MOLECULAR CHARACTERIZATION OF CRISPR- CAS SYSTEM IN CLINICAL STAPHYLOCOCCUS AUREUS ISOLATED FROM IRAQI PATIENTS.

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Abstract

The aim of study was detection the CRISPR-Cas system genes in *Staphylococcus aureus* in attempt as a new strategy for the control of resistance of *S. aureus*. **Material and methods**: The isolation of *S. aureus* requires, a total of 50 specimens (abscesses, cellulitis, and other forms of soft tissue infection) were collected from Al-Hussein Teaching Hospital in Al-Muthanna governorate, followed by Kirby-Bauer's Disc, with some tweaks, will be used to determine antibiotic susceptibility. Antibiotics that were utilized were penicillin, azithromycin, Ciprofloxacin, tetracycline, gentamicin, doxycycline, vancomycin, ofloxacin, chloramphenicol, ampicillin, cefoxitin, and oxacillin. Finally Molecular uncovering of crispr-Cas system, **The results**: Our results revealed that 25 S.aureus isolate obtained from 50 specimen as following 6 (12%)from cellulitis, 7(14%) from abscesses and12 (24%) from soft tissue infections S.aureus isolates showed various response to antibiotics depending on antimicrobial resistance percentages the highest resistance noticed in penicillin(100%) and Ampicillin (100%) followed by Chloramphenicol (80%), Vancomycin(60%), Cefoxitin (60%), Ofloxacin (45%), however the isolates showed more sensitive to following antibiotics in addition All 25 isolates found was not harboured to CRISPER CAS-F1, Furthermore the results reported that all 25 isolates found were not harboured to Cas6 System-II. **Conclusion**: a high occurrence of multidrug-resistant *S. aureus* alongside the complete absence of CRISPR-Cas systems, emphasizing the importance of ongoing antimicrobial surveillance and the development of alternative molecular control approaches

Keywords: S. aureus, molecular characterization, CRISPR-Cas systems, Iraqi

Introduction

The pathogen that is Gram-positive Numerous disorders, such as food poisoning, pneumonia, toxic shock syndrome, suppurative infections, and staphylococcal scaled-skin syndrome, are caused by Staphylococcus aureus. High rates of morbidity and mortality have been linked to nosocomial infections caused by bacteria resistant to multiple medications, particularly methicillin-resistant strains (MRSA) (Espedido and Gosbell, 2012). Methicillin-resistant S. aureus (MRSA) is one of the resistant forms of aureus that are gaining more attention due to drug resistance. Given its potential for zoonotic transmission, MRSA poses a serious threat to human health (Parvin et al., 2021). Antimicrobial resistance arises from clonal evolution, plasmid transfer, mutations, and irrational use. Antimicrobial resistance genes (ARGs) are acquired by Staphylococcus through mobile genetic elements (MGEs) such plasmids (Achek et al., 2018), and horizontal gene transfer (HGT) enables the spread of ARGs across the bacterial community (Shehreen et al., 2019).

It has been established that the CRISPR-cas system serves as a defense mechanism for bacteria against foreign gene invasion. A CRISPR array and six to twenty nearby genes make up the system's architecture (Shabbir et al., 2019). A short direct repeat (DR) sequence of 21–48 base pairs (bps) with a spacer sequence of 26–72 bp make up the CRISPR array (Cao et al., 2016). Three steps are used by this system: interference, biogenesis, and adaptability. MGEs that mirror the CRISPR spacer sequence

are recognized by CRISPR, which also prevents their transfer (Zhao et al., 2018). This system is classified into three types: I, II, and III based on the signature gene (Shabbir et al., 2019). A type III CRISPR-cas system is present in S. aureus (Cao et al., 2016).that includes cas proteins such as csm3, csm2, csm6, csm4, csm5, Cas2, Cas1, Cas6, and Cas10 (Li et al., 2016). Csm is a CRISPR-cas interference complex of type III-A. Spacer acquisition involves Cas2 and Cas1. While csm1–5 share a csm effector complex that directly interferes with the targeted sequence, Cas6 is required for the first processing of crRNA (Cao et al., 2016).

Moreover, rapid screening of transform ants on agar plates is made possible by the use of the CRISPR/Cas9 technology in genetic engineering, which does away with the need for tedious sequential double crossover operations. As a result, this shortens the time needed for research (Liu et al., 2017). The CRISPR-Cas system in clinical S. aureus isolates from Iraqi patients has not yet been molecularly characterized, despite the fact that numerous research have been carried out regarding virulence factors in S.aureus and their role in the pathogenicity of this bacterium. Additionally, there is an alternative perspective on the research. The primary hypothesis of this work is that the pathogenesis of Staphylococcus aureus is significantly influenced by the CRISPR-Cas system. The aim of study is detection the virulence factors as CRISPR CAS system gene in S.aureus

Materials and Methods

Clinical specimens for S. aureus isolation

Fifty cellulitis, abscess, and soft tissue infection specimens were obtained from Al-Hussein Teaching Hospital in the governorate of Al-Muthanna in order to isolate *S.aureus* (Lemaire, 2008). Finally, after incubating at 37 ºC for 24 hours, all samples were cultivated in nutritional broth medium. Each sample was diluted serially and subculture on blood agar and mannitol agar medium before being incubated at 37 ºC for 24 hr.

Antimicrobial susceptibility

The antibiotic discs available commercially will be used in a modified version of the Kirby Bauer's disc diffusion procedure to determine antibiotic susceptibility. Stains were classified as sensitive, intermediately resistant, or resistant depending on the size of the inhibitory zone. The antibiotics used were penicillin(P,10 µg) , azithromycin(AZM, 15 µg), ciprofloxacin(CIP,5 µg), tetracycline(T,30 µg), gentamicin (G, 10µg), doxycycline (DO,30 µg), vancomycin (VA,30 µg), ofloxacin(OF,5 µg), chloramphenicol(C,30 µg), ampicillin(A, 10 µg), cefoxitin (CX,30 µg) and oxacillin(OX,1 µg).

Molecular characterization of the CRISPR-Cas system

Oligonucleotide Primers

Forward and reverse oligonucleotide primers used to amplify enterotoxin genes and cas genes are indicated table (1 and 2. Lyophilized primers were purchased from Alpha DNA Company), reconstituted in free nuclease distilled water to the manufacturer's recommended concentration of 100 picomole /µl, and finally diluted to yield 10 picomole / µl of each primer by adding 10 µl of the stock solution to 90 µl of the working solution, vortexed, and stored at -20◦C.

Table (1): Sequence of oligonucleotide primers used for amplification of CRISPR

Gene	Sequences (5 ⟶ 3)	Product size	Ref
CRISPR-Cas-f2	TATAGAACTATTTGGCGTAATG		
CRISPR-Cas-r2	GTAATCTTGCTTCTTCATAACT		

Preparation of reaction mixture

The amplification mixture for polymerizing enterotoxin genes and cas genes was prepared as in table (2).

Table (2): Preparation of PCR reaction mixture

No.	Content	Volume for single tube (µl)
1	Green master mix	12.5
2	Forward Primer	1.5
3	Reverse Primer	1.5
4	DNA template	5
5	Nuclease free water	4.5

Agarose gel electrophoresis

Samples were put into the wells of an agarose gel (1%) immersed in 0.5X TBE solution for analysis of PCR products using a horizontal electrophoresis apparatus. One to two hours of 50V electrophoresis were performed. Ten microliters of an ethidium bromide stock solution were used to stain the electrophoresis gel. U.V transilluminator at 365 nm was used to observe DNA bands alongside (1500 bp and 10000 bp DNA ladders as markers) (Maniatis et al., 1982).

Results and Discussion

Isolation of *S. aureus* from clinical specimens

Colonies of this bacteria showed clustered, paired, or solitary cells that Gramme staining identified as Gram-positive cocci (Sizar and Unakal,2020).on Mannitol salt agar which this the colonies is surrounded by yellow medium as in figure (1).

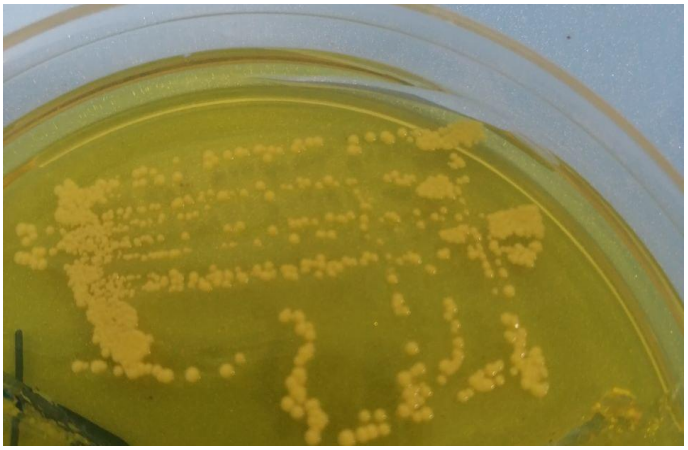

Figure (1): *S. aureus* colonies on Mannitol salt agar

A total of 50 specimens (15cellulitis, 15 abscesses and 20 soft tissue infections) were collected from Al-Hussein Teaching Hospital in Al-Muthanna governorate, Then, for 24 hours at 37ºC, the samples were grown in nutrient broth medium. Our results revealed that 25 S.aureus isolate obtained from 50 specimen as following 6 (12%) from cellulitis, 7(14%) from abscesses and12 (24%) from soft tissue infections.

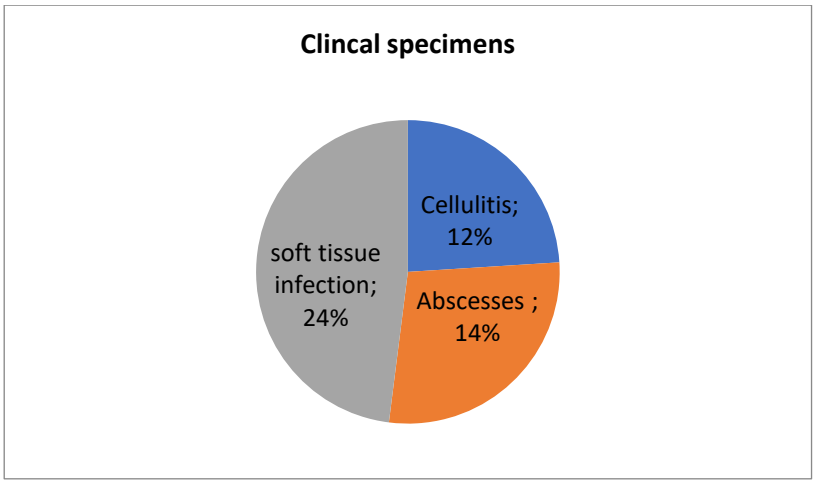

Figure (2): The isolation of *S.aureus* clinical specimen percentage %

Staphylococcal species isolated from clinical or environmental samples are cultured on mannitol salt agar. Considering it contains roughly 7.5% sodium chloride, the development of most bacteria other than staphylococci and phenol red indicator will be inhibited. Due to the generation of fermentation acids that aid in decreasing the pH of the medium, S. aureus ferment the mannitol and generate yellow zones in the reddish agar, changing the colour of the phenol red to yellow (Harley, 2016). This test distinguishes it from S. epidermidis, which leaves red zones in colony growth. Small, spherical, and yellow colonies of S. aureus can be seen on the surface of Mannitol salt agar (Carroll et al., 2016) These findings agreed with (Moran et al.,2006) who studied Methicillin-resistant *S. aureus* infections among Patients with acute, purulent skin and soft-tissue infections ,422 patients with skin and soft-tissue infections were enrolled. Infections were classified as an abscess in 81 percent of patients, an infected wound in 11 percent, and as cellulitis with purulent exudate in 8 percent S. aureus was isolated from skin and soft-tissue infections in 320 patients (76 %); 249 of *S. aureus* isolates (78 %) were MRSA.

Similar results were found by Mohanty and colleagues (2018), who found that out of 1702 total samples, 1622 (95.29%) tested positive for the presence of at least one bacterial pathogen, with *S. aureus* accounting for 365 (20.9%) of those positive results.S. aureus strains identified from various sources including,soft tissue infection-195(12.02%), abscess- 33(2.03%) and cellulitis-137(8.5%).

Furthermore different studies reported same findings as total of 109 S. aureus strains isolated from patients visited/admitted to hospitals with infections comprised of microbial keratitis (n = 18), eyelid abscess (n = 8), endophthalmitis (n = 5), Steven Johnson syndrome with bacterial keratitis (n = 9), suture-related infections (n = 3), and other ocular infection (n = 5); pus from eye (n = 4), wound infection (n = 16) (Aggarwal et al.,2019) also Eighty-five patients with periorbital (n = 58) or orbital (n = 27) cellulitis were identified. found 57 (67%) methicillin-resistant *S.aureus* (MRSA) isolates(Foster et al.,2018).

One of the several enzymes and poisons produced by *S. aureus* is called Panton-Valentine leukocidin (PVL). Staphylococcus aureus secretes the polymorphonuclear leukocyte pore-forming toxin (PVL), which is made up of two subunits, LukS-PV and LukF-PV (Waldenburger et al.,2014) Although PVL is thought to be the primary toxin in SSTIs, In necrotic SSTIs and necrotic haemorrhagic pneumonia, it is unclear if this is the predominant pathogenic component. Besides scalded skin syndrome toxin (SSST), coagulase, protein A, and toxic shock syndrome toxin (TSST1) are also involved in the pathogenicity of SSTIs (Gould, 2009). Commonly known as "commensal bacteria," *Staphylococcus*

spp (Park et al.,2019) However, when the immune system is compromised, they can cause a wide variety of disorders, including osteomyelitis, pneumonia, endocarditis, and infections related to implantable medical devices. Staphylococcal resistance to antibiotics has become a major problem in recent decades, resulting in illnesses that are resistant to treatment and placing a heavy financial load on healthcare systems around the world (Williams et al.,2019).

One of the most common causes of both hospital- and community-acquired infections, *S.aureus* is a major public health concern. Most bacterial skin infections, including those at surgical sites, purulent cellulitis, cutaneous abscesses, and many others, are caused by Staphylococcus aureus, even though the organism can infect any tissue in the body (Heilmann et al.,2019).

The recent finding that community-acquired methicillin-resistant *S. aureus* (MRSA) cutaneous infections typically exceed hospital-acquired MRSA infections emphasizes the necessity of creating viable therapeutic alternatives. According to Rodrigues et al. (2018), the initial stage of staphylococcal biofilm formation—adherence to organic and inorganic surfaces—is caused by bacterial cell wall constituents like teichoic acid. (Baur et al., 2012), Clumping is caused by the host's cytokeratin, fibrinogen, fibronectin, staphylococcal protein A; and fibronectin binding proteins A and B (Herman-Bausier et al., 2017). Biofilm formation can cause keratinocyte mortality, impede neutrophil and macrophage removal of pathogens, and even facilitate macrophage cytotoxicity (Blicharz et al., 2019). By generating substances such extracellular fibrinogen-binding protein, extracellular complement-binding protein, and complement 4 binding protein, *S.aureus* can stop C3b-mediated opsonization and start a complement-independent inflammatory response used in phagocytosis (Williams et al., 2019).

S.aureus isolates showed various response to antibiotics depending on antimicrobial resistance percentages the highest resistance noticed in penicillin (100%) and Ampicillin (100%) followed by Chloramphenicol (80%), Vancomycin (60%), Cefoxitin (60%), Ofloxacin (45%), however the isolates showed more sensitive to following antibiotics; Gentamicin (40%), Ciprofloxacin (40%), Doxycycline (35%), Azithromycin (30%), Tetracycline (30%)

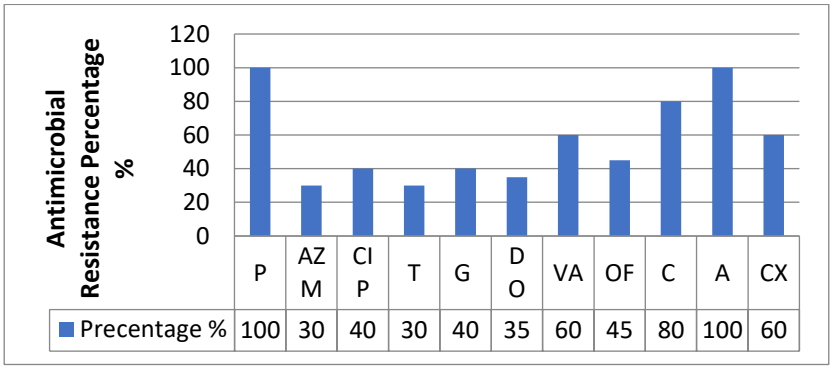

Figure (3): Antimicrobial Resistance Percentage %

These findings showed agreements and disagreement with different studies as found total of 944 *S. aureus* isolates from from clinical specimens, High sensitivity of S. aureus was observed for quinupristin/dalfopristin (100%), tigecycline (98.2), imipenem (98%), nitrofurantoin (97.6%), linezolid (97.3%), teicoplanin (97.1%) and vancomycin (95.1%). High resistance was recorded against penicillin G (91.9%), trimethoprim/sulfamethoxazole (56.9%) and tetracycline (33.2%) (Gitau et al.,2018). Meanwhile *S. aureus* strains originating from pyomyositis, furuncles and osteomyelitis cases were resistant to 4/17 tested antibiotics (benzyl penicillin, rifampincin, tetracycline, and trimetroprim/sulfamethoxazol), 136 isolated S. aureus strains, 34 (25%) were resistant to oxacillin (MRSA), while none of the strains showed resistance to vancomycin (VRSA). The oxacillin-resistant strains were all isolated from abscesses and ulcers (Sina et al.,2013).

Penicillin G and ampicillin (100%) were shown to be highly resistant to S. aureus isolates in our investigation. This result is consistent with research conducted (Rutare, 2013). The mechanism of resistance, such as the permeability barrier, efflux pumps, mutational or recombination changes in the target enzymes, acquired resistance by drug-resistant target enzymes in trimethoprim/sulfamethoxazole, and alteration of the target with decreased affinity for the antibiotic in penicillin, may be responsible for this resistance (Pantosti et al., 2007). Furthermore, the other hand the results noticed the sensitive Gentamicin (40%), Ciprofloxacin (40%), Doxycycline (35%), Azithromycin (30%), Tetracycline (30%) which agreed with (Naimi et al.,2017) during determination the susceptible to strains of S. aureus isolated from pus, urine, tracheal secretions, and blood, Vancomycin was effective against all strains tested. In sum, 100 (95.2%) strains were susceptible to rifampicin, 96 (91.4%) susceptible to clindamycin, 94 (89.5%) susceptible to imipenem, 83 (79.0%) susceptible to gentamicin, 81(77.1%) susceptible to doxycycline, 77 (77.1%) susceptible to amoxicillin + clavulanic acid, 78 (74.3%) susceptible to cefazolin, 71 (67.6%) susceptible to tobramycin, 68 (64.8%) susceptible to chloramphenicol, 60 (57.1%) were susceptible to trimethoprim-sulfamethoxazole, 47 (44.8%) susceptible to ciprofloxacin, 38 (36.2%) susceptible to azithromycin and erythromycin, 37 (35.2%) susceptible to ceftriaxone and 11 (10.5%) were susceptible to cefixim.

Multiple methods exist for this bacterium species to develop resistance to drugs. The horizontal transmission of mobile genetic resistance elements, the alteration of antibiotic targets, and the activation of endogenous efflux pumps are all significant mechanisms (Foster, 2017). Medical management and therapeutic decisions benefit from a thorough understanding of antibiotic action and resistance mechanisms, as well as reliable global data on antibiotic resistance rates. Additionally, it can aid in the development of effective infection control measures and the suppression of the spread of bacterial resistance.

DNA extraction and preparation

After DNA extraction by Presto™ Mini gDNA Bacteria Kit, DNA concentration was between 10 and 87 ng/ml; Gel electrophoresis was done to confirm the purity of extracted DNA.

Detection of the Cas gene by polymerase chain reaction

In this study, the PCR technique was applied to confirm the presence of Cas6 System-II genes. Furthermore, the results reported that all 25 isolates found were not harboured to Cas6 System-II this agreed with different studies that no cas6-positive *S. aureus* was detected from 80 clinical isolates and 88 genomes sequenced by the Danish State Serum Institute (Li et al., 2016).

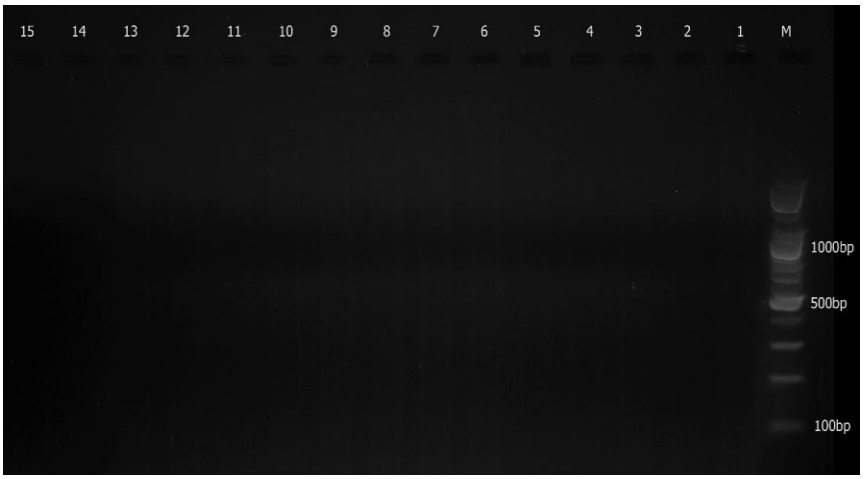

Figure (4): Agarose gel electrophoresis of PCR products obtained by using Cas6 System II -specific primer. lanes 1-15 represent the negative results, Lane M represent 100bp DNA ladder.

Table (3): Identification of Sta. aureus among all studied samples using Cas6 System II-specific primer.

Results	N	Percentage	P value
Positive	0	(0)	<0.0001*
Negative	25	(100)	
Total	100	100%	

* Represents a significant difference at p<0.05.

From the other side, also the results showed All 25 isolates found was not harboured to CRISPER CAS-1 as Cruz-López and his colleague (2021), Results from a screening of 1,385 S. aureus sequences for the CRISPR-Cas system revealed that 0.83 percent of S. aureus strains include CRISPR-Cas (6/716).

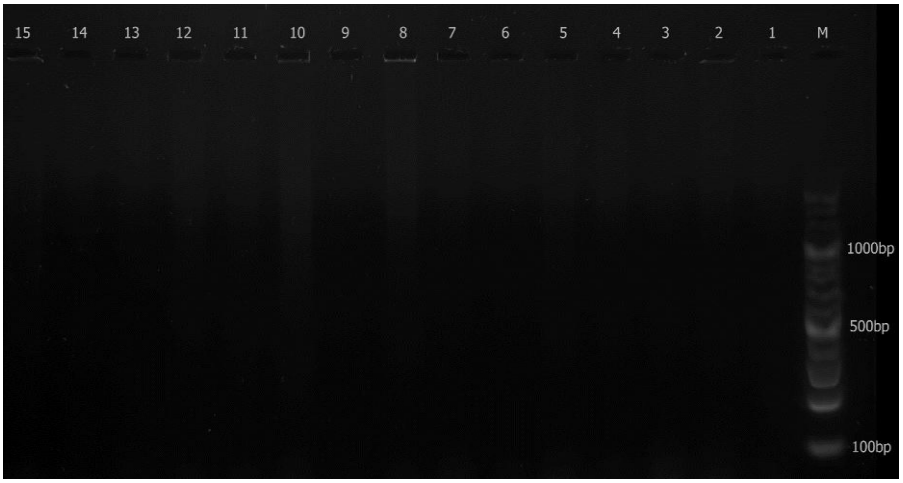

Figure (5): Agarose gel electrophoresis of PCR products obtained by using CRISPR-Cas-1-specific primer. lanes 1-15 represent the negative results, Lane M represent 100bp DNA ladder.

Table (4): Identification of *S.aureus* among all studied samples using CRISPR-Cas-1-specific primer.

Results	N	Percentage	P value
Positive	0	(0)	<0.0001*
Negative	25	(100)	
Total	100	100%	

* Represents a significant difference at p<0.05.

Although just a few studies have investigated for CRISPR-Cas systems in this genus, they were found in 0.94 percent (6/616) of Staphylococcus spp. isolates (7.89% (3/39) of the S. aureus strains analyzed by Zhao et al. (2018) and Cao et al. (2016) .The CRISPR-Cas activity of 129 isolates of Staphylococcus species was investigated. Eight percent (10/129) of the 129 isolates of *S.aureus*, , *Staphylococcus haemolyticus*, that were gathered from nine different countries were found to be CRISPR-Cas system carriers (Rossi et al., 2019). Three components make up CRISPR: (1) the cas genes; (2) a collection of CRISPR genes that have spacer sequences (SSs) between tandem repeats (TRs); and (3) an upstream leader sequence at the CRISPR locus; the cas genes are divided into a "effector complex" made up of the remaining cas genes and a "adaptation module" made up of the cas1 and cas2 genes(Koonin et al.,2017).

There are currently two main groupings, six kinds, and thirty-three subtypes in the CRISPR-Cas system. Class 1 systems employ multi-protein complexes (Koonin et al., 2017). Furthermore, due to DNA degradation, only one multidomain protein is utilized in Class 2 systems (Shmakov et al., 2017). DNA deterioration occurs in three stages (Hsu et al., 2014): (1) the primo-infectious stage of adaptation (Nuñez et al., 2014), (2) the expression stage of reinfection, and (3) interference in endonuclease digestion of mobile genomic elements (MGEs) Cas, which is directed by crRNA (Hille et al., 2018). Short sequences (roughly 30 nucleotides) within an MGE are identified by protospacer adjacent motifs (Jiang and Doudna, 2017). Nevertheless, it has only been discovered in a few types of Staphylococcus aureus. The Gram-positive bacteria Staphylococcus aureus colonizes 30% of the population asymptomatically and is the cause of various serious diseases (Craft et al., 2019). Sequenced bacterial genomes are currently used as data sources to search the CRISPR-Cas system in important medical microorganisms like *S. aureus*.This study was limited by the relatively small sample size and the collection of isolates from a single hospital, which may restrict the generalizability of the findings. In addition, only selected CRISPR-Cas systems were investigated, and no molecular analysis of antibiotic resistance genes or genomic sequencing was performed, limiting deeper insight into resistance mechanisms and genetic diversity

Conclusion

This study confirms the high prevalence of *S. aureus* in soft tissue infections and demonstrates a marked level of antimicrobial resistance, particularly against penicillin and ampicillin. The observed variability in antibiotic susceptibility highlights the importance of continuous resistance surveillance. Molecular analysis revealed the complete absence of CRISPR-Cas (type I-F and Cas6 system II) in all isolates, suggesting a limited role of CRISPR-based defense mechanisms in these strains and indicating that alternative molecular approaches may be required for controlling S. aureus in clinical and food safety contexts.

Conflict of Interest

The authors declare no conflict of interest.

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