Nat. Volatiles & Essent. Oils, 2021; 8(4): 15215-15225

NVEO
Natural Volatiles &
Essential Oils

Molecular Investigation Of Some Erythromycin Resistance Genes In Staph Aureus Isolated From Different Clinical Infections In Diyala, Iraq

Zainab Amer Hatem[1] , Hadi R. Rasheed Al-Taai[2] and Abbas Abood F. Al-Dulaimi [3]

1,2 Department of Biotechnology , College of Sciences, Diyala University, Iraq.

3 Department of Biology, College of Education for Pure Sciences, Diyala University, Iraq.

Abstract

Staphylococcus aureus is an important infectious pathogen in health sector and communities. It causes various infections ranged between simple to life threating infections. This study was carried out during the period from the beginning of December 2019 to the end of August 2020,. Out of 300 specimens, 75isolates of S.aureuswere recovered..All isolates were tested toward the different class of clinically important antibiotics (15) by using agar diffusion method. The results of resistance were as following: Cefotaxime (100%) ceftriaxone (92%), Imipenem (12%). while the resistance to Fluoroquinolones include Norfloxacin (64%), Ciprofloxacin (56%), Amikacin was (70.6%), Gentamycin (74.6%) ,vancomycin is (48%) ,azithromycin (84%),erythromycin (80**%)** . tetracycline (88%) but resistance to doxycycline is (60%) ,colistin (100%), Trimethoprim (68%),Chloramphenicol (36%) . In this investigation, antibiotic susceptibility testing of the Saureusisolates showed that 9(12%), 24(32%), 42(56%)of the isolates were MDS, MDR,XDR .The polymerase chain reaction (PCR) assay wasapplied to determine the major erythromycin-resistant genes (ermA, ermB, ermC) . this genes detected in 15 isolates and results show that the ermA, ermB,ermCgene found in 5(33.3%) ,5(33.3%),15(100%) .It is well known that many bacterial species exhibit variable genetic variations as a result of the difference in the clinical source from which they were isolated. Within these bacterial species, Staphylococcus aureus represents a crucial bacterial organism that can be used to connect its genetic variation to the clinical source .This study was conducted to identify the genetic polymorphism of two bacterial samples of S.aureus that were isolated from two clinical sources (wounds and burns) in Diyala province.TheermC gene-based genetic investigation revealed a remarkable deletion in both investigated samples since S12 and S14 showed one deletion mutation of A54del. This deletion manifested in clear phylogenetic positioning for both samples in the ermC gene-based tree

Keywords: S.aureus, Antibiotic Resistance ,erythromycin genes ,ermA ,ermB ,ermC

Introduction

Staphylococcus aureus species are known as human pathogens which cause skin and soft tissue infections, acute septicemia, pneumonia and toxic shock syndrome(Goudarziet al.,2016) . S aureus is the most common microorganism isolated from wounds, and colonization requires careful management because of its ability to become resistant to antibiotics. Thus, wounds are at risk of

colonization with S. aureus(Pireset al., 2018) These organisms are resistant to most of drugs and constant against most of disinfectants agentsNowadays, antibiotic resistance of S. aureus is a major problem in society(Talebiet al.,2019).The stability and worldwide spread of this pathogen isdue to its' ability to rapidly acquire and loss resistanceand virulence determinants from other members ofthe genus Staphylococci through horizontal transferof mobile genetic elements (MGEs) (Bitruset al., 2017).Some studies have also demonstratedthe role of horizontal gene transfer in rapid acquisitionand dissemination of antibiotics resistance determinantsin S. aureus(Sabetet al., 2014).some pathogenic bacteria become resistant tomultiple kinds of antibiotics. S.aureusis becoming a main public health problem because of the continuous elevation in antibiotic resistance (Oliveira , 2011).Resistanceto tetracycline, chloramphenicol and erythromycinare carried by small plasmids while, large plasmidscarry multiple drug resistance genes to aminoglycosides,
beta-lactams and macrolides. Additionally, largerplasmids also integrate with other MGEs such astransposons and confer resistance to spectinomycin, trimethoprim, erythromycin, beta lactams and vancomycin
(Planet et al., 2017)Macrolides including erythromycinare the antibiotics used against Gram-positive and someGram-negative bacteria. Three mechanisms in Grampositivebacteria that result in resistance to erythromycinare as follows: (I) modification in the ribosomal targetsite, mediated by the methyltransferases encoded byerm(erythromycin resistance methylase) genes, (II) effluxpump encoded by msrA/B (macrolide specific resistancegenes) and (III) ereA/B (erythromycin esterases) genes (Zmantaret al.,2008) .Among these mechanisms, the ermencoded methy-lases are the major factor of resistance to macrolides. Among many reported and sequenced ermgenes, three major genes of ermA, ermBand ermCare present in staphylococci(Maravicet al.,2004).

Aim of the study

This study aimed to determine the prevalence of erm genes (ermA ,ermB ,ermC) which were recovered from various clinical samples from hospitalized patients in Diyala hospitals **.**

Isolation and identification of bacterial isolates

A total of (300) clinical specimen from both genderwith different age were collected from the beginning of December 2019 to the end of August 2020, from patientsin different hospitals of Baquba city. The isolates wereidentified by their colony characteristic, gram-stain andconfirmed by the pattern of biochemical profiles usingVitek 2-GN system.

Antibiotic Susceptibility testing

To estimate potential resistance of S.aureusisolates against 15 items of antibiotics from differentclasses, all isolates had been subjected to antibiogram
test according to CLSI (2017), forceftriaxone,Cefotaxime,imipenem,Norofloxacin, ciprofloxacin ,Amikacin,gentamycinvancomycin,azithromycin, erythromycin, tetracycline,doxycycline,colistin,trimethoprim,Chloramphenicol,. Detection of S.aureusphenotypes based on the drug resistance patterns.Multidrug-resistant (MDR) phenotype is defined asS.aureus, which is resistant to more than oneantimicrobial agent in three or more antimicrobialcategories. Extensively drug-resistant (XDR) phenotypeis defined asSaureus, which is resistant to morethan one antimicrobial agent in all the antimicrobialcategories, except in two or less.

DNA Extraction and polymerase chain reaction

(PCR) amplification: Genomic DNA was extracted from isolates using extraction Kits of Genomic DNA, Purification depending on instruction of manufacturing company (Promga USA).All erythromycin -resistant isolates were screened by standard PCR conventional using specific primers for ermA, ermB, ermC genes as shown in table (1). PCR reaction tubes were transferred into thermal cycler that was programmed as following: initial denaturation for 5 mints at 95°C, (the conditions for each cycle were: 30 sec. at 95 C̊ , 30 sec. at 55C and 30 sec. at 72°C), and final extension at 72°C for 5 mints. Amplified PCR products were detected by agarose gel electrophoresis.

Table (1): The primers used forErythromycingenes detection

Primer	Oligo sequence (5'-3')	Product size bp	Annealing temp°C	Reference
ermA	F-5`-TATCTTATCGTTGAGAAGGGATT-3` R- 5`-CTACACTTGGCTTAGGATGAAA-3`	139	55	Goudarzi,et al.,2016
ermB	F- 5`-CCGTTTACGAAATTGGAACAGGTAAAGGC-3` R- 5`-GAATCGAGACTTGAGTGTGC-3`	360	55	Goudarzi, et al.,2016
ermC	F- 5`-CTTGTTGATCACGATAATTTCC-3` R- 5`-ATCTTTTAGCAAACCCGTATTC-3`	190	55	Goudarzi, et al.,2016

DNA Sequencing of PCR amplicons

The resolved PCR amplicons were commercially sequenced from termini, forward, and reverse, according to instruction manuals of the sequencing company (Macrogen Inc. Geumchen, Seoul, South Korea). Only clear chromatographs obtained from ABI (Applied Biosystems) sequence files were further analyzed, ensuring that the annotation and variations are not because of PCR or sequencing artifacts. By comparing the observed DNA sequences of local samples with the retrieved DNA sequences of the bacterial database, the virtual positions, and other details of the retrieved PCR fragments were identified.

Statistical Analysis

The Statistical Analysis System- SAS (2012) program was used to detect the effect of difference factors in study parameters. Least significant difference –LSD test (Analysis of Variation-ANOVA) was used to significant compare between means. Chi-square test was used to significant compare between percentage (0.05 and 0.01 probability) in this study.

Results and Discussion

A total of 75 clinical isolates of gram positive bacteria primary identified as Staphylococcus aureus were collected from different clinical sources.The source of these isolates were as follows:burn 24(32%) Wounds 18(24%) blood 15(20%) , Nasal carriage 11 (14.66%), urine 7(9.33%)

Antimicrobial Sensitivity Test

Seventy-five ofS.aureusisolates were screened for their resistance to 15 different types of antibacterial agents. Results in Table 2 shows that isolate varied in their resistance and sensitivity to the antibiotics. It was found high resistance tobeta lactams, aminoglycosidesandflouroquinolones. Profile of antibiotic resistance to other antibiotics is shown in table (2). In this investigation, antibiotic susceptibility testing of the S,aureusisolates showed that 25(33.3%), 33(44%), 17(22.6) of the isolates were MDS, MDR, XDR.For many years, a number of S. aureus isolates have evolved resistance to both synthetic and traditional antimicrobial chemotherapy and their prevalence outside the hospital is of potential epidemiological threat . This trend does not only increase morbidity and mortality but also higher cost of healthcare The spread of resistance to antimicrobial agents in S. aureus is largely due to the acquisition of plasmids and or transposons (Ismail et al.,2015).this present study agree with (Shamkhi ,2019) that found a highest resistance to Azithromycin 88 (91.67%), Tetracycline 89 (92.71%), Erythromycin86 (89.58%), and Trimethoprime-slphamethoxazole 61(63.54%).Study by (Al-hamedawy and Mahmoud,2019) in Iraq revealed that resistance percentage to Cefotaxim, Ceftriaxone, Ciprofloxacin,norofloxacin, vancomycin ,erythromycin is (73.9%),(73.9%),(50%),(50%),(6.5%),(32.6%).Finally, S. aureus isolates showed the lowest rates of resistance toward imipenem with a sensitivity rate that reached to 88%. A previous study by Abd-Alamer and Al-Khozai (2016) also showed low resistance (20%)

Table (2): Antibiogram susceptibility of Staphylococcus aureus isolatestoward antistaphyllococcalagents (n=75).

Antimicrobial agent	Resistant isolates No. & %	Sensitive isolates No. & %	P-value
Cefotaxime	**75 (100%)**	**0%**	0.0001 **
Ceftriaxone	**69(92%)**	**6 (8%)**	0.0001 **
Imipenem	**9 (12 %)**	**66 (88%)**	0.0001 **
Norfloxacin	**48 (64%)**	**27(36%)**	0.0153 *
Ciprofloxacin	**42(56%)**	**33(44%)**	0.298 NS
Azithromycin	**63 (84%)**	**12(16%)**	0.0001 **
Erythromycin	**60(80%)**	**15(20%)**	0.0001 **
Gentamycin	**56 (74.6%)**	**19 (25.3%)**	0.0001 **
Amikacin	**53 (70.6%)**	**22 (29.3%)**	0.0003 **
Vancomycin	**36 (48%)**	**39 (52%)**	0.729 NS
Tetracycline	**66 (88%)**	**9 (12%)**	0.0001 **
Doxycycline	**45(60%)**	**30(40%)**	0.0833 NS
Trimethoprim	**51 (68%)**	**24 (32%)**	0.0018 **
Colistin	**75(100%)**	**0**	0.0001 **
Chloramphenicol	**27 (36%)**	**48(64%)**	0.0153 *
* (P≤0.05).** (P≤0.01).			

Molecular detection of ermgenes

the frequency of erm genes is variable in different studies. Among 15 isolates that resistant to erythromycin genes the results achieved by using PCR revealed that 15(100%) isolates carried ermCgenes (Fig.3), while5 (33.3%) isolates have ermB genes (Fig.2). The number of isolates that have ermAare 5(33.3%) (fig.1). The percentage of gene in the current study was agreed with(Liet al.,2019) who found that ermC found in (90%) in isolates in china . In another study erm C was the most common gene detected in Iran, Turkey and Brazil (Ghanbariet al., 2016) reported erm B was the most common genes detected from S. aureus isolates. our study disagreed with Zmantaret al. (2011) reported ermB was the most common genes detected from S. aureus isolates. the percentage ermA higher than previous study in Serbia wasnoted ermAappearedin25.5% of isolates(Mišicet al.,2017).

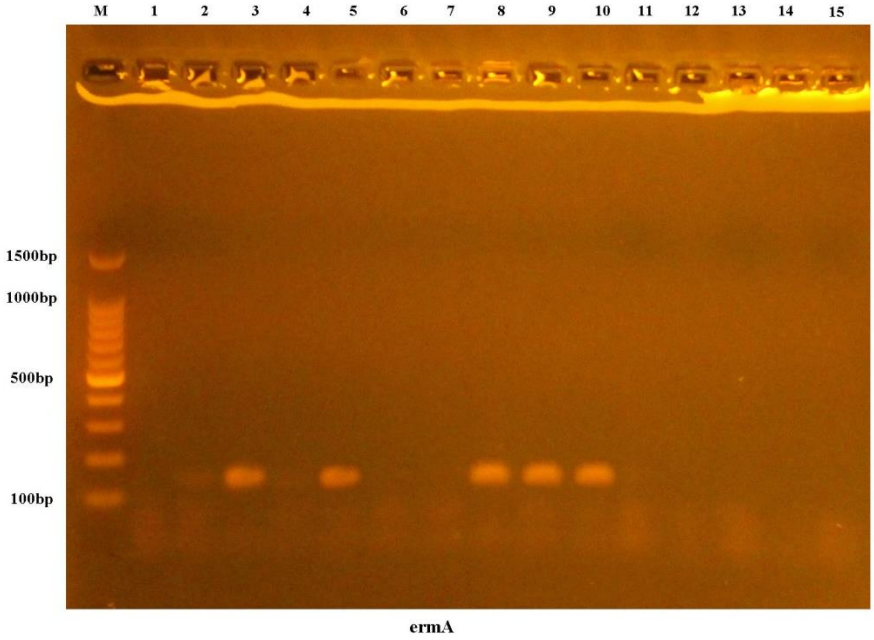


Fig (1) Results of the amplification of ermA gene of Staphylococcus aureus samples were fractionated on 1.5% agarose gel electrophoresis stained with Eth.Br. M: 100bp ladder marker. Lanes 1-15 resemble 139bp PCR products.

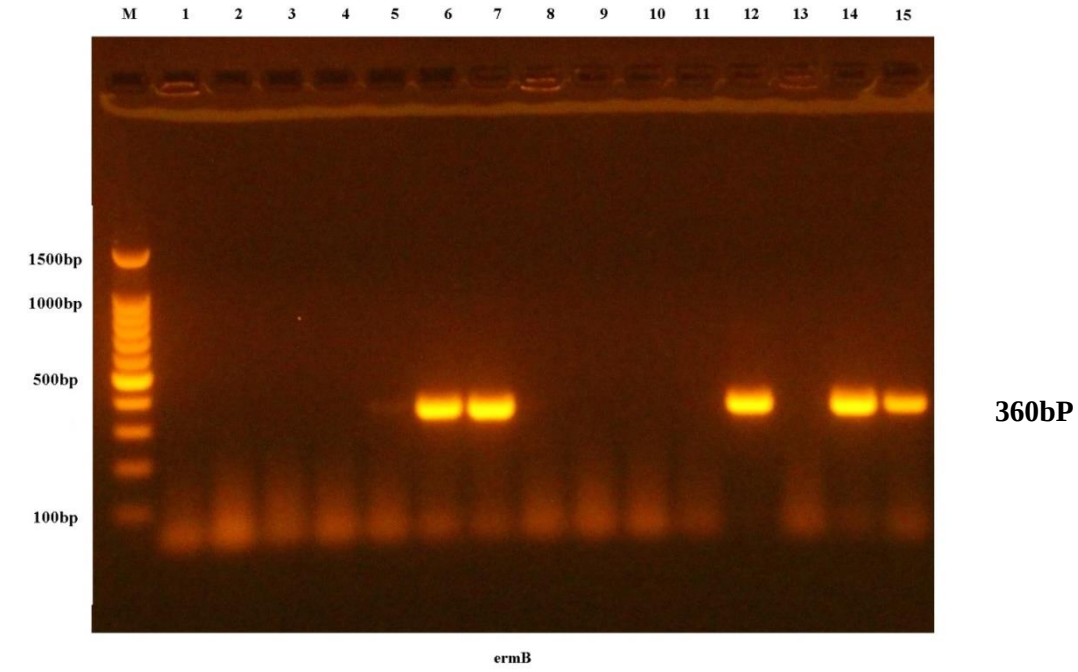


Fig (2) Results of the amplification of ermB gene of Staphylococcus aureus samples were fractionated on 1.5% agarose gel electrophoresis stained with Eth.Br. M: 100bp ladder marker. Lanes 1-15 resemble 360bp PCR products.

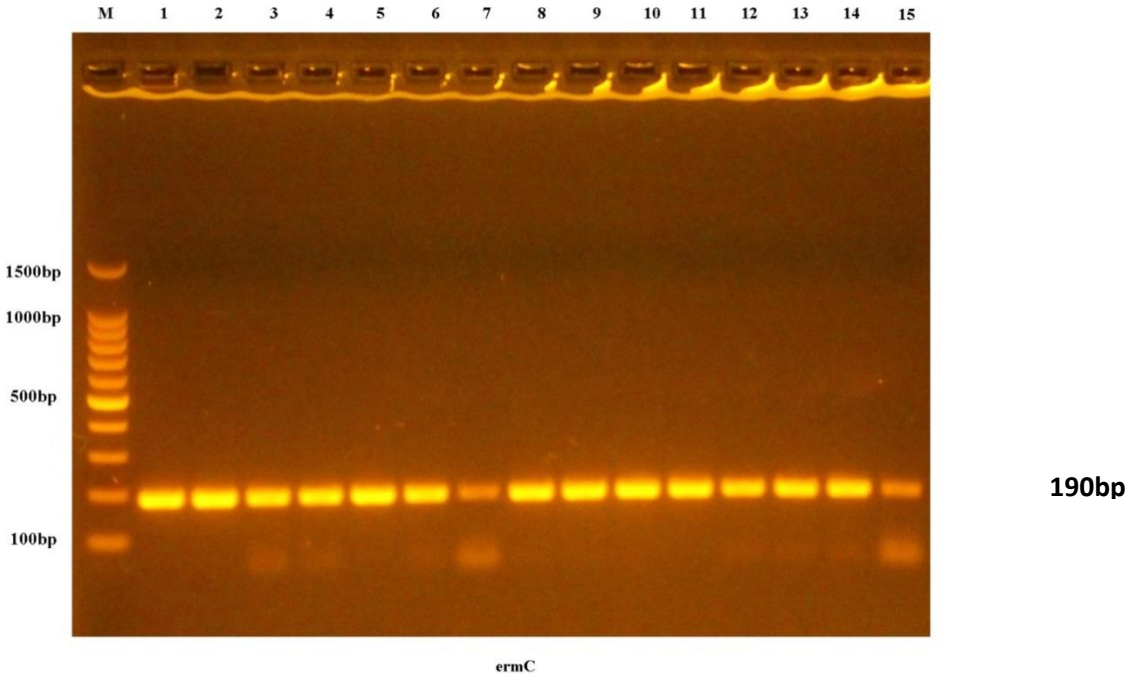
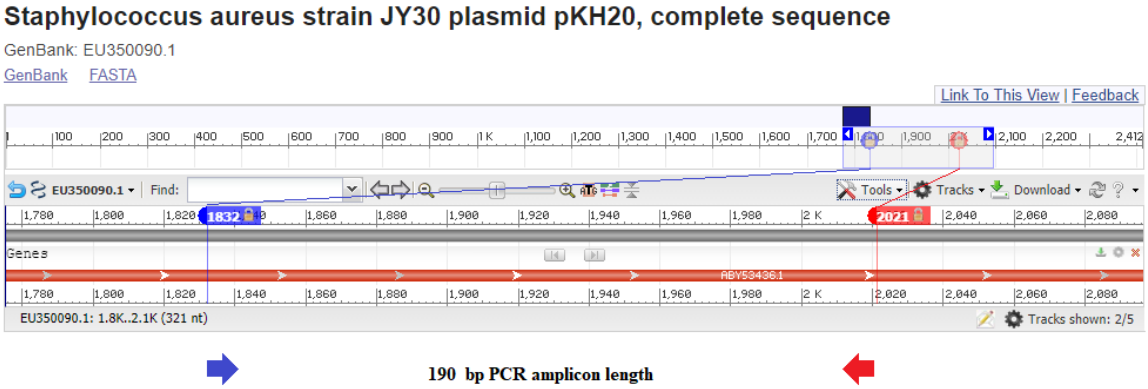


Fig (3) Results of the amplification of ermC gene of Staphylococcus aureus samples were fractionated on 1.5% agarose gel electrophoresis stained with Eth.Br. M: 100bp ladder marker. Lanes 1-15 resemble 190bp PCR products.

Sequencing and phylogeny analysis ofermCgene

The sequencing reactions indicated the exact positions after performing NCBI blastn analysis. This engine showed about 99% sequences of similarity between the sequenced samples and this target. NCBI BLASTn engine indicated the presence of remarkable homology with the expected target that covered a portion of the ermClocus within the S. aureus sequences. By comparing the observed DNA sequences of these bacterial samples with the retrieved DNA sequences (GenBank acc. EU350090.1), the exact positions and other details of the retrieved PCR fragment were identified Fig (4)



Fig(4) The exact position of the retrieved 190 bpamplicons that partially covered a portion of ermClocuswithin the S. aureus genomic sequences (acc. no. EU350090.1). The blue arrow refers to the starting point of this amplicon while the red arrow refers to its endpoint.After positioning the 190 bpamplicons' sequences within the ermClocusof S. aureus sequences, the details of these sequences were highlighted, starting from the position of the forward primer to the position of the reverse primer within the same sequences (Table3).

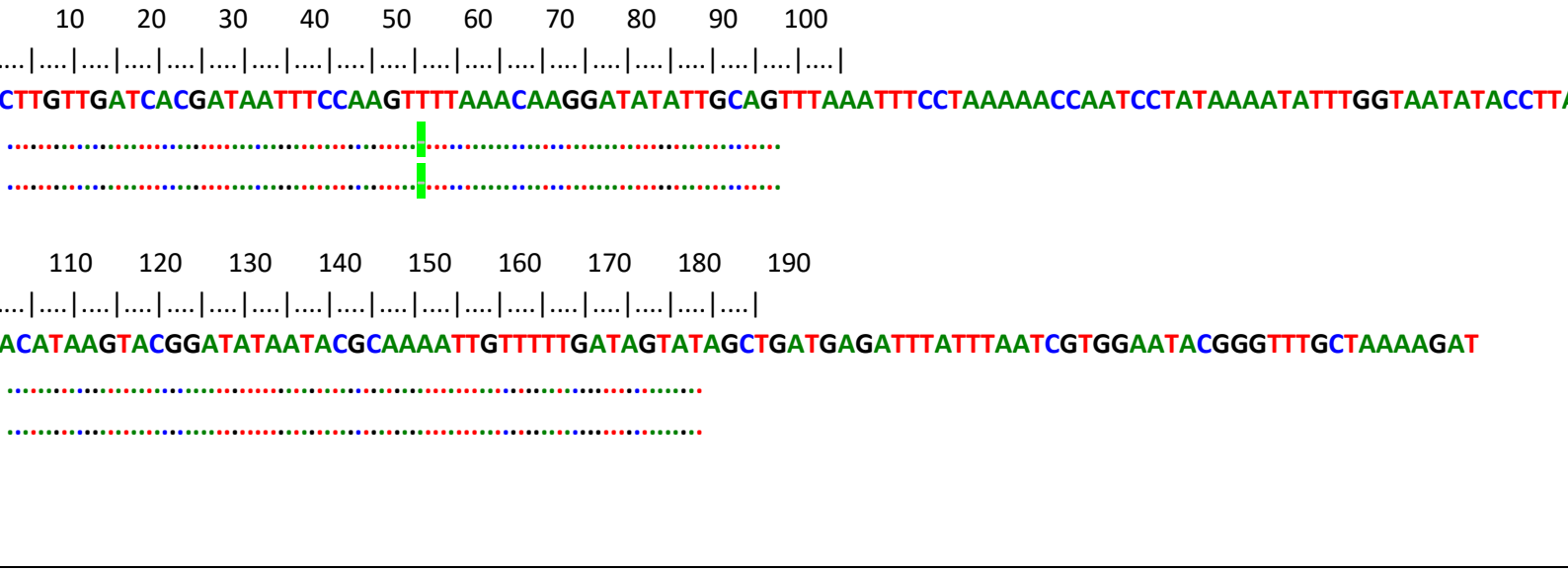
Table(3). The position and length of the 190 bp PCR amplicons that used to amplify a portion of the ermClocuswithin the S. aureus genomic sequences. The amplified sequences were positioned within the NCBI reference DNA sequence of the GenBank acc. no. EU350090.1.

Amplicon	Reference locus sequences (5' - 3')	length
ermCsequences	CTTGTTGATCACGATAATTTCCAAGTTTTAAACAAGGATATATTGCAGTTTAA ATTTCCTAAAAACCAATCCTATAAAATATTTGGTAATATACCTTATAACATAA GTACGGATATAATACGCAAAATTGTTTTTGATAGTATAGCTGATGAGATTTA TTTAATCGTGGAATACGGGTTTGCTAAAAGAT	190bp

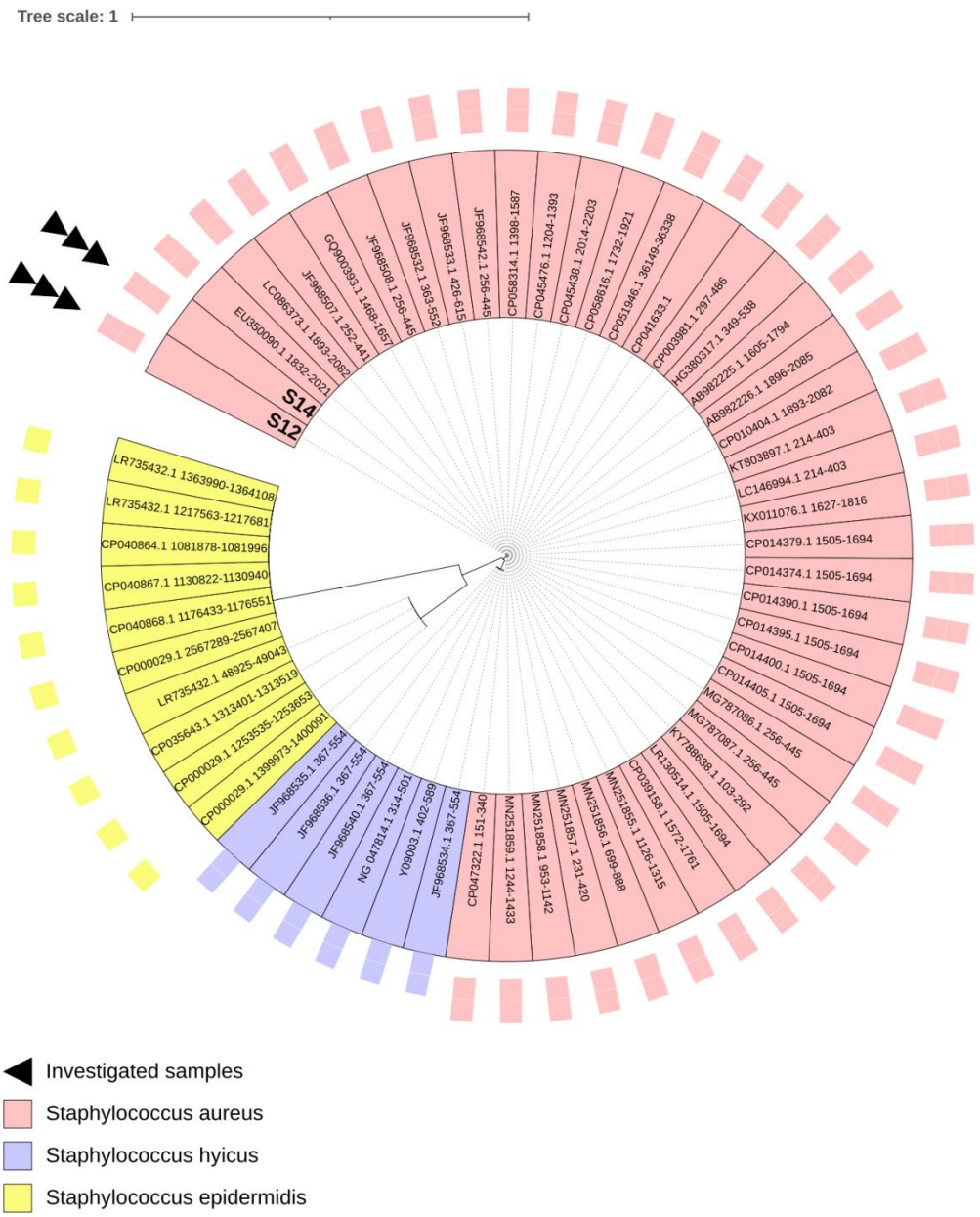
* Refers to the forward primer sequences (placed in a forward direction)

**Refers to the reverse primer sequences (placed in a reverse complement direction

The alignment results of the 190 bp samples revealed the detection of one indel (insertion-deletion) mutation in both investigated samples (S12 and S14) in comparison with thethe alignment results of the 190 bp samples revealed the detection of one indel (insertion-deletion) mutation in both investigated samples (S12 and S14) in comparison with thereferring sequences of the GenBank acc. EU350090.1(Fig.5). These nucleic acid mutations were represented by one deletion detected at position 54 in the ermCamplicons



Fig(5) DNA sequences alignment of two bacterial samples with their corresponding reference sequences of the ermClocuswithin the S. aureus genomic sequences. The symbol “ref” refers to the NCBI reference sequences, while “S” refers to sample code.A comprehensive phylogenetic tree was generated in the present study, which was based on the observed nucleic acid variation detected in the investigated sample. This phylogenetic tree has contained these amplicons, alongside other relative reference sequences. The total number of the aligned nucleic acid sequences in this comprehensive tree was 57. This generated comprehensive tree indicated the presence of three highly-related bacterial organisms, S. aureus, S. hyicus, and S. epidermis. The currently generated neighbor-joining comprehensive tree showed extremely close genetic distances between both incorporated sequences of S. aureus, S. hyicusFig(6)



Fig(6) The comprehensive phylogenetic tree of genetic variants of the ermClocuswithin the S. aureus bacterial isolates. The variably colored numbered refer to Genbank acc. numbers of deposited reference sequences. The number “1” at the top portion of the tree refers to the degree of scale range among the comprehensive tree categorized organisms. The symbol “S” refers to the code of the investigated sample .Within this tree, our investigated samples resided in one phylogenetic group, S12 / S14 group, in the major phylogenetic clade of the main incorporated S. aureus sequences. the reason for the same positioning of S12 and S14 belonged to the A54del mutation that was observed in both investigated samples. Noteworthy, both S12 and S14 are positioned in the vicinity of the GenBank accession number of EU350090.1, which is a Korean isolate of the same organism. However, both resolved groups were not found to be far away from each other as both of them were still having close genetic distances. However, the currently observed A54del was not associated with the origin of isolation of these bacterial isolates as both samples isolated from

different sources having the same deletion. This notion entailed no possible role for ermC-based amplicons in inducing any possible adaptation for S. aureus to each clinical stress within the host. For this reason, this ermC– based observation indicated a limited role of the generated phylogenetic tree in the discrimination between these bacterial sequences.

References

- Abd-AlamerHand Al-Khozai, Z. (**2016)**. Phages isolation Staphylococcus aureus resistant to methicillin and can be used in bioremediation against host. Journal of Al-qadisiyah for Pure Science. **21**(4): 84–105.
- Al-hamedawyH , Mahmoud S.(2019). Synergistic Effect of Linezolid, Tigecycline, and Vancomycin on Staphylococcus Aureus Isolated From Iraqi Patients with Diabetic Foot Ulcers . Iraqi Journal of Science, Vol. 60, No.1.
- Bitrus A, Zunita Z, Bejo S, Othman S, Nadzir N. (2017). In vitro transfer of methicillin resistance determinants mec A from methicillin resistant Staphylococcus aureus (MRSA) to methicillin susceptible Staphylococcus aureus (MSSA). BMC microbiology. 17(1):83. https:// doi.org/10.1186/s12866-017-0994-6.
- CLSI. Performance Standards for Antimicrobial Susceptibility Testing. 27th ed. Wayne, PA: Clinical and Laboratory Standards Institute. (2017).
- Forstall G, Knapp C and Washington J. (1991). activity of new quinolones against ciprofloxacin-resistant staphylococci. Antimicrobial agents and chemotherapy, 35(8)
- Ghanbari F, Ghajavand H, Havaei R, Jami MS, Khademi F, Heydari L, Shahin M, Havaei SA (2016). Distribution of erm genes among Staphylococcus aureus isolates with inducible resistance to clindamycin in Isfahan, Iran. Adv. Biomed. Res. 5.
- Goudarzi G, Tahmasbi F, Anbari K, Ghafarzadeh M.(2016). Distribution of genes encoding resistance to macrolides among staphylococci isolated from the nasal cavity of hospital employees in Khorramabad, Iran. Iran Red Crescent Med J; 18: 25701.
- Ismail H, Bello H, Mustafa A, Adamu A .(2015).Multidrug Resistance Pattern of Staphylococcus Aureus Isolates in Maiduguri Metropolis Vol. 1, No. 2.
- Li X, Huang T, Xu K. et al.(2019). Molecular characteristics and virulence gene profiles of Staphylococcus aureus isolates in Hainan, China. BMC Infect Dis **19,** 873. https://doi.org/10.1186/s12879-019-4547-5.
- Maravic G. Macrolide resistance based on the Erm-mediated rRNA methylation.(2004). Curr Drug Targets Infect Disord. **4**(3):193–202. [PubMed: 15379730].
- Mišic´ M, ukic´ J, Vidanovic´ D, Šekler M, Matic´ S, Vukašinovic´ M and Baskic´ D .(2017). Prevalence of Genotypes That Determine Resistance of Staphylococci to Macrolides and Lincosamides in Serbia. Front. Public Health 5:200. doi: 10.3389/fpubh.2017.00200.
- Oliveira DC, de Lencastre H. Methicillin-resistance in Staphylococcus aureus is not affected by the overexpression in trans of the mecA gene repressor: a surprising observation (2011). PLoS One. 6(8):e23287. doi: 10.1371/journal.pone.0023287. Epub Aug 2. PMID: 21829724; PMCID: PMC3149077.
- Pires B, De Oliveira F , De Oliveira B, Fuly P ,Ferreira-Carvalho B, De Paula Gand Teixeira L. (2018). Monitoring and Molecular Characterization of Staphylococcus aureus Isolated from Chronic Wounds. Advances in skin and wound care, 31: 399-405.

- Planet P, Narechania A, Chen L, Mathema B, Boundy S, Archer G, Kreiswirth B. (2017). Architecture of a species: phylogenomics of Staphylococcus aureus. Trends in microbiology. 25(2):153-66. https://doi.org/10.1016/j. tim.2016.09.009
- Sabet N, Subramaniam G, Navaratnam P. and Sekaran S. (2014). In vitro mecA gene transfer among Staphylococcus aureus in Malaysian clinical isolates. African Jouranl of Biotechnology, 11: 385-390.
- SAS. 2012. Statistical Analysis System, User's Guide. Statistical. Version 9.1th ed. SAS. Inst. Inc. Cary. N.C. USA.
- Shamkhi G.J.(2019) .The effect of natural bioactive inhibitors on efflux pump in methicillin resistant Staphylococcus aureus . PhD. Thesis, Genetic Engineering & Biotechnology for Postgraduate Studies, University of Baghdad, Iraq.
- Talebi G, Hashemia A, Goudarzi H, Shariati A, Bostanghadiri N, Sharahi J.Y, &Abbsi E. (2019). Survey of ermA, ermB, ermC and mecA genes among Staphylococcus aureus isolates isolated from patients admitted to hospitals in Tehran, Iran by PCR and Sequencing. Biomedical Research-tokyo, 30, 259-263.
- Zmantar T, Chaieb K, Ben Abdallah F, Ben Kahla-Nakbi A, Ben Hassen A, Mahdouani K, et al.(2008). Multiplex PCR detection of the antibiotic resistance genes in Staphylococcus aureus strains isolated from auricular infections. Folia Microbiol (Praha). **53**(4):357–62. doi: 10.1007/s12223-008-0055-5. [PubMed: 18759121].
- Zmantar T, Kouidhi B, Miladi H, Bakhrouf A. (2011). Detection of macrolide and disinfectant resistance genes in clinical Staphylococcus aureus and coagulase-negative staphylococci. BMC Res. Notes 4(1):453.