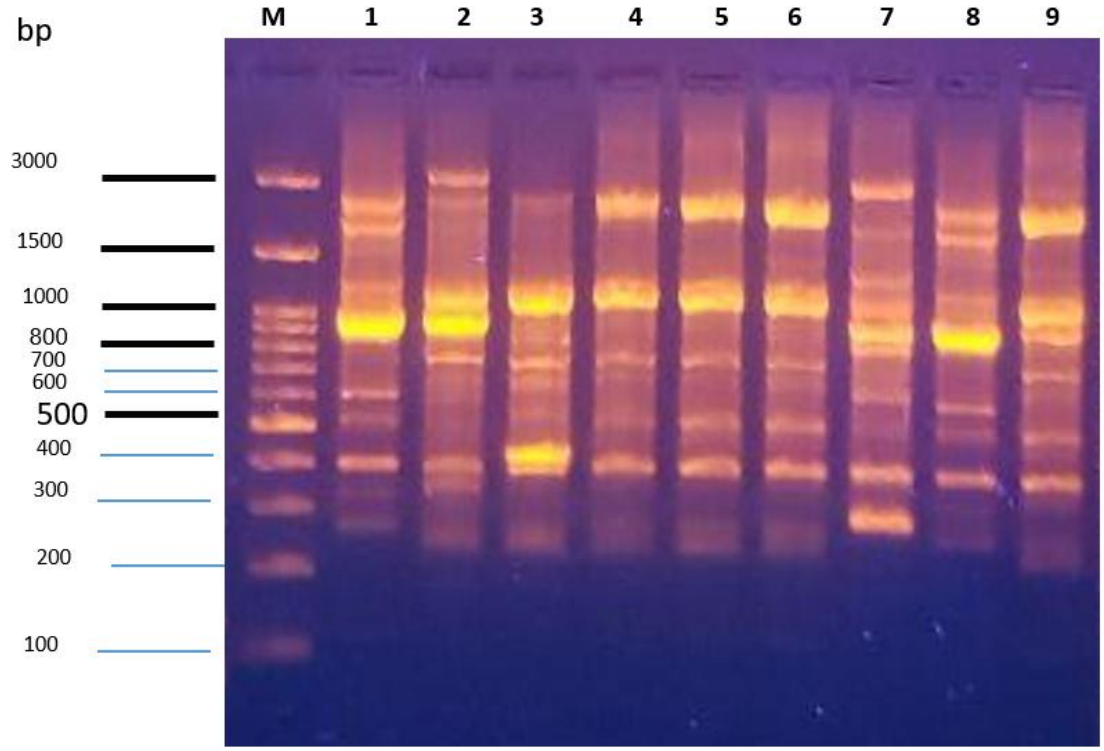

Fig.4: Agarose gel electrophoresis (1.5%) showing the banding pattern of RAPD640-PCR for the nine *St.aureus* MRSA clinical strains using RAPD-640 primer. Lanes (1-9): RAPD640-PCR DNA banding pattern for the nine *St.aureus* MRSA clinical strains namely SKLS1to SKLS9 serially. M: 100 bp DNA ladder.

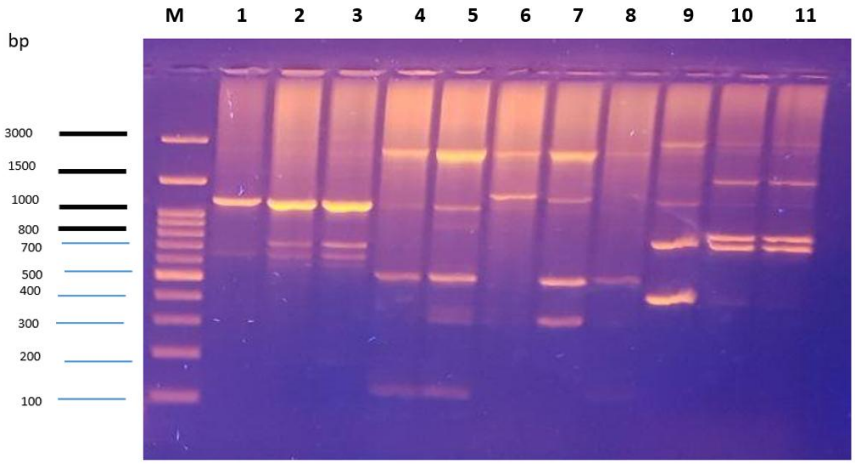

Fig.5: Agarose gel electrophoresis (1.5%) showing the banding pattern of RAPD640-PCR for the eleven *St.aureus* MRSA clinical strains using RAPD-640 primer. Lanes (1-11): RAPD640-PCR DNA banding pattern for the eleven *St.aureus* MRSA clinical strains namely SKLS10to SKLS20 serially. M: 100 bp DNA ladder.

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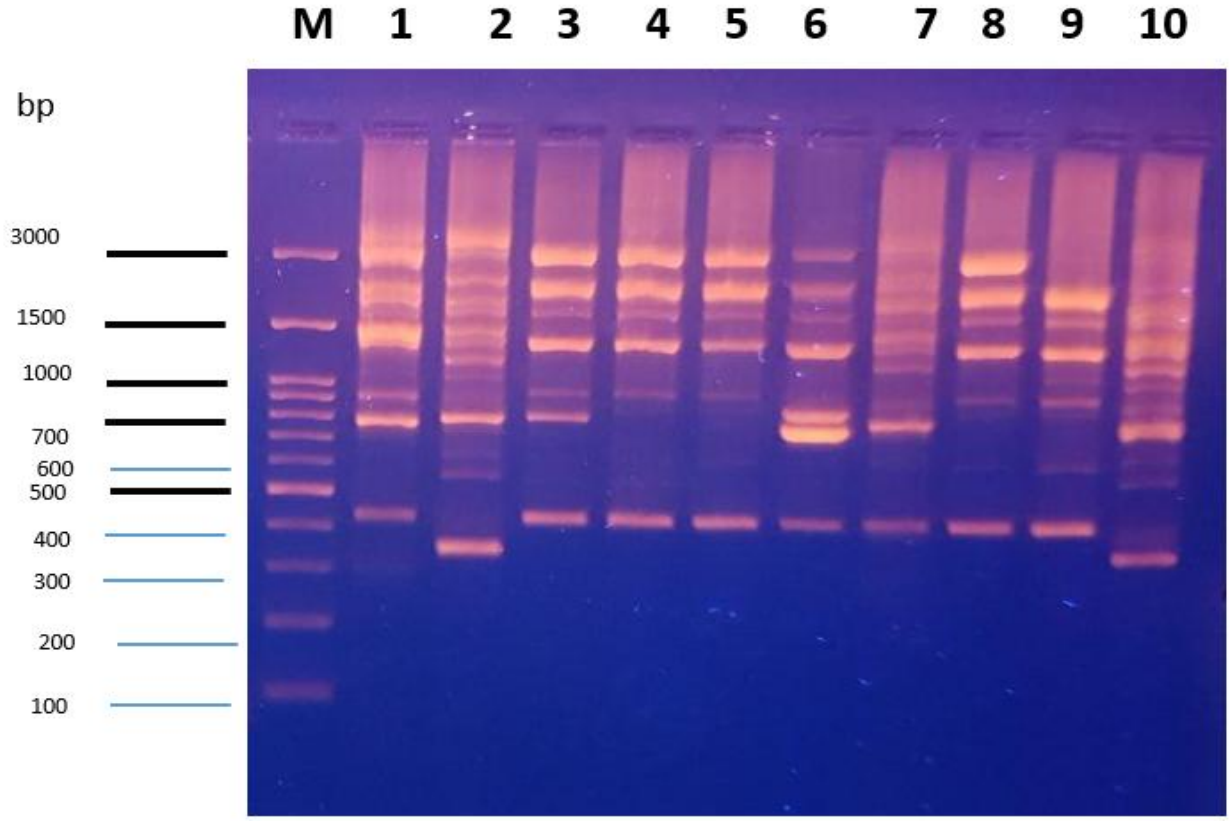

Fig.6: Agarose gel electrophoresis (1.5%) showing the banding pattern of RAPD640-PCR for the ten *St.aureus* MRSA clinical strains using RAPD-640 primer. Lanes (1-11): RAPD640-PCR DNA banding pattern for the ten *St.aureus* MRSA clinical strains namely SKLS21to SKLS30 serially. M: 100 bp DNA ladder.

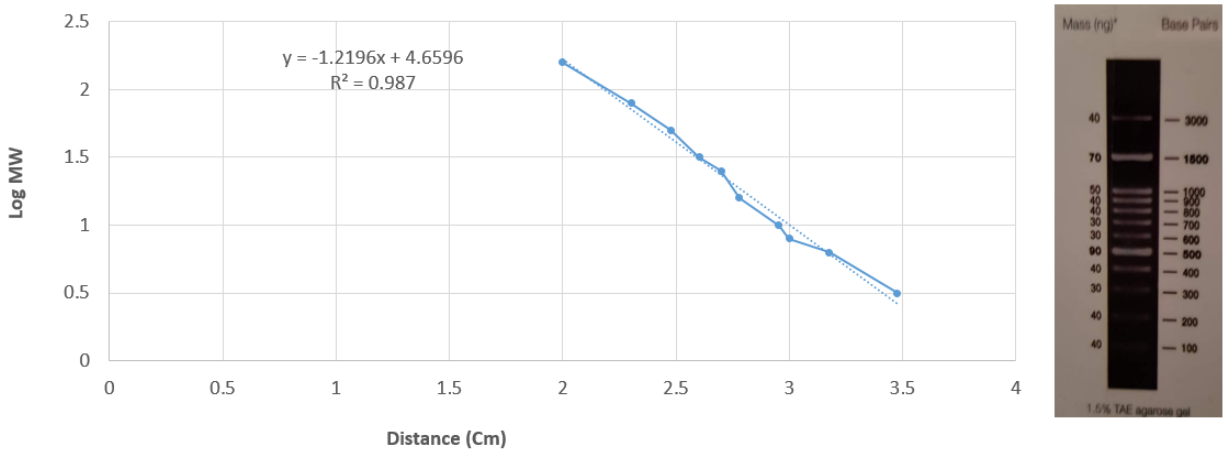

Fig. 7:A DNA standard curve using 100 bp DNA ladder (abm, Canada). The R^2 value was close to 1.0 that indicates the very small difference between the predicted values and the experimental values. MW: molecular weight of DNA ladder bands.

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Table 1: RAPD640-PCR profile and molecular weight of DNA bands for the thirty skin lesions pathogen *St.aureus*MRSA clinical strains

DNA band size (bp)	*St.aureus* MRSA strains with DNA band size (SKLS)*	RAPD640-PCR profileno in relation to the strain
1500, 2800, 2300, 900, 600, 500, 400, 300, 150, 250	SKL1	P1
3000, 2800, 2300, 900, 700, 600, 400, 450, 250	SKL2	P2
1200, 1000, 2800, 700, 500, 400, 250	SKL3, SKL4, SKL5, SKL6	P3
3000, 1200, 1000, 2800, 900, 600, 500, 400, 300	SKL7	P4
1200, 1000, 2800, 2300, 600, 500, 400, 250	SKL8	P5
1000, 2800, 900, 700, 500, 400, 250	SKL9	P6
1200, 700	SKL10	P7
1200, 700, 800	SKL11, SKL12	P8
1200, 2800, 500, 400, 300	SKL13, SKL14	P9
1200, 2800	SKL15	P10
1200, 2800, 500, 300	SKL16	P11
1200, 2800, 500	SKL17	P12
1200, 2800, 700, 400	SKL18	P13
1200, 2800, 700, 600	SKL19, SKL20	P14
3000, 1500, 2800, 2300, 2500, 1200	SKL21	P15
3000, 1500, 2800, 2300, 900, 800, 600, 500, 2500, 1200, 300	SKL22	P16
3000, 1500, 2800, 2300, 900, 800, 400, 1200	SKL23, SKL24, SKL25, SKL26, SKL27, SKL28	P17
1500, 2300, 900, 800, 400, 1200	SKL29	P18
3000, 1500, 2800, 2300, 1000, 900, 800, 700, 600, 500, 2500, 1200, 300	SKL30	P19

*SKLS: Skin lesion strain

Discussion

Staphylococcus aureus MRSA clinical strains are the most leading pathogens in human skin lesions worldwide.It is compulsory to type the *St.aureus* MRSA clinical strains involved in human infections due to their methicillin resistance.Billions of dollars are dismissed annually as consequence of the cosmopolitan multidrug resistance phenomenon displayed by the MRSA clinical strains. The pattern of multidrug resistance of MRSA clinical strains is most probably correlated with peculiar patterns of certain virulence genes(11). Remarkably, the occurrence of MRSA clinical strains in the present study regarding antibiotic sensitivity (phenotypically) was 100% (n=30/30). In parallel, the

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frequency of occurrence of *mecA* gene among the tested MRSA clinical strains by using the molecular approach for *mecA* detection was 100%% (n=30/30). Our data were in a partial agreement with the previous data in Sudan(12), where 90.2% (n=111/123) of the clinical strains harbored the *mecA*gene,.Elhassan et al. suggested that the phenotypic methicillin resistance is mostly ascribed to the existence of *mecA* gene .In contrast, our datawere in discordance with thepreviously published data in Ecuador(13,14,15 ,16) where 6.1% (n=9/148) of the *St.aureus* clinical strains did harbor the*mecA*gene.

With regard to the frequency of *tst-1* gene in our *St.aureus* MRSA clinical strains, it was 100% (n=30/30). The inspectionin the foregoing studies pointed out a low to intermediary grade of prevalence of the *tst-1* gene in *St.aureus* MRSA clinical strains. For example, less than 20% level of prevalence of *tst-1* gene among *St.aureus* MRSA clinical strains in preceding studies (17,18,19,20) was observed. However, 48 and 68% levels of occurrence of the *tst-1* gene among *St.aureus* MRSA clinical strains were evidencedfrom some preceding studies (21 ,22). There exists an obvious inconsistency among previously reported *St.aureus* MRSA clinical strains traced from various countries but, the existence of *tst-1* gene does not certainlyverify the existence of the toxin on a protein level. This would necessitate theobligatorydemand to trace the expression of the*tst-1* gene in our *St.aureus* MRSA clinical strains in prospective studies.

Our data revealed 100% (n=15/30) occurrence of *etb* gene among *St.aureus* MRSA clinical strains. Unlike our finding, a low level of occurrence (0.5-1.7%) of *etb* gene among *St.aureus* isolates in various countries like,Germany, China, and Nigeria;(23 ,24)was reported previously. Whilst, 16.7% frequency of occurrenceof *etb* gene was observed in *St.aureus*strains from clinical specimens in Iran (25). Regarding the frequency of occurrence of *eta* gene among our *St.aureus* MRSA clinical strains, it was 16.66% (n=5/30). A previous study did observe a frequency of occurrence 76.7% for *eta* gene among *St.aureus* clinical strains isolated from clinical specimens in the north of Iran

The frequency of occurrence of *Hla* and *Hlb* genes among our *St.aureus* MRSA clinical strains was 100% (n=30/30) and 60% (18/30), respectively. A previous study did report a frequency of occurrence of 88 and 85.33% of *Hla* and *Hlb* genes, respectively in St.aureus clinical strains isolated from goats (26). Moreover, the prevalence of *Hla* and *Hlb* genes in coagulase negative Staphylococci (CONS) was 87.9 and 47.3%, respectively (27).Nasaj et al. 2020).

Another study did report a prevalence level of 92.8%, 34.7% for *Hla* and *Hlb* genes, respectively among *St.aureus* clinical strains isolated from samples in a referral burn hospital in Tehran, Iran (28).

With regard to the frequency of occurrence of *erm-a* and *erm-b* genes in our *St.aureus* MRSA clinical strains, 16.66 (n=5/30) and 26.66% (n=8/30) were evidenced. A previous study did state the 22.22 and 11.11% frequency of occurrence for *erm-a* and *erm-b*, respectively among nine *St.aureus* MRSA clinical strains isolated from Al-Zahra Hospital in Isfahan, Iran (13). Our data were in a close agreement regarding to higher frequency of occurrence of *erm-b* when compared to that of *erm-a* gene (29).

Clearly, there is a great discrepancy in the distribution of the present testedeight virulence genes among a panel of *St.aureus*MRSA clinical strains worldwide. This conclusion would outline the

possible role of antibiotic resistance genes in acquiring , maintaining, and missing the virulence genes cassette; encountered in the pathogenesis.

RAPD640-PCR technique did prove a powerful technique to discriminate the present thirty *St.aureus*MRSA clinical strains into 19 clusters.

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