Molecular Detection of CRISPR-Cas System in *Staphylococcus epidermidis* Isolated from Different Sources in Iraq

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Abstract

The goal of the present study was to isolate and identify (CRISPR-Cas) elements in S. epidermidis (MRSE) from 300 samples collected from various sources. Culture techniques, biochemical tests and VITEK2 system were used for confirming of *Staphylococcus epidermidis* isolation. The partial sequencing of the 16s rRNA gene was evaluated and compared with those in the Gene Bank to find differences in the sequence using the BLAST tool (http://www.ncbi.nlm.nih.gov). On the other hand, the CRISPR-Cas system was examined in all isolates of multidrug-resistant *S. epidermidis* in which the SECR1 and SECR2 elements were not found. The cas6 element was found in all of the tested bacterial strains. Susceptibility tests were done using the disc diffusion method after identification. Antibiotic resistance testing on S. epidermidis isolates revealed the highest percentage of multi-resistance to ampicillin, ceftriaxone, chloramphenicol, cefalexin, vancomycin, nalidixic acid at 100%. Moreover, *S. epidermidis* isolates showed intermediate effect against amoxillin-clavulanic acid, tobramycin, neomycin, oxacillin, tetracycline and methicillin. However, *S. epidermidis* isolates were found to be the most sensitive to erythromycin, norfloxacin and rifampicin. The CRISPR-Cas system can be found in *S. epidermidis* isolates from a variety of local sources and that the appearance of the CRISPR system *in S. epidermidis* isolates plays a unique role in antibiotic suitability.

Introduction

Staphylococcus epidermidis, also known as coagulase-negative (CNS) and Gram-positive *Staphylococcus*, is one of the emerging pathogens as it has become the most common public bacteria found on mucosal surfaces and skin, with the ability to cause nosocomial and inframammary infections in many countries .(1,2)

Antibiotic resistance in microorganisms is a significant public health concern. Infection rates of resistant pathogens continue to rise against practically all antimicrobials, prompting the development of various antimicrobial resistance tactics (3). Furthermore, the rising threat of antimicrobial resistance has highlighted the interconnectedness of humans, animals and the environment, as well as their roles in the transmission and dissemination of resistance genes (4,5). In recent decades, the clinical importance and the emergence of methicillin-resistant *Staphylococcus epidermidis* strains have created many challenges in the treatment process.(6)

The CRISPR-Cas (clustered regularly interspaced short palindromic repeats–CRISPR-associated proteins) system has been discovered in various bacterial strains since the genomic sequences of *Staphylococci sp* were completed (7). The CRISPR-Cas system is prevalent in numerous bacterial strains as an adaptive immune system against plasmids or phages. Cas genes and CRISPR loci, which are repetitive elements intercalated with short spacer sequences that match plasmid or phage genomes, make up the CRISPR system. The bacteria's immunity to these foreign DNA components is determined by the identification of spacers to phage or plasmid sequences (8). Antibiotic resistance genes are frequently linked to foreign nucleic acid elements, and CRISPR can prevent infection by foreign nucleic acids, suggesting that CRISPR may be linked to antibiotic susceptibility (9). As a result, the goal of the present study was to see if there's a link between CRISPR and antibiotic susceptibility in multidrug-resistant *S. epidermidis* (MRSE).

Materials and Methods

Samples collection and isolation of *Staphylococcus epidermidis*

A total of 300 samples were collected from the following sources: normal skin (75 swabs) was taken from the cows, sheep, and goats. Milk (75 samples) was taken from cows, sheep, and goats with clinical and subclinical mastitis. Skin with lesion and wounds (75 swabs) were taken from skin of cows, sheep, and goats and 75 swabs were taken from workers' or dealers' hands. The samples were taken from various locations in Al-Basrah and Al-Muthanna provinces. In the event of milk samples with subclinical mastitis, the California Mastitis Test (CMT) was performed pre-sampling (10). The samples were collected in sterile vials and maintained in an icebox before being transported to the microbiology laboratory.Each sample from milk and swab samples were immediately inoculated onto mannitol salt agar (MSA) and incubated at 37oC for 24h to identify S.epidermidis as described previously (11). Mannitol fermented colonies were then subcultured from parent cultures onto MSA medium and incubation at 37 oC for 24-48 h to purify the colonies. Gram stain and the coagulase tube were used to execute the experiment (12,13). The positive isolates were considered presumptive for *Staphylococcus sp* and sent to the VITEK 2 compact system (bioMérieux company/France) for confirmation.

DNA extraction and PCR analysis

All suspected isolates were cultured at 37°C for 48h on BHI broth. The supernatant the was then discarded and the pellet cells were collected after centrifuging 3ml of culture overnight at 14000g for 2 min. Following the manufacturer's instructions, the bacterial DNA was isolated using the Genaid microbial DNA isolation Kit (Genaid Biotech Ltd, China).

Identification *of Staphylococcus epidermidis* using PCR

Oligonucleotide primers used in the current study are presented in Table 1. The tuf gene was utilized to confirm and identify *S. epidermidis* isolates by amplifying a specific area of previously isolated DNA samples. Using the operational pre-mix pcr (Bioneer/Korea) with 2µl(10 pmol/l) for each primer and 4µl of DNA template, the PCR reaction was finished in 30µl reaction mixtures. Each reaction tube's ultimate volume was adjusted by adding 22µl of nucleus-free water. Initial denaturation at 94°C for 5 min was followed by a 25 cycle of denaturation at 94°C for 30 sec, annealing at 55°C for 30 sec, and extension at 72°C for 30 sec, followed by a final extension at 72°C for 10 min. The PCR amplicons were then run on a 1.5 percent agarose gel stained with ethidium bromide to ensure that the amplification was effective (14). A gel documentation system was utilized to visualize an agarose gel. A fragment of about 370bp was found to be positive for *Staphylococcus spp* bacterium.

16S rRNA Gene Sequencing and Phylogenetic Analysis

The 16S rRNA region was PCR amplified using 27F and 1392 R primers for comprehensive detection of isolates (Table 1). The PCR amplification reaction was carried out in a total volume of 30µl, containing operational Pre-mix pcr from Bioneer/Korea and 4µl of genomic DNA, followed by 2µl of each primer, with the final volume being adjusted by adding 22µl of nucleus-free water. The following program was used to perform the amplification steps in a thermal cycler (USA): The PCR conditions were produced by first denaturing at 94°C for 5 min, followed by 25 PCR cycles at 94°C (30 sec), 55°C (30 sec), and 72°C (30 sec). The last elongation cycle was carried out at 72°C for 30 min. The PCR amplification products are visualized using a gel documentation system after electrophoresis on a 1.5% agarose gel stained with ethidium bromide. As a molecular mass marker, the DNA ladder was used. Macrogen Inc., Seoul, South Korea was sequenced the PCR product. The blast analysis was then used to distinguish between similar sequences in the NCBI database (https://www.ncbi.nlm.nih.gov). The phylogenetic tree was created using the MEGA-11 software and the neighbor-joining method.(15)

Identification of *Staphylococcus epidermidis* CRISPR-Cas Genes

All oligonucleotide primers used in the current study are presented in Table 1. A total of 70 DNA samples were used in the PCR. Pre-mix PCR was utilized to perform the PCR amplification reaction in a total volume of 30µl with reaction chemicals and volume similar to those employed in the 16S rRNA PCR reaction. The PCR conditions are as follows: an initial denaturation step at 94 °C for 5 min followed by 32 cycles of another denaturation step at 94 °C for 30 sec ; an annealing step at 55 °C for 30 sec to Cas genes, 58 °C for SECR1 and SECR2 genes and an elongation step at 72 °C for 1:30 min, followed by a final elongation step at 72 °C for 10 min . Electrophoresis on a 1.5% agarose gel was used to separate the PCR amplicons .(14)

Table 1: The sequences of oligonucleotide primer used in the current study.

Primer name	Sequence (5'-3')	Size (bp)	References
tuf gene (TStaG422 - TStag765)	GGC CGTGTTGAACGTGGTCAAATCA TCACCA TTTCAG TACCTTCTGGTAA	370bp	16
16S rRNA gene (27 F-1392 R)	AGAGTTTGATCCTGGCTCAG GGTTACCTTGTTACGACTT	~1350bp	17
Cas6	AGGAAGTATTTTACATGGTGT AACCTGAAAATTCGCCAAAC	568 bp	18
SECR1	CTATTTTCCTTCGCAGTAAC TCTTGTAGTGAGGGAACGTC	Different size	18
SECR2	TGTCTTGAGAACTAGGAATACC CGTTTACAGGAGAAATGGTG	Different size	18

Antibiotics Susceptibility Test by Disk Diffusion Method.

The disc diffusion method was applied to determine antibiotic susceptibility as described previously (19). The inoculum was made by saline suspending isolated colonies from mannitol salt agar plate, adjusted the suspension with saline and vortexed mixer to match the 0.5 McFarland turbidity standards. The excess inoculum was removed by pushing hard on the inside wall of the tube with a sterile cotton swab soaked in the adjusted solution. The mueller-hinton agar plate's dried surface was inoculated by streaking the swab over the entire surface. This operation was repeated two more times, turned the plate 60 degrees each time to achieve a uniform dispersion of the streaks. To ensure an even distribution of inoculum, the streaking procedure was repeated two more times and rotated the plate approximately 60° each time.
Using sterile forceps, the antimicrobial discs were firmly deposited on the surface of the inoculated agar plate. Within 15 min of applying the discs, the plates were incubated at 35°C overnight. The plates were examined and the diameters of the zones of inhibition were measured after 16 to 18 h of incubation. According to the established rules of the FDA, the results were classified as susceptible, intermediate or resistant.(20)

Statistical analysis

The rate of antibiotics resistance was compared by one-way ANOVA test using GraphPad Prism 8 software.

Results

Prevalence of S. epidermidis according to different techniques

After bacterial culture on MSA and Gram staining, 40 (13.3%) of the 300 isolates examined were suspected of being *S. epidermidis* (Table 2). Based on the coagulase test, the percentage of viable S. epidermidis isolates was 20 (50%). According to the VITEK 2 system results, there were 14 (82.3%) *Staphylococcus spp*. isolates out of 20 coagulase negative samples. On the other hand, the PCR results revealed that there were 14 (100%) *Staphylococcus spp* isolates. While the sequencing analysis results revealed that only 6 samples (42.8%) were identified as *S. epidermidis*. The number and percentage of *Staphylococcus spp* that Obtained by PCR

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were 14 (100 %) while the sequencing analysis results showed only 6 samples (42.8%) were confirmed as *S. epidermidis*. An agarose gel electrophoresis was performed to examine the PCR results. Distinct bands with expected sizes (370 bp) and (1350 bp) were detected on the gel (Figure 1 and 2).

The 1350-bp amplified products were acquired for each sequence and the BLAST tool was used to compare them to the existing global sequences in GenBank (Table 4). The connection between the examination sequence and their adjacent relatives was evaluated using the software MEGA-11 to construct the phylogenetic tree (Figure 3).

Table 2: The Number and Percentage of *Staphylococcus spp.* Recovered from Different Sources of Samples.

Type of samples	Conditions	Total No	MSA+No. (%)	Coagulase test -No. (%)	VITEK +No. (%)
Milk samples cows, sheep and goats	Clinical Mastitis	75	7	0	0
	Subclinical Mastitis		6	5	3
Swabs samples	Normal skin	75	9	6	3
cows, sheep, goats and Hands of workers or dealers	Skin lesions and wounds	150	18	9	8
Total No. (%)		300	40 (13.3)	20 (50)	14 (70)

Table 3: Identification of *S. epidermidis* by PCR and 16SrRNA Sequences Analysis.

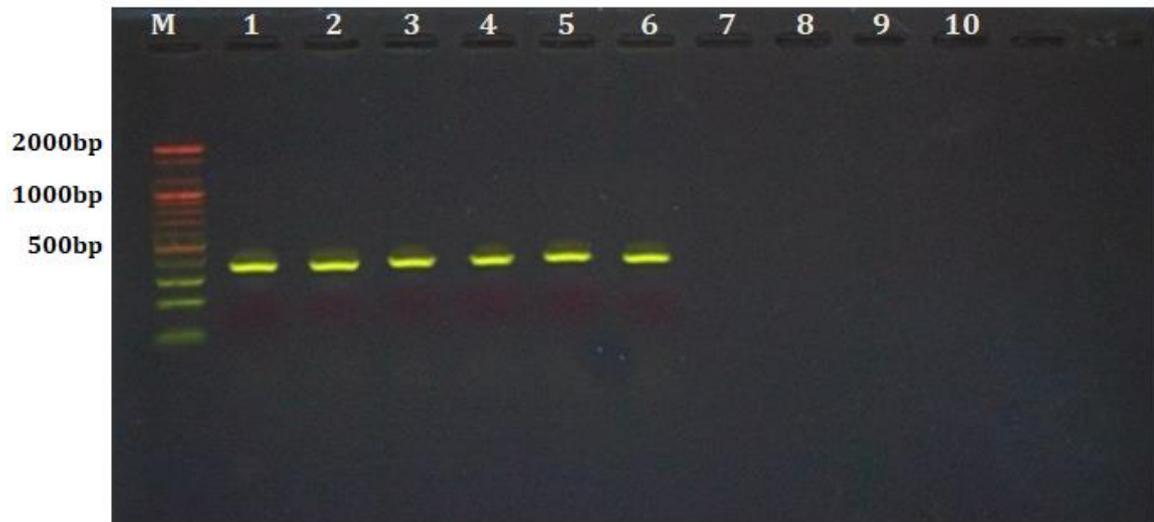| Type of samples	Conditions	PCR +No. (%)	Sequences +No. (%)
Milk samples cows, sheep and goats	Clinical Mastitis	-	0
	Subclinical Mastitis	3	2
Swabs samples	Normal skin	3	1
cows, sheep, goats and Hands of workers or dealers	Skin lesions and wounds	8	3
Total No. (%) 14		14 (100)	6 (42.8)

Figure 1: **PCR Amplification of *Staphylococcus sp.* The *tuf* gene. The product was electrophoresis on 1.5% agarose. Lane M: DNA ladder, Lanes 1-6, positive PCR amplification results, which give band in size (370 bp).**
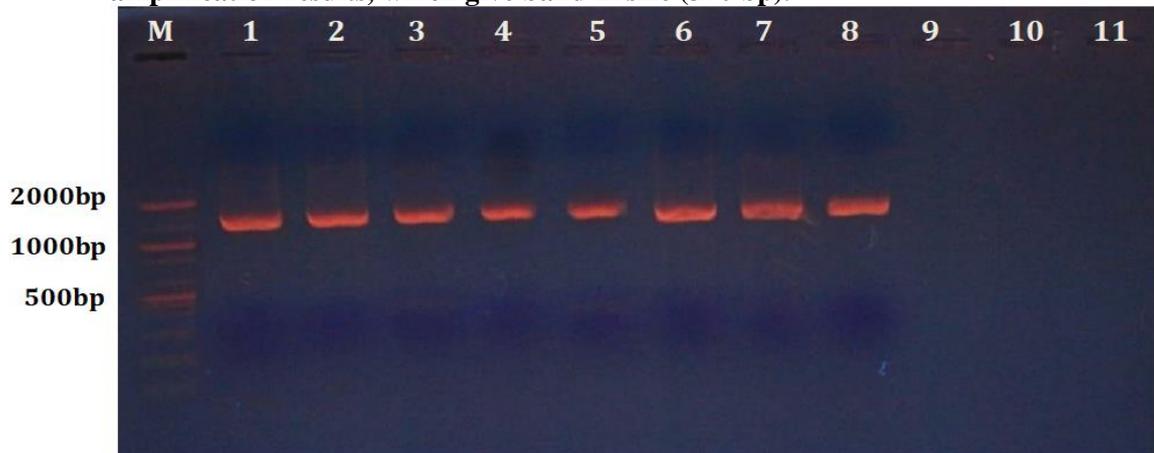

Figure 2: **Gel electrophoresis of PCR product of the 16S rRNA gene. The product was electrophoresis on 1.5% agarose. Lane M: DNA ladder (100bp), positive PCR amplification results in Lanes 1-8, which give band in size (~1350 bp).**

 Table 4: The identification results of 6 strains of *S.epidermidis*   based on 16S rRNA Sequences analysis

Sample No.	Sources	Identities	GenBank ID	Country
211	*Staphylococcus epidermidis strain JST6*	96%	MH311997.1	Czech
	Staphylococcus epidermidis strain P2A1	96%	MT792522.1	Czech
	Staphylococcus epidermidis strain 309-13	96%	MG557813.1	China
225	*Staphylococcus epidermidis strain SS16*	96%	MK347055.1	Pakistan
	Staphylococcus epidermidis ,isolate: T7-3M	96%	AB617572.1	South

|				
	Staphylococcus epidermidis strain 3-9	96%	MN938189.1	Korea
				China
230	*Staphylococcus epidermidis strain T14.3*	99%	MN198059.1	Serbia
	Staphylococcus epidermidis strain BM36	99%	MZ350502.1	Italy
	Staphylococcus epidermidis strain CDC121	99%	CP034115.1	South Korea
234	*Staphylococcus epidermidis strain LQ-1*	98%	KM013932.1	China
	Staphylococcus epidermidis strain T14.3	98%	MN198059.1	Serbia
	Staphylococcus epidermidis strain SB018	98%	KY623311.1	USA
29	*Staphylococcus epidermidis strain CIFRI P-TSB7*	96%	JF784023.1	India
3	*Staphylococcus epidermidis strain CIFRI P-TSB7*	96%	JF784023.1	India
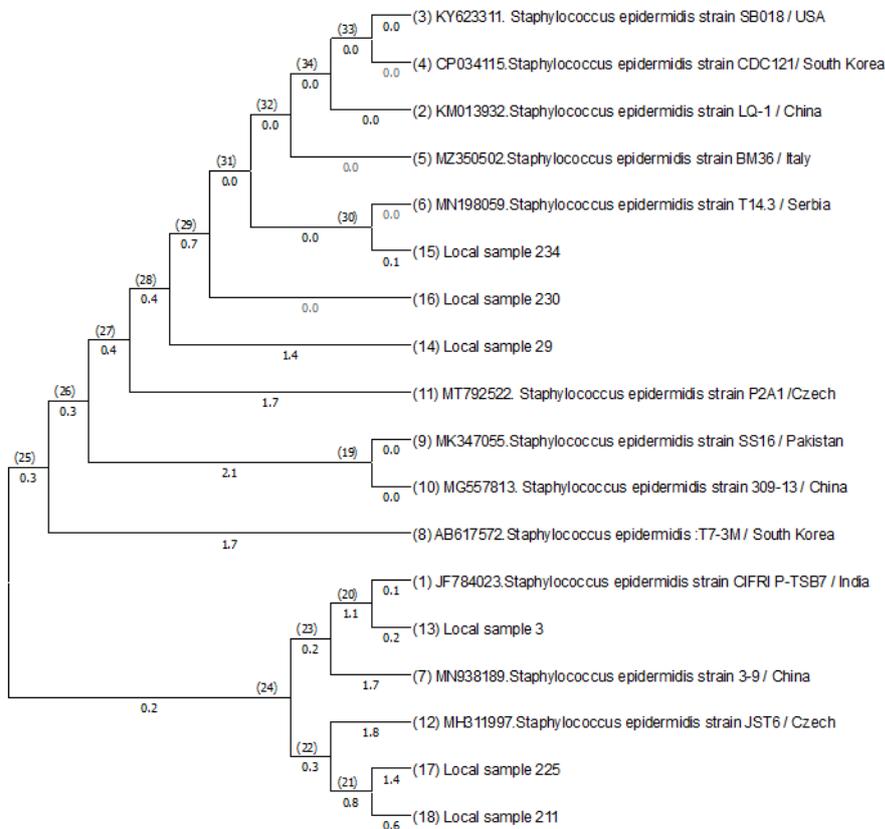
Figure 3: Phylogenetic tree of the *S. epidermidis:* Phylogeny showing the genetic relationship of 6 local strains of *S. epidermidis* isolated from different sources in Iraq and with other international strains which were extracted from GenBank database.

Molecular Detection of CRISPR-Cas in *Staphylococcus epidermidis* Isolates

CRISPR elements were studied in all *Staphylococcus epidermidis* isolates (Table 5). The SECR1 and SECR2 elements were no

detected. However, the cas6 element was detected in all investigated bacterial strains. Furthermore, the accepted cas6 element PCR bands have an average size of 568bp (Figures 4).

Table 5: Number of CRISPR markers detection among *S. epidermidis* isolates

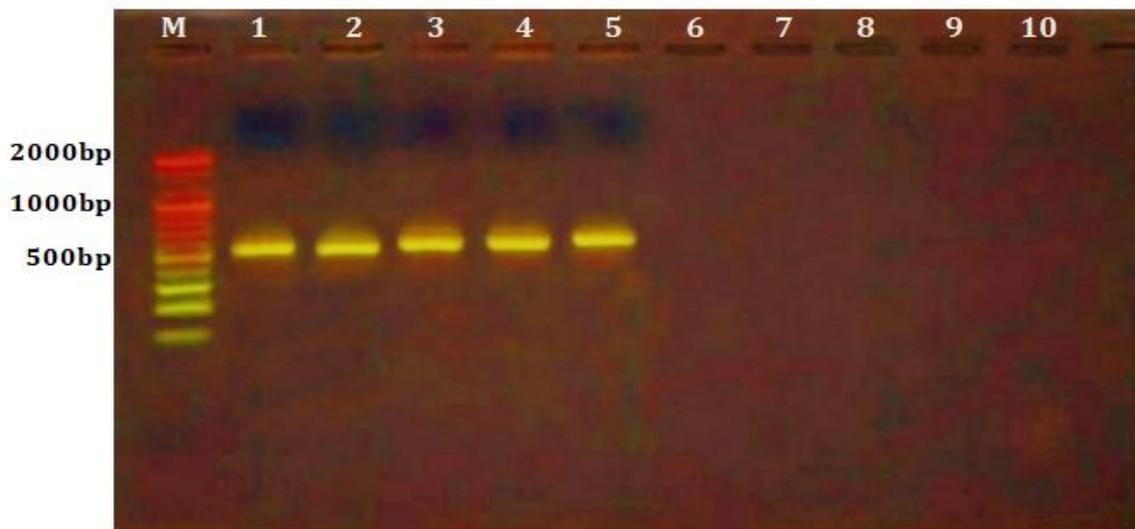| Source of samples	Condition	Positive Samples	Type of CRISPR		
			*Cas*6	*SECR1*	*SECR2*
Milk samples cows, sheep and goats	Clinical Mastitis	0	0	0	0
	Subclinical Mastitis	2	2	0	2
Swabs samples cows, sheep and goats	Normal skin	0	0	0	0
	Skin lesions and wounds	4	4	0	4
Swabs samples	Hands of workers or dealers	0	0	0	0
Total No. (6)		6 (100%)	6 (100%)	0%	0%

Figure (4): PCR products of the *cas* 6 gene for *Staphylococcus epidermidis* Isolates. Lane M: DNA Ladder; Lanes (1-5) positive PCR amplification results, which give band in size (568bp).

Antibiotics Susceptibility Test

All *S. epidermidis* isolates (100%) were susceptible to ciprofloxacin, clindamycin, norfloxacin, rifampicin, erythromycin, and streptomycin. The sensitivity level against tobramycin, neomycin, and tetracycline was 50%. The isolates revealed the highest resistance level (100%) against chloramphenicol, cefalexin, ceftriaxone, ampicillin, and vancomycin. The resistance level against nalidixic acid was 83% and 50% against both neomycin and tetracycline,

respectively. Some isolates revealed intermediate effects against amoxillin-clavulanic acid, oxyacillin and methacillin and resistance at 100%. However, some isolates revealed 50 % against tobramycin (Figure 5 and 6). The correlation between the cas6 space, the susceptibility and resistance to the tested antibiotics was presented in Figure 7.
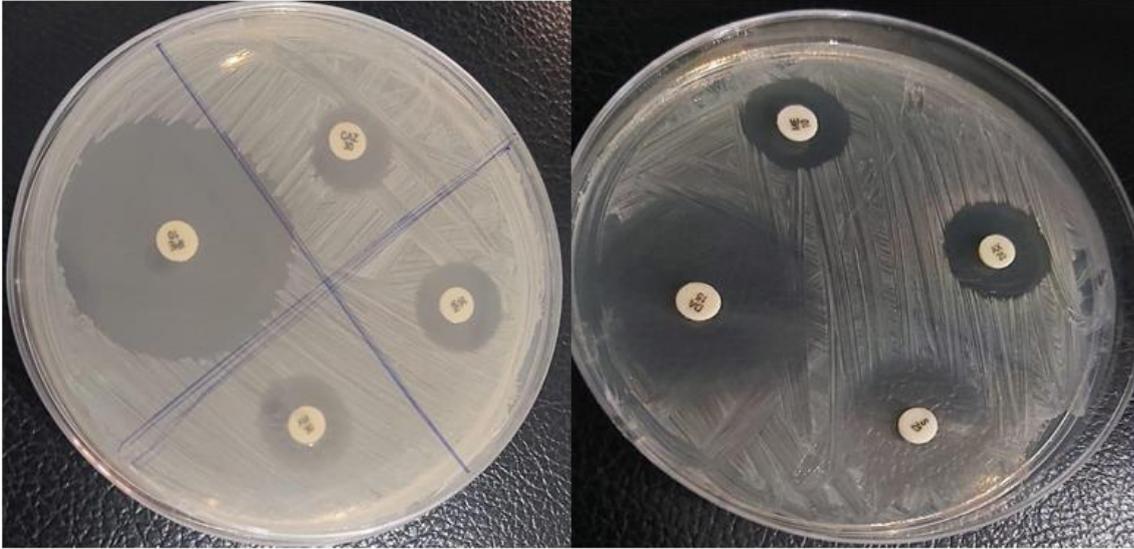

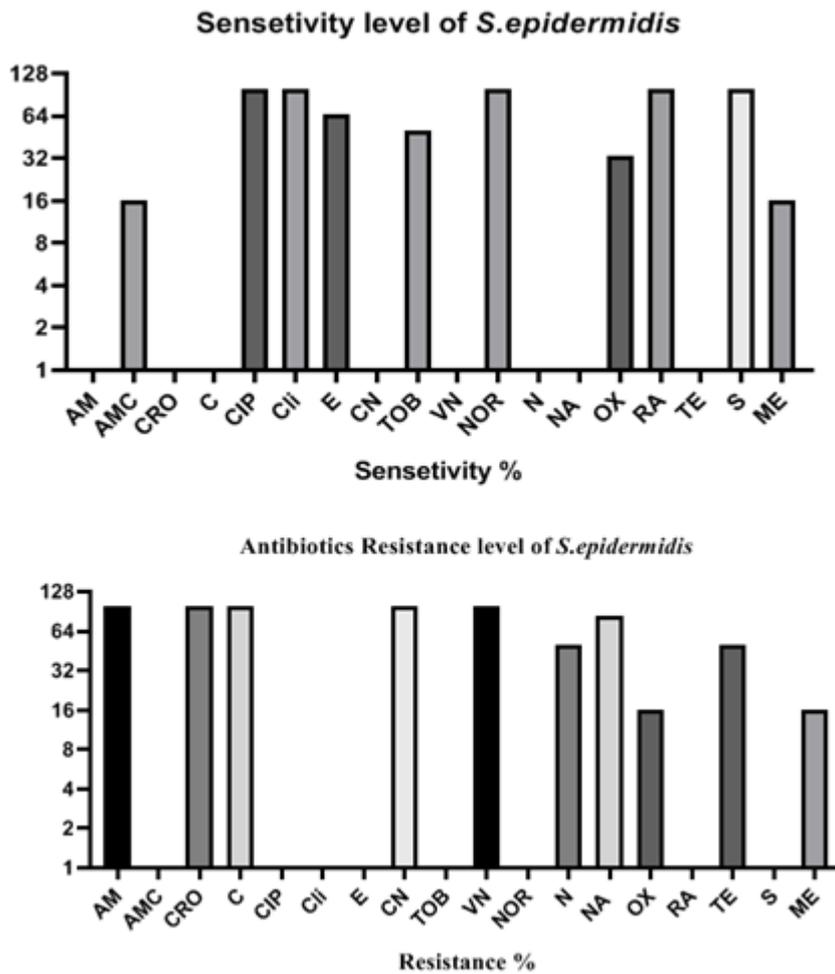**Figure 5: Antimicrobial Susceptibility Test Against *S. epidermidis* Isolates.**

Figure 6: Antibiotic Susceptibility and resistance of *S. epidermidis*: The frequency of resistance strains in *S. epidermidis* strains isolated from different sources is high. A: Graph demonstrating the significant sensitivity level against ciprofloxacin, clindamycin, norfloxacin, rifampicin, erythromycin, and streptomycin of these clones to a variety of antibiotic classes; p-value 0.0001. B. figure showing the significant resistance level of chloramphenicol, cefalexin, ceftriaxone, ampicillin, and vancomycin, followed by 83% and 50% against nalidixic Acid neomycin and tetracycline respectively at p-value 0.0001.

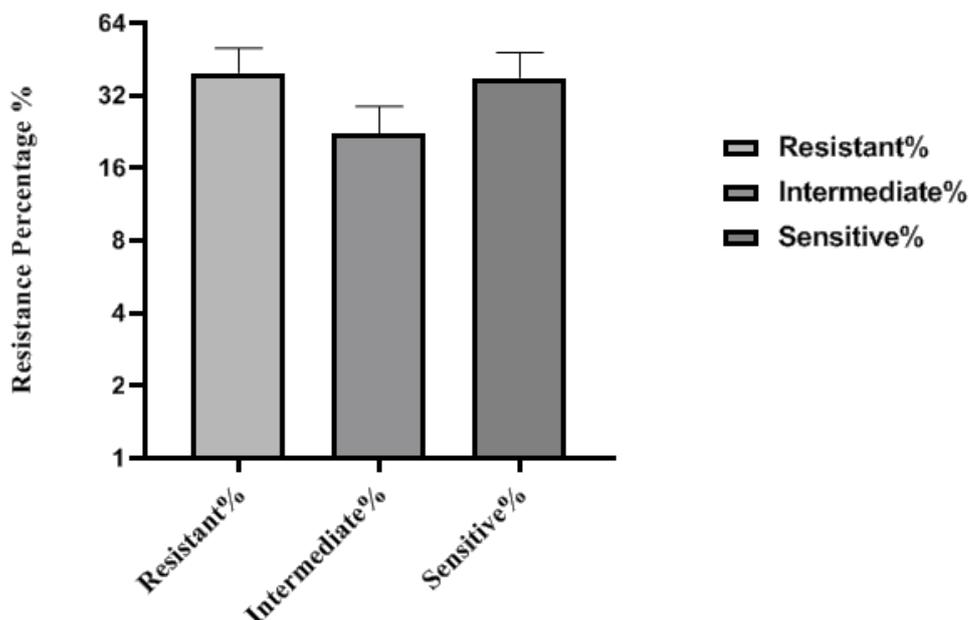**Resistance level of *S.epidermidis* in persent of *cas* gene**

Figure 7: The frequency of resistance strains in *S. epidermidis* strains isolated from different sources. The resistance and intermediate effect of the 6 clone were significant in present of cas6 space; p-value 0.0001.

Discussion:

Bacterial CRISPR loci encode a sequence-specific defence mechanism against intruder nucleic acids that can be programmed to prevent horizontal gene transfer and subsequently antibiotic resistance gene transfer. The impact of CRISPRs on the emergence of virulence, on the other hand, remains uncertain.(21)

The isolation and molecular identification of *S. epidermidis* from several local sources was the first stage of this work. The results of the culturing were consistent with the results of the tests (22). They stated that mannitol salt agar were utilised as standard media for the isolation of bacteria from the *Staphylococci* groups. Furthermore, the tube coagulase test may detect this enzyme directly from blood samples, but the test's sensitivity range was stated to be between 62 and 100% due to common culturing and preparation dilution procedures (23,24). As a result, doing additional studies on any suspected colonial cultures that are similar to *staphylococcus* requires a significant amount of effort and time.(25)

On the other hand, VITEK 2 was utilised in this investigation as a fast test for identifying *S. epidermidis* isolates, it was identified 14 (82.3%) of the 17 isolates as Staphylococcus epidermidis. While, the remaining three were

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either misidentified or unclearly identified. This conclusion is consistent with the results of the previous study (26). Moreover, the first VITEK system in the identification of coagulase-negative staphylococci was discovered that it was the species recognised with the lowest accuracy (63%) of all strains tested (27). Likewise, if the technique fails to identify this species from other coagulase-negative staphylococci molecular methods should be used to confirm the conclusion .

The use of PCR as a conclusive test was specific and sensitive enough. Additionally, PCR procedures have the ability to reduce the number of other confirming tests and complete all isolation processes in a short amount of time (28). *Staphylococcus spp* were identified using a PCR assay with specific primers for the tuf gene cluster, which WAS located in the short tandem repeat region on the bacterial chromosome and displays significant variation amongst groups of *Staphylococci,* and allowed for the rapid identification of these species with high sensitivity and specificity .(29,30)

Furthermore, the significance of employing the tuf gene sequence for the identification of all Staphylococcal species isolated from clinical samples was recorded (31, 32). The results of the sequences had to be analysed by Basic Local Alignment Search Tool (BLAST) to form a comparison with the NCBI information data and to find out the differences in the sequence. The samples and corresponded with reference strains and their accession numbers for complete detection of *Staphylococcus epidermidis* at a genus level (ID) was illustrated in Table 4. The comparison bacterial strains that were previously registered and published in the database revealed that all 6 isolates classified as *S. epidermidis* homology ranged from 96% to 99% (GenBank). As a result of these

findings, the Iraqi isolate was given the closest proximal national isolate and its identity level.   On the other hand, the phylogenetic analysis classified all isolates into two main roots, each of which has multiple sub-roots or branches (Figure 3). The distribution of national samples among Iraqi samples S234, S230 and S29 showed clear similarity with samples from the United States, South Korea, China, Italy, Serbia, the Czech Republic, and Pakistan. S234 showed extreme similarity with national samples from Serbia and having the same ancestor .

The second root involved the *S. epidermidis* local strains S3, S225, and S211, with S3 showed a stronger association with Indian samples. Furthermore, S225 and S211 have the same progenitor as the Czech national sample, although they have distinct branches. According to the findings, 16S rRNA gene identification is a useful tool in phylogenetic studies as well as a reference method for bacterial identification and taxonomic studies (33,34). On the other hand, all S.epidermidis isolates were examined for the presence of the CRISPR-Cas system and three primers used to identify the target elements (Cas6, SECR1, and SECR2), respectively. The SECR1 and SECR2 elements were not detected in any of the tested bacterial strains (Table 5). However, the cas6 gene was found in all of them(Figures 4).

According to our findings, the acquisition of the cas6 spacer in local *S. epidermidis* isolates may make them more sensitive to particular antibiotics. This finding is in agreement with previous study, in which the appearance of CRISPR in *Staphylococci ssp*. like a double-edged sword may aid bacteria in preventing horizontal transfer of antibiotic resistance genes to reduce drug resistance or virulence and protecting bacteria from phage infection

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(18). The presence of CRISPR-Cas systems in *S. epidermidis* was first reported by (35), who showed in their study that four clinical isolates of S. epidermidis may have a conjugation plasmid that was limited by the CRISPR system, implying a broader and more critical role for the CRISPR-Cas system in preventing horizontal gene transfer (HGT). Furthermore, this similarity was found to be in agreement with the findings of (36), who explain the role of CRISPR-Cas systems in the limiting of horizontal gene transfer and their usefulness as a molecular clock to depict the history of staphylococcal genetic exchange.

Conclusion

We concluded that the CRISPR-Cas system may provide a novel way for the development of distinctive antibiotics, which could eradicate multidrug-resistant pathogens and discriminate between pathogenic and beneficial bacteria.

Acknowledgements

Conflict of Interest

The authors report no conflicts of interest.

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الكشف الجزيئي لنظام CRISPR-Cas في المكورات العنقودية الجلدية المعزولة من مصادر مختلفة في العراق.

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الخلاصة

هدفت الدراسة الى عزل وتوصيف نظام (CRISPR-Cas) في *Staphylococcus epidermidis* *المقاومة* للأدوية المتعددة (MRSE) من مجموعة متنوعة من المصادر السريرية. ولتحقيق هذا الهدف، تم جمع 300 عينة من مصادر مختلفة. حيث تم استخدام تقنيات الاستزراع والاختبارات الكيميائية الحيوية ونظام VITEK2 لتأكيد عزل المكورات العنقودية الجلدية. وتم تحديد التسلسل الجزئي لجين 16s rRNA ومقارنته مع تلك الموجودة في بنك الجينات للعثور على اختلافات في التسلسل باستخدام أداة BLAST (http://www.ncbi.nlm.nih.gov). من ناحية أخرى، تم فحص نظام CRISPR-Cas في جميع عزلات *S. epidermidis* المقاومة للأدوية المتعددة حيث لم يتم العثور فيها على عناصر SECR1 و SECR2. تم العثور على عنصر cas6 في جميع السلالات البكتيرية التي تم اختبارها. تم إجراء اختبارات القابلية باستخدام اختبار انتشار القرص بعد التشخيص. حيث كشف اختبار مقاومة المضادات الحيوية لعزلات *S. epidermidis* عن وجود نسبة عالية من المقاومة المتعددة تجاه الأمبيسلين ، سيفترياكسون ، الكلورامفينيكول ، سيفالكسين ، فانكومايسين ، حمض الناليديكسيك وبنسبة 100٪. علاوة على ذلك، أظهرت عزلات *S . epidermidis* تأثيرا متوسطا ضد حمض Amoxillin-clavulanic و Tobramycin و Neomycin و Oxacillin و Tetracycline و Methacillin. ومع ذلك ، تم العثور على عزلات *S. epidermidis* كانت أكثر حساسية للإريثروميسين والنورفلوكساسين والريفامبيسين. وجدنا أن نظام (CRISPR-Cas) يمكن العثور عليه في عزلات S. epidermidis من مجموعة متنوعة من المصادر المحلية ، وأن ظهور نظام كريسبر في عزلات *S. epidermidis* يمكن ان يكون له دورا فريدا في الحساسية تجاه المضادات الحيوية.