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والبحث العلمي
جامعة بابل
كلية الطب

العلاقة بين التعبير الجيني لجين Panton Valentine Leukocidin والمضادات الحيوية المختلفة لعزلات المكورات العنقودية الذهبية المقاومة للميثيسيلين

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Ministry of Higher Education and Scientific Research
University of Babylon College of Medicine

Association Between *Panton Valentine Leukocidin* Gene Expression and Different Antibiotics in Methicillin Resistant *Staphylococcus aureus* Isolates

A Thesis

Submitted to the Council of College of Medicine-University of Babylon as a Partial Fulfillment of the Requirements for the Degree of Doctor of Philosophy inScience/ Medical Microbiology

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2023 A.D **1445 A.H.**

بسم الله الرحمن الرحيم

(وَمَنْ يَتَّقِ اللَّهَ يَجْعَلْ لَهُ مَخْرَجًا وَيَرْزُقْهُ مِنْ حَيْثُ لَا
يَحْتَسِبُ وَمَنْ يَتَوَكَّلْ عَلَى اللَّهِ فَهُوَ حَسْبُهُ إِنَّ اللَّهَ بَالِغُ
أَمْرِهِ قَدْ جَعَلَ اللَّهُ لِكُلِّ شَيْءٍ قَدْرًا)

صدق الله العلي العظيم

سورة الطلاق اية (2-3)

بسم الله الرحمن الرحيم

(وَمَنْ يَتَّقِ اللَّهَ يَجْعَلْ لَهُ مَخْرَجًا وَيَرْزُقْهُ مِنْ حَيْثُ لَا
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صدق الله العلي العظيم

سورة الطلاق اية (2-3)

DEDICATION

To whom on His light I was guided and depended on … Allah the most Merciful all compassionate

For earing an to the man who illuminated my path and my idol honest living for us and for supporting and encouraging me to believe in

My self … … … My father

To the big heart and the source of kindness

My mother…

to source of support in my life

My Husband…

To my son, you are the light that lights my life

(Bassem).

To my angel, the biggest gift in my life

(Ayah).

Life has its trying moments; for your trust, cordiality and unique support; I say a big thank you

My brothers and sisters….

SARA 2023

Summary:

During the current study, 100 specimens of different skin infections (burn, wound, impetigo, boils, acne, abscess, folliculitis, atopic dermatitis, secondary infection) were collected from two hospitals including, Al- Hilla Teaching Hospital, Imam Sadiq Hospital in addition to private clinics during a period from July to the November 2022.

This study aimed to isolate and differentiate methicillin resistant *Staphylococcus aureus*, community acquired methicillin resistant *Staphylococcus aureus* (CA-MRSA) and hospital acquired methicillin resistant *Staphylococcus aureus* (HA-MRSA) , the association between Panton Valentine Leukocidin Gene Expression and different antibiotic in methicillin resistant *Staphylococcus aureus* isolates.

According to the cultural morphology and biochemical characteristics the *S. aureus* bacteria were isolated and identified. VITEK 2 compact system was used to confirm the diagnosis phenotypically. Out of 24/100isolates were identified as *S. aureus*, and the distribution of *S. aureus* isolates was as follows: 7(28%) isolates from burns, 10(33%) isolates from impetigo, 5(23%) isolates from wound, 2(16%) isolates were isolated from boils and acne, abscess, folliculitis, infected atopic dermatitis, secondary infection 0(0.00%) for each.

According to the origin of isolates, hospital (inpatient) and community (outpatient), out of 24 *S. aureus* isolates, 15 isolate (62.5%) from inpatients 9 isolates (37.5%) recovered from outpatients.

Antibiotic susceptibility test and phenotypic screening of resistance to cefoxtine and oxacillin was done by VITEK 2 system to confirm *S.aureus* as methicillin resistant *Staphylococcus aureus*, the results of this study demonstrated that all of the isolates 24(100%) were resistant to cefoxitin and oxacillin, However, molecular characterisation of the *mec*A gene as

an indicator of genetically methicillin-resistant *Staphylococcus aureus* revealed that 19 (79.16%) of the isolates were methicillin-resistant.

The results of antibiotic susceptibility test (VITEK 2 compact system) using 21 antibiotics revealed, obvious levels of resistance against the used antibiotics. The resistance to antibiotic was as follow: (100%) against Benzylpenicillin, Amoxicillin/Clavulanic Acid, Ceftriaxone, Meropenem respectively, Tetracycline (80%), Doxycycline (75%), Erythromycin (70%), Clindamicin (65%), Lincomycin (65%), and Fusidic Acid (60%). Also methicillin-resistant *Staphylococcus aureus* isolates showed the lowest rates of resistance toward Gentamicin(35%), Amikacin (30%), Rifampicin (20%), Ciprofloxacin, Levofloxacin, Moxifloxacin (15%). Finally, methicillin-resistant *Staphylococcus aureus* isolates showed the lowest rates of resistance toward Linezolid, Teicoplanin, Vancomycin, Tigecycline, Trimethoprim/Sulfamethoxazole with sensitivity rates reach up to 100%.

Using the *pvl* gene and Staphylococcal Cassette Chromosome (SCCmec), all confirmed methicillin-resistant *Staphylococcus aureus* isolates were subjected to polymerase chain reaction techniques in a monoplex pattern to distinguish between community acquired methicillin resistant *Staphylococcus aureus* (CA-MRSA) and hospital acquired methicillin resistant *Staphylococcus aureus* (HA-MRSA).

The prevalence of methicillin-resistant *Staphylococcus aureus* in the community (CA-MRSA) was 12 (63.15%) and 11 (57.89%) in hospital isolates (HA-MRSA), with 12/19 isolates (63.15%) found to be positive for the *pvl* gene, 12/19 isolates (63.15%) belonging to SCCmec type II, and 13/19 isolates (68.42%) discovered to be positive for SCCmec type IV.

Different concentrations of clindamycin were used to test their effect on the expression of the *pvl* gene using q-Real Time-polymerase chain reaction (qRT-PCR). The results revealed that the different concentrations of clindamycin (0.5, 1, 5, 10) g/ml decreased the level of gene expression of PVL (lukS-pv and lukF-pv) genes (0.84,0.73,0.35,0.24) respectively.

In the current study the PCR products of *pvl* and SCC*mec*A IV of (10) isolates were subjected to DNA sequencing and analyzed to confirm the nucleotide sequences and identity with other world strains using NCBI-Blast-query nucleotide-online program and it produced the exact percentage of identity results by further world strains and extended from (99-100%). In the present study genotypic variations in *pvl* and SCC*mec* A IV within (10) isolates were studied for the first time in Iraq.

According to the obtained results, can be concluded, the dissemination of community acquired methicillin resistant *Staphylococcus aureus* (CA-MRSA) and hospital acquired methicillin resistant *Staphylococcus aureus* (HA-MRSA)and increasing the resistance burden between community and hospital environment. And using protein-synthesis inhibitors is an important consideration in the selection of antimicrobial agents to treat skin infection caused by toxin-producing gram-positive pathogens.

الخلاصة:

خلال الدراسة الحالية (100) عينة سريرية من اصابات جلدية مختلفة شملت:- (الحروق، الجروح، القوباء، الدمامل، حب الشباب، الخراج، التهاب الجريبات، التهاب الجلد التأتبي، اصابات ثانوية) تم جمعها من مستشفيين :- مستشفى الحلة التعليمي، مستشفى الأمام الصادق بالإضافة الى عيادات طبية خلال الفترة من تموز الى تشرين الثاني من سنة 2022.

هدفت هذه الدراسة الى عزل وتمييز المكورات العنقودية الذهبية المقاومة للميثيسيلين، المكورات العنقودية الذهبية المقاومة للمثيسيلين المكتسبة من المجتمع و المكورات العنقودية الذهبية المقاومة للمثيسيلين المكتسبة من المستشفى، العلاقة بين التعبير الجيني لجين (Panton Valentine Leukocidin) ومضادات حيوية مختلفة في عزلات المكورات العنقودية الذهبية المقاومة للميثيسيلين.

اعتمادا على الصفات المظهرية الزرعية والكيموحياتية تم عزل وتشخيص بكتريا المكورات العنقودية الذهبية، وبأستخدام (VITEK 2 compact system) تم تأكيد التشخيص ظاهريا. من اصل (100) عزلة تم تشخيص (24) عزلة على انها بكتريا المكورات العنقودية الذهبية وكان توزيع عزلات المكورات العنقودية الذهبية كالتالي:- 7 (28 ٪) من الحروق، 10(33٪) من القوباء،5 (23٪) من الجروح، 2(16٪) من الدمامل. وحب الشباب، الخراج، التهاب الجريبات، التهاب الجلد التأتبي، اصابات ثانوية كانت 0(0.00%) لكل منهما.

وبالأعتماد على أصل العزلات ،المرضى الراقدين في المستشفى ومرضى العيادات الخارجية من اصل (24) عزلة من بكتريا المكورات العنقودية الذهبية تم عزل 15 (62.5٪) عزلة من المرضى الراقدين في المستشفى، 9 (37.5٪) عزلة من مرضى العيادات الخارجية. تم اجراء اختبار فحص الحساسية الدوائية والفحص المظهري لمقاومة السيفوكستين والأوكساسيلين بواسطة(VITEK 2 compact system) للتأكد من المكورات العنقودية الذهبية على انها المكورات العنقودية الذهبية المقاومة للمثيسيلين ، واظهرت نتائج هذه الدراسة على ان جميع العزلات 24(100٪) كانت مقاومة لسيفوكستين والأوكساسيلين، ومع ذلك، فإن التوصيف الجزيئي لجين (*mec*A) كمؤشر على المكورات العنقودية الذهبية المقاومة للميثيسيلين وراثيا كشف أن 19 (79.16٪) من العزلات كانت مقاومة للميثيسيلين.

كشفت نتائج اختبار الحساسية الدوائية بأستخدام21 مضادًا حيويًا (VITEK 2 compact system) عن مستويات واضحة من المقاومة للمضادات الحيوية المستخدمة. وكانت المقاومة

للمضادات كالتالي:- (100٪) ضد Benzylpenicillin، (100٪) ضد Amoxicillin/Clavulanic Acid، (100٪) ضد Ceftriaxone ، (100٪) ضد Meropenem، (80 ٪) ضد Tetracycline ،(75٪) ضد Doxycycline ،(70 ٪) ضد Erythromycin، (65 ٪) ضد Lincomycin , Clindamicin ،(60 ٪) ضد Fusidic Acid ، كما أظهرت عزلات المكورات العنقودية الذهبية المقاومة للميثيسيلين أقل معدلات المقاومة (35٪) Gentamycin، (30٪) Amikacin ، (20٪) Rifampicin (15٪) Ciprofloxacin, Levofloxacin, Moxifloxacin. وأخيراً أظهرت عزلات المكورات العنقودية الذهبية المقاومة للميثيسيلين أقل معدلات مقاومة تجاه Linezolid، Teicoplanin، Vancomycin، Tigecycline، Trimethoprim/Sulfamethoxazole وبنسب حساسية تصل إلى (100%).

باستخدام (*pvl* gene) و(Staphylococcal Cassette Chromosome (SCCmec)، تم إخضاع جميع عزلات المكورات العنقودية الذهبية المقاومة للميثيسيلين لتقنيات تفاعل البلمرة المتسلسل في نمط أحادي للتمييز بين المكورات العنقودية الذهبية المقاومة للميثيسيلين المكتسبة من المجتمع (CA-MRSA) و المكورات العنقودية الذهبية المقاومة للميثيسيلين المكتسبة في المستشفى (HA-MRSA). بلغ معدل انتشار المكورات العنقودية الذهبية المقاومة للميثيسيلين في المجتمع 12(63.15٪) و11 (57.89٪) في عزلات المستشفيات، مع وجود 19/12 عزلة (63.15٪) إيجابية بالنسبة (*pvl* gene)، تم اكتشاف أن 19/12 عزلة (63.15٪) تنتمي إلى (*SCCmec* type II)، و19/13 عزلة (68.42٪) كانت إيجابية لجين (*SCCmec* type IV)

تم استخدام تراكيز مختلفة من الكليندامايسين لاختبار تأثيرها على التعبيرالجيني لجين (*pvl*) باستخدام تفاعل البلمرة المتسلسل (q-Real Time-polymerase chain reaction). أظهرت النتائج أن التراكيز المختلفة من الكليندامايسين (10، 1،5، 0.5) جم / مل أدت إلى انخفاض مستوى التعبير الجيني لجينات (PVL (lukS-pvl and lukF-pvl) (0.84, 0.73, 0.35,0.24)على التوالي.

في الدراسة الحالية، خضعت منتجات (pcr) لجين (pvl) و(SCCmecA IV) المكونة من (10) عزلات لتسلسل الحمض النووي وتحليلها للتأكد من تسلسل النيوكليوتيدات وهويتها مع سلالات عالمية أخرى باستخدام برنامج (NCBI-Blast-query nucleotide-online)وقد وصلت نسبة التطابق الحمض النووي الدقيق للعزلات مع سلالات عالمية أخرى من (99-

100٪). تمت في هذه الدراسة دراسة التغايرات الوراثية في (pvl) و(SCCmec A IV) ضمن (10) عزلات لأول مرة في العراق.

وفقا للنتائج التي تم الحصول عليها، يمكن استنتاج انتشار المكورات العنقودية الذهبية المقاومة للميثيسيلين المكتسبة من المجتمع (CA-MRSA) والمكورات العنقودية الذهبية المقاومة للميثيسيلين المكتسبة في المستشفى (HA-MRSA) وزيادة عبء المقاومة بين بيئة المجتمع والمستشفى. ويعد استخدام مثبطات تخليق البروتين أحد الاعتبارات المهمة في اختيار العوامل المضادة للميكروبات لعلاج العدوى الجلدية الناجمة عن مسببات الأمراض إيجابية الجرام المنتجة للسموم

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Sara AL-Ghazal

Supervision

I certify that this thesis which is entitled " **Association Between *Panton Valentine Leukocidin* Gene Expression and Different Antibiotic in Methicillin Resistant *Staphylococcus aureus* Isolate "** was prepared by "**Sarah Aqeel Hassan Ahmed**"under my supervision at the College of Medicine, University of Babylon as partial fulfillment of the requirements for the degree of doctor of philosophy in medical microbiology.

Supervisor

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College of Medicine

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/ / 2023

In view of the available recommendations. I forward this thesis for debate by the examination committee.

Professor

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/ /2023

Committee Certificate

We the examining committee, certify that we have read this thesis and examined the student Sarah Aqeel Hassan Al-Ghazal with its contents and that, according to our opinion, is accepted (**excellent**) as a thesis for the degree of doctorate of Philosophy in Medical Microbiology .

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List of Abbreviations

Abbreviation	Meaning
CA-MRSA	Community-associated MRSA
CAP	Community-acquired pneumonia
CA-MSSA	Community acquired methicillin-susceptible *S. aureus*
ccr	Cassette chromosome recombinase
ETs	Exfoliative toxins
ETA	Exfoliative toxins A
ETB	Exfoliative toxins B
EDINs	Epidermal cell differentiation inhibitors
Hla	α-hemolysin
HA-MRSA	Hospital-associated MRSA
HAP	Hospital-acquired pneumonia
HCAP	Health care-associated pneumonia
LPS	lipopolysaccharide
MRSA	Methicillin Resistance *Staphylococcus aureus*
MSSA	Methicillin-Sensitive *S. aureus*
MSCRAMMs	Microbial surface components recognizing adhesive matrix molecules
MHC-II	Major histocompatibility complex class II
MIC	Minimum inhibitory concentration
MAC	Membrane Attack Complex
PBP2a or PBP2′	Penicillin-binding protein 2a or 2′
PVL-SA	Panton-Valentine leukocidin-producing *S. aureus*
PFTs	Pore-forming toxins

PSMs	Phenol-soluble modulins
PFPs	Pore-forming proteins
PVL	Panton-Valentine leukocidin
spa A	Protein A
SEs	Staphylococcal enterotoxins
SSSS	Staphylococcal scalded skin syndrome
SCC	Staphylococcal Cassette Chromosome
SSTI	Skin and soft-tissue infections
TSS	Toxic shock syndrome
TCRs	T cell receptors

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1.1.Introduction:

Methicillin-resistant *Staphylococcus aureus* (MRSA) is an important bacterial pathogen in both community and health care related settings in different parts of the world. It is one of the major human pathogens that can cause broad variety of human diseases ranging from mild skin and soft tissue infections(SSTIs) to severe life-threatening invasive infections; such as: endocarditis, osteomyelitis, necrotizing pneumonia, bacteremia, septicemia, meningitis, food poisoning, and toxic shock syndrome(Kobayashi *et al*., 2015).

Methicillin-resistant *Staphylococcus aureus* strains carry a unique and transmissible genetic component known as Staphylococcal cassette chromosome mec (SCCmec) that harbors the *mec*A gene at the 30 end of a chromosomal open reading frame named orfX. It encodes a penicillin-binding protein (PBP2a) with a reduced affinity for beta-lactam antibiotics. Consequently, these strains are resistant to all beta-lactam antibiotics, with the exception of fifth-generation cephalosporins (Abdelwahab *et al*., 2023). On the basis of SCCmec elements, MRSA strains are classified as hospital acquired(HA), community-acquired (CA) (Peechanika *et al*., 2021).

Community-acquired strains Methicillin-resistant *Staphylococcus aureus* (CA-MRSA) are genetically different from the hospital-acquired MRSA (HA-MRSA) strains. They have been recognized as a novel pathogen group. The CA-MRSA strains are usually characterized by limited antibiotics resistance (except to b-lactams). They possess different exotoxin gene profiles (e.g., Panton-Valentine leucocidin *pvl* genes) and carry the type IV staphylococcal cassette chromosome (SCCmec IV). Presence of PVL has some major clinical therapeutic impacts, such as the association with deep-seated abscess, multiple lesions, recurring SSTI episodes, multiple antibiotic resistances and outbreaks of SSTIs (Yueh *et al*., 2022). Many studies have

shown significant association of *pvl* gene with CA-MRSA isolates compared with HA-MRSA isolates despite its controversial significance (Samaranayake, 2019).

Methicillin-resistant *S. aureus* produces many virulence factors, including secreted pore-forming toxins (PFTs) of three major classes that are critical for bacterial spread and survival in the host: α-hemolysin (Hla), phenol-soluble modulins (PSMs), and bicomponent leukocidins (Blake *et al*., 2018).

Panton–Valentine leukocidin is a bi-component, pore-forming toxin produced by co-transcribed genes, lukF-PV and lukS-PV, that targets and lyses human macrophages, polymorphonuclear leukocytes and monocytes and also incites the human inflammatory immune response (Coombs *et al*., 2020).

Methicillin-resistant *S. aureus* strains harboring the pathogenic marker Panton–Valentine leukocidin (PVL) are isolated more frequently, the presence of PVL is relevant, as it is associated with a more severe clinical presentation, often with recurrences, deep and multiple lesions and frequent transmission to contact persons, irrespective of methicillin resistance. In addition, PVL-positive *S. aureus* often displays multiple resistances to commonly used antibiotics to treat *S. aureus* infections(Sabrina *et al*., 2019).

Panton–Valentine leukocidin positive community-acquired strains Methicillin-resistant *Staphylococcus aureus* is closely associated with skin and soft tissue infections (SSTIs). Thus, the pathogenicity of CA-MRSA strains is considered higher than that of HA-MRSA strains (Nakaminami *et al*.,2020).

The hallmark signs of PVL-SA include recurrent boils and necrotizing SSTIs in otherwise healthy patients, particularly when other members of the same household or close community also present with similar signs . The most common SSTIs seen with PVL-SA are furunculosis, carbuncles, folliculitis, cellulitis, abscesses and skin necrosis, which tend to recur despite several courses of antibiotics and delay the diagnosis of PVL-SA disease. It is

also important to appreciate that associated pain/erythema can be out of proportion to the severity of the clinical signs (Shallcross *et al*., 2013).

This study aimed to isolate and differentiate methicillin resistant *Staphylococcus aureus*, community acquired methicillin resistant *Staphylococcus aureus* (CA-MRSA) and hospital acquired methicillin resistant *Staphylococcus aureus* (HA-MRSA) , the association between Panton Valentine Leukocidin Gene Expression and different antibiotics in methicillin resistant *Staphylococcus aureus* isolates.

This aim is achieved by using the following objectives:

1. Isolation and identification of methicillin resistant *Staphylococcus aureus* (MRSA) by traditional methods
2. Confirmatory diagnosis using VITEK 2 compact system and testing the sensitivity of these bacteria to antibiotics.
3. Confirmation of MRSA by *mec*A gene by PCR .
4. Molecular detection of *pvl* gene by PCR.
5. Discrimination between HA-MRSA and CA- MRSA.
6. Study the effect of antibiotic on expression of *pvl* gene.

1.2. Literature Review:

1.2.1. History of *Staphylococcus aureus*

In 1880, the Scottish surgeon Sir Alexander Ogston first described staphylococcus in pus from a surgical abscess in a knee joint(Algammal *et al*., 2020) and he involvement in wound infections was established in the 1881 report , who described the formation of new abscesses in guinea pigs and mice injected with pus taken from his patients. He called the clusters of bacteria he observed in the abscesses "staphyle" come From the Greek staphyle (bunch of grapes) and kokkos (berry) because the arrangement of the bacterial cells resembled a cluster of grapes, In 1884, the German physician Friedrich Julius Rosen Bach differentiated the staphylococci by the color of their colonies: *Staphylococcus aureus* (from the Latin aurum, gold) and *Staphylococcus albus* (Latin for white). *Staphylococcus albus* was later renamed *S. epidermidis* because of its ubiquity on human skin (Salman, 2022).

Staphylococcus aureus belongs to the genus Staphylococcus, Firmicutes; is positive for Gram stain, ~0.8 μm in diameter, arranged in a "string of grapes" under a microscope(Naureen *et al.*, 2022), an aerobic or anaerobic; and grows optimally at 37°C, and at pH7.4 . The colonies on blood agar plate are thick, shiny, and round with a diameter of 1~2 mm, most of them are hemolytic, forming a transparent hemolytic ring around the colonies on blood agar plates(Gonzalez-Perez *et al*., 2019; Sato *et al*., 2019). Moreover, *S. aureus* does not form spores or flagella, but possesses a capsule, can produce golden yellow pigment, and decompose mannitol, additionally, it has also been found that tests of plasma coagulase, lactose fermentation and deoxyribonuclease are positive in *S. aureus* (Chino *et al*., 2017; Tayeb-Fligelman *et al*., 2017).

This bacterium has the ability to infect almost every organ system in the human body, often with fatal consequences. This remarkable adaptability is largely due to the wide range of virulence factors they produce, many of which are encoded in plasmids, transposons, prophages, and pathogenicity islands (Silva *et al*., 2020) , These factors include cell surface components (e.g., protein A, fibronectin-binding protein, collagen-binding protein, and clumping factor), and exoproteins (e.g., enterotoxins, exfoliatins, toxic shock syndrome toxin, and Panton-Valentine leucocidin (PVL).

There is a wide range of *S. aureus* infections in the human. The clinical infections of *S. aureus* are classified into community and nosocomial categories based on origin of infection. These two types are distinct in clinical manifestations of the infections, antibiotic susceptibility and the genetic background of the infecting *S. aureus* strains. For decades, *S. aureus* has been predominately a nosocomial pathogen and is a leading cause of mortality and morbidity in hospitals. However, the community *S. aureus* infections are in rise. The important clinical *S. aureus* infections are bacteraemia, infective endocarditis, skin and soft tissue infections, osteoarticular infections and pleuropulmonary infections. Other clinical infections are epidural abscess, meningitis, toxic shock syndrome and urinary tract infections (Tong *et al.*, 2015).

1.2.2.Epidemiology:

S. aureus are found on the skin and mucous layers, and people are the major reservoir for this organism. It is assessed that up to half of all grown-ups are colonized, and roughly 15% of the populace tirelessly carry *S. aureus* within the front nares. A few populaces tend to have higher rates of *S.aureus* colonization (up to 80%), such as wellbeing care laborers, people who utilize needles on a standard premise (i.e., diabetics and intravenous

(IV) sedate clients), hospitalized patients, and immune compromised people. *S. aureus* can be transmitted person-to-person by coordinate contact or by fomites (Tong *et al*., 2015).

1.3. Pathogenicity of *S. aureus*

S. aureus is one of the most common pathogenic bacteria, as its pathology is dependent on a group of virulence factors that affect the host and cause the disease. (Alfatemi, *et al*., 2014). *S.aureus* causes disease by third major mechanisms: (1) Invasion and inflammation. This includes mechanisms for colonization, synthesis of extracellular molecules that facilitate adherence, and the ability to evade host defenses. (2) Toxin production. Exotoxins which produces by *S. aureus* are responsible for damaging host tissue and promoting dissemination. (3) A third virulence mechanism that is important in certain infections there is the ability to form biofilms (Bien *et al*., 2011).

S. aureus is an important cause of pneumonia. It was initially implicated as a devastating respiratory complication of influenza during the 1918 pandemic . It thereafter remained an infrequent but well-documented cause of community-acquired pneumonia (CAP), even in the absence of preceding influenza infection. *S. aureus* has had a more predominant role in hospitalized patients with respiratory infections and has been implicated in each of the three other major subsets of pneumonia: hospital-acquired pneumonia (HAP), ventilator-associated pneumonia (VAP), and health care-associated pneumonia (HCAP). It is also a common pathogen in patients with cystic fibrosis (Tong *et al*., 2015).

Staphylococcus aureus are one the most common bacterial infections in humans and are the causative agents of multiple human infections, including bacteremia, infective endocarditis, skin and soft tissue infections (e.g.,

impetigo, folliculitis, furuncles, carbuncles, cellulitis, scalded skin syndrome, and others), osteomyelitis, septic arthritis, prosthetic device infections, pulmonary infections (e.g., pneumonia and empyema), gastroenteritis, meningitis, toxic shock syndrome, and urinary tract infections (Tong *et al.*, 2015) .

Methicillin Resistance *Staphylococcus aureus* (MRSA) has also caused many diseases, including endocarditis and chronic osteoarthritis .Although, MRSA has many mechanisms to increase pathogenicity and virulence, including biofilm production and host cell adhesion (Krismer and Peschel, 2011., Al-Khafaji, 2018), burn patients are exposed to various infections due to skin damage and weak innate immunity, as bacterial infections are the main problems for burn patients in hospitals and one of the main causes of death for burn patients(Chen *et al.*, 2021). *S. aureus* is one of the most common bacteria isolated from burn infections. MRSA including hospital-associated MRSA (HA-MRSA) and community-associated MRSA (CA-MRSA) have been reported as important bacterial causes of burn wound infections(Tajik *et al*., 2019).

S. aureus have an arsenal of virulence factors that play significant roles in the wide variety of infections and diseases in humans including animals. These factors can also support and provide protection for *S. aureus* to evade the host immune system recognition and their actions. One of the most important virulence factors is surface proteins which promote and encourage the binding and the attachment processes of this bacterium to the host cells surfaces. To avoid recognition from the host immune cells, the surface proteins combined with the blood proteins that lead to aid the bacterium to survive and cause damage to host tissues (Gnanamani *et al*., 2017).

Coagulase, hyaluronidase, deoxyribonuclease, and lipase are some of the enzymes that *S. aureus* can synthesize to enhance its pathogenicity and

disseminate within the host (Tam and Torres, 2019). Moreover, enterotoxins, toxic shock syndrome toxin1 (TSST1), exfoliative toxins (ETs), hemolysins, epidermal cell differentiation inhibitors (EDINs), and Panton–Valentine leukocidin (PVL) have all been identified as extracellular protein toxins that enhance pathogenicity(Oliveira *et al*., 2018). Interestingly, some of these toxins were detected in MRSA infections more frequently than non-MRSA cases (Liu *et al*., 2010, Ezeamagu *et al*., 2018)

S. aureus infections are particularly problematic due to frequently occurring antibiotic resistance in *S. aureus* isolates, among which methicillin-resistant *S. aureus* (MRSA) are the most important clinically Infections by MRSA are accompanied by increased mortality, morbidity, and hospital stay, as compared to those caused by methicillin-sensitive *S. aureus* (MSSA). MRSA could induce severe infectious diseases in human including; pyogenic endocarditis, suppurative pneumonia, otitis media, osteomyelitis, pyogenic infections of the skin, soft tissues, and septic arthritis (Ippolito *et al* ., 2010, Algammal *et al*., 2020).

1.4. Virulence Factors of *S. aureus*

S. aureus have an arsenal of virulence factors that play significant roles in the wide variety of infections and diseases in humans and animals. These factors can also support and provide protection for *S. aureus* to evade the host immune system recognition and their actions. One of the most important virulence factors is surface proteins which promote and encourage the binding and the attachment processes of this bacterium to the host cells surfaces. To avoid recognition from the host immune cells, the surface proteins combined with the blood proteins that lead to aid the bacterium to survive and cause damage to host tissues(Gnanamani *et al*., 2017).

Another vital factor is protein A, which is located in the cell wall of *S. aureus* which accurately anchored to the peptidoglycan pentaglycine bridges of the bacteria. It is also known as an IgG-binding protein which combines with the Fragment crystallisable (Fc) region of the antibody in order to cover the surface of the bacterium with IgG antibody to make the recognition of this organism by the immune cells difficult and hard to detect (Shettigar and Murali, 2020).

The clumping factor A (ClfA) is another *S. aureus* virulence factor that is expressed by the surface of the bacteria cells. Also, it is known as fibrinogen binding protein that promotes the clotting process of blood cells and the damage process of tissues. In addition, the polysaccharide capsule is considered as one of the most essential virulence factor which can contribute to assist the *S. aureus* surviving within the host cells by inhibiting the phagocytosis process by macrophage and dendritic cells, moreover, *S. aureus* cells are capable of secreting several important toxins and enzymes such as; coagulase; DNAase; leukocidin; hemolysins; exfoliative toxin in order to promote bacterial penetration and help the bacterium to evade in to the host tissues(Lacey *et al*., 2016, Gnanamani *et al*., 2017).

Moreover, *S. aurues* can generate another harmful type of toxins such as Panton- Valentine leukocidin (PVL) which cause pneumonia in children and Toxic Shock Syndrome Toxin-1 (TSST-1) which is associated with some cases of septicemia due to the use of particular types of tampons. A number of *S. aureus* strains have the ability to produce a pigment that known as staphyloxanthin which acts as an important virulence factor. This strain has an antioxidant role against reactive oxygen species used by the host immune cells, in order to help the bacterium to escape from killing action of immune cells. Furthermore, *S. aureus* are capable of forming biofilms of various surfaces which associated generally with most indwelling medical devices

problems such as heart valves and knee replacements and via this biofilm community resistance can be acquired to antibacterial agents through horizontal gene transfer (Kane *et al*., 2018, Shettigar and Murali, 2020).

1.4.1. Pore forming toxin

Pore-forming toxins (PFTs) are a common class of bacterial virulence factors found in many human pathogens including *Streptococcus pneumoniae*, *Mycobacteria tuberculosis*, *Escherichia coli* and *Staphylococcus aureus*. These toxins belong to a larger family of proteins known as pore-forming proteins (PFPs) which include the mammalian immunity proteins perforin and complement C9 of the Membrane Attack Complex (MAC). Two groups of PFTs exist, classified on the basis of the structure of their transmembrane components: α-PFTs whose pores consist of α-helices, and β-PFTs which form β-barrel pores (Bischofberger *et al*., 2012).

The best-studied PFTs to date are arguably those secreted by *S. aureus*, a ubiquitous human pathogen that causes a wide-range of diseases such as pneumonia, sepsis, and skin and soft tissue infections (SSTI). The success of *S. aureus* can be attributed its broad array of host-targeting virulence factors, most notably its arsenal of seven different β-barrel PFTs: α-hemolysin (α-toxin, Hla) and six bicomponent leukocidins (Tong *et al*., 2015).

Methicillin-resistant *S. aureus* produces many virulence factors, including secreted pore-forming toxins (PFTs) of three major classes that are critical for bacterial spread and survival in the host: α-hemolysin (Hla), phenol-soluble modulins (PSMs), and bicomponent leukocidins (Blake *et al*., 2018).

1.4.1.1. Bicomponent leukocidins

S. aureus leukocidal activity was discovered contemporaneously with its hemolytic activity. However, for many years following this observation, it was unclear if this was a secondary function of α- toxin, or if it was caused by a separate biological product (Alonzo and Torresm, 2013). The independence of the hemolytic and leukocidal features of *S. aureus* was first demonstrated by Julianelle in 1922, then confirmed by Panton and Valentine in 1932. In these studies, a careful analysis of multiple *S. aureus* strains revealed that hemolytic activity did not necessitate leukocidal activity. Interestingly, this investigation correlated leukocidal activity with acute pathology in humans, and hemolytic activity with virulence in rabbits. It is notable that the difficulty in assigning the toxic effects of *S. aureus* supernatants to individual toxins is partially attributable to the number of leukocidins that *S. aureus* can secrete, not all of which were identified until the 21 st century. The biology of these toxins is further complicated by the fact that different leukocidins can target the same cell types, as well as the same receptors (Alonzo and Torresm, 2013 ,Spaan *et al*., 2014).

Unlike α-toxin, leukocidins are hetero-oligomeric, consisting of two subunits that are encoded within a single open reading frame, with γ-hemolysin AB (HlgAB) as the sole exception . This was demonstrated for the first time by Woodin through the fractionation of the Panton-Valentine Leukocidin (PVL) using ion-exchange chromatography, reflected in the names of the subunits F and S, for fast and slow fractions, respectively. Most importantly, this study was the first to demonstrate that the two fractions, or subunits, must be combined for maximal cytolytic activity. While reports suggested that the leukocidin oligomer consisted of six or seven subunits, it is now accepted and confirmed that the assembled leukotoxin is an octamer of four F-subunits and four S-subunits in alternating fashion, Like Hla, the bi

component leukocidins are generally secreted as water soluble monomers. The S component confers cell-type specificity by binding to cellular receptors, inducing a conformational change to allow dimerization with F-components. These dimers then oligomerize to form the pre-pore prior to insertion of the β-barrel transmembrane channel. The exception to this is LukAB, which is either secreted as a dimer or dimerizes shortly after secretion, and can also adhere to the bacterial cell wall(Seilie *et al*., 2017).

1.4.1.2.Panton-Valentine Leukocidin (LukF-PV and LukS-PV)

Panton-Valentine Leukocidin toxin is a bicomponent, synergohymenotropic cytolysin comprising two non-associated, secretory water-soluble monomers LukS (284 amino acids; molecular weight 25.88 kDa) and LukF (301 amino acids; molecular weight 34.88 kDa). It is encoded by two contiguous and transcribed genes, lukS-PV and lukF-PV, which are located in the genomes of a number of icosahedral or elongated head-shape temperate lysogenic bacteriophages, that is, FSa2958, FSa2MW, FPVL, F108PVL, FSLT, and FSa2USA, respectively (Himanshu *et al*., 2021). It was initially discovered by Van deVelde in 1894 due to its ability to lyse leukocytes. It was named after Sir Philip Noel Panton and Francis Valentine when they associated it with soft tissue infections in 1932 (Boyle-Vavra and Daum, 2007).

Exotoxins such as PVL constitute essential components of the virulence mechanisms of *S. aureus* Nearly all strains secrete lethal factors that convert host tissues into nutrients required for bacterial growth (Brisset *et al*., 2020). It is a virulence factor, which causes a range of diseases known as collectively known as PVLSA disease, typically presenting as recurrent skin and soft tissue infections (SSTIs) despite antibiotic treatment (Saeed *et al*., 2018).

Panton-Valentine Leukocidin may be produced by different strains of SA, both methicillin-sensitive SA (MSSA) and methicillin-resistant SA (MRSA). The proportion of PVL-SA is higher in infections caused by MRSA (74–100%) than in those caused by MSSA (9–46%), with the proportion being dependent on the prevalence of MRSA in the respective regions, rarely, *pvl* can lead to very serious invasive infections, including necrotizing haemorrhagic pneumonia (which has a mortality of up to 75%) (Ritz *et al*., 2012, Shallcross *et al*., 2013).

Methacillin resistant *staphylococcus aureus* strains harboring the pathogenic marker Panton–Valentine leukocidin (PVL) are isolated more frequently, the presence of PVL is relevant, as it is associated with a more severe clinical presentation, often with recurrences, deep and multiple lesions and frequent transmission to contact persons, irrespective of methicillin resistance. In addition, PVL-positive *S. aureus* often displays multiple resistances to commonly used antibiotics to treat *S. aureus* infections(Sabrina *et al*., 2019).

1.4.1.2.1. Risk factors

Patients with PVL-SA are often young adults with very little or no previous exposure to healthcare settings(Shallcross *et al*., 2013). Factors increasing the risk of acquiring PVL-SA-related disease have been categorized into the five by the US Centre for Disease Control and Prevention. These are: (1) close contact; (2) contaminated items, (3) crowding, (4) cleanliness, and (5) cuts and other compromised skin integrity. They can be similar to features for other cutaneous infections. There have been reported outbreaks in a variety of different communities, with certain groups being recognized as at risk. These include children, prisoners, those who play close contact sports, men who have sex with men and the military.

Close contact dominates as an important factor in disease transmission (Sheikh *et al*., 2015).

1.4.1.2.2. Pathogenesis

The pathogenesis of PVL-SA diseases arises from the cytolytic pore-forming action of the PVL toxin, which induces cell apoptosis through the intrinsic pathway of cells. PVL is a cytotoxin composed of two proteins: LukS-PV and LukF-PV, which are encoded by two genes (lukS-PV and lukF-PV) carried on temperate bacteriophages(Zhang *et al*., 2017, Saeed *et al*., 2018).

These two components are secreted by SA and act synergistically to create one hetero-octameric unit, which binds to the C5AR and C5A2 human complement receptors of human polymorphonuclear neutrophils (PMNs), monocytes and macrophages. Initially, the LukS-PV subunit binds to the human complement receptor C5AR, which then results in the secondary binding of LukF-PV to form the assembly of one bi-component toxin that creates pores in the cell membranes. This eventually leads to cell membrane lysis and cell death (Spaan *et al*., 2013, Zhang *et al*., 2017).

Although USA300 is a widely accepted globally hypervirulent strain, whether or not its virulence is directly related to PVL gene expression is controversial. Neutrophil lysis is widely accepted to have a role in PVL-SA disease; however, initially in trials on rabbits (in which neutrophils are highly sensitive to PVL gene expression) it was found that lysis had a limited impact on virulence and there may be a role for the expression of other virulence factors, such as the a toxin from *agr* gene expression (Li *et al*., 2010). This was further reflected in human trials, in which it was found that PVL-positive infections did not result in a worse clinical outcome regardless of methicillin resistance; in fact, other genes were also noted to produce a statistically significant clinical impact on disease response, including the aforementioned type IV SCCmec element and the *agr* group of genes. Regardless, it has been

shown in both animal and human studies that there is consistently a link between PVL positivity and its hallmark clinical manifestations of SSTIs and severe pneumonia, especially in younger populations(Qu *et al*.,2022).

1.4.1.2.3.Clinical presentation

PVL-SA presents as a broad range of disease, ranging from asymptomatic nasopharyngeal carriage of PVLSA to severe necrotizing disease (Pantelides *et al*., 2012). These can be divided into dermatological and non dermatological presentations.

1.4.1.2.3.1. Dermatological presentations

The hallmark signs of PVL-SA include recurrent boils and necrotizing SSTIs in otherwise healthy patients, particularly when other members of the same household or close community also present with similar signs . The most common SSTIs seen with PVL-SA are furunculosis, carbuncles, folliculitis, cellulitis, abscesses and skin necrosis, which tend to recur despite several courses of antibiotics and delay the diagnosis of PVL-SA disease. It is also important to appreciate that associated pain/erythema can be out of proportion to the severity of the clinical signs (Shallcross *et al*., 2013).

1.4.1.2.3.2.Nondermatological presentations

PVL-SA can lead to invasive infections in previously immune competent patients. In those with community acquired pneumonia, haemoptysis should be a major alerting sign for necrotizing pneumonia, with further imaging to elucidate this. Haemoptysis is typically preceded by a period of influenza-like prodromal in these patients (typically children and young adults). Around a quarter of patients have a prior history of skin lesions, either personal or in a close contact. Other invasive infections include necrotizing fasciitis and purpura fulminans. PVL-SA disease can present as severe musculoskeletal

infections such as osteomyelitis, pyomyositis and septic arthritis, especially in children. Compared with PVL-negative SA infections, these musculoskeletal infections have been associated with higher complication rates, elevated levels of inflammation and prolonged hospital stay, and overall are more likely to require surgical treatment (Bhatta *et al*., 2017).

1.4.1.2.4. Treatment

Once diagnosed, PVL-SA infection must be treated appropriately, without delay. Initial empirical antimicrobial coverage generally includes an anti staphylococcal regimen and an anti-toxin agent able to block the production of the toxin (Saeed *et al*., 2018).

There have been reported experience of community PVL-MSSA cases failing treatment with commonly used antibiotics such as flucloxacillin. This can be explained by studies showing that suboptimal tissue concentration of flucloxacillin enhances the transcription of the PVL toxin gene, thus up regulating the release of PVL increasing toxicity. By contrast, clindamycin, linezolid and fusidic acid have been found to significantly down regulate the release of the PVL toxin through suppression of translation, thought to be due to better soft tissue penetration (Shallcross *et al*., 2013, Saeed *et al*., 2018).

After primary treatment of the patient's disease, preventative measures are crucial to stop infections from recurring and to interrupt the transmission through the community. Decolonization plays a key role in achieving this(Saeed *et al*., 2018). All patients along with their close contacts and/or family members living in the same household) should undergo a 5-day course of topical decolonization with chlorhexidine 4% wash once daily and mupirocin nasal ointment applied three times daily, without prior PVL gene screening (Creech *et al*., 2015). To further prevent the spread of PVL-SA, personal hygiene measures in relation to close contacts must be followed. Fomites such as toiletries, towels and clothes should be kept separately from those of others and washed frequently, while shared household areas and

shared personal/household items should be regularly vacuumed and/or cleaned with a household detergent. Washing hands frequently with liquid soap and water, especially after changing plasters/dressings and touching infected skin should be encouraged. To date, there are no reports of a significant association between livestock-associated MRSA and antibiotic-resistant PVL-MRSA strains; however, standard hygiene precautions should be adhered to when dealing with animals and handling raw meat (Anjum *et al*., 2019).

Repeated decolonization has been found to greatly reduce the chances of reinfection, with patients being 89% less likely to be reinfected after the fifth course of topical decolonization (Hanitsch *et al*., 2020). If mupirocin resistance is found, neomycin-based nasal ointments can be used, and if there is allergy to chlorhexidine, octenidine antimicrobial wash lotion can be used.

1.5. Pore Forming Toxin (PV L) & Gene Expression

Panton–Valentine leucocidin (PVL) are pore-forming toxins that possess cytolytic properties are expressed by most *S. aureus* strains, and they have the ability to interact with host immune signalling, thus contributing to staphylococcal pathogenesis. PVL has been linked to specific types of human *S. aureus* infections, including primary skin and soft tissue infections and severe diseases such as necrotizing pneumonia, which has a high mortality rate, and recurrent complicated osteomyelitis. Moreover, PVL is frequently detected in clinical practice because it is produced by many community acquired methicillin-resistant *S. aureus* (CA-MRSA) clones that are spreading throughout the world. It has been shown previously that sub inhibitory concentrations of some antibiotics influence the expression of virulence factors by *S. aureus*, which may affect the pathogenesis of infection. Based on several series of in vitro data, recent guidelines recommend the use of antibiotics that inhibit the expression of virulence factors for the treatment of severe infections due to PVL-producing *S. aureus* (Otto *et al*., 2013).

It is approved that the real-time polymerase chain reaction (Rt-PCR) is dramatically faster than conventional PCR and other diagnostic methods. Due to its high sensitivity, high specificity, very low risk of contamination, and simplicity the Rt-PCR has considered as an appealing method in clinical microbiology laboratories (He *et al*., 2018) .

1.6. Resistance to Antibiotics

The multiple antimicrobial resistances in *S. aureus* has raised a significant global veterinary and public problem. *S. aureus* is an extremely versatile pathogen, could produce genes of resistance quickly. The development of bacterial resistance predates the time of antibiotic usage. But discoveries of resistance in *S. aureus* were recorded from the early 1940s, When *S. aureus* resistant to penicillin was the first recorded since then, this bacteria has acquired worldwide prominence as the greatest reason for infection associated with nosocomial, community, and livestock The mechanism for generating resistance in bacteria included the incorporation of compound structures such as the alteration of the target drug site, enzymatic inactivation and mutation at the target drug site, and horizontal gene transfer of resistance determinants (Bitrus *et al*., 2018).

Penicillin-resistant *S. aureus* can produce penicillinase, which can hydrolyze the penicillin β-lactam ring, leading to resistance to penicillin. Later, scientists developed a new penicillinase-resistant semisynthetic penicillin named methicillin, which is resistant to the hydrolysis of β-lactamase (Tyagi *et al.*, 2021).

After being applied to the clinic in 1959, methicillin effectively controlled the infection of penicillin-resistant *S. aureus* (Sharma *et al*., 2021). However, only 2 years after methicillin was applied, in 1961, British scientist Jevons reported the isolation of an MRSA strain; this resistance was

produced by a gene encoding the penicillin-binding protein 2a or 2′ (PBP2a or PBP2) (*mec*A) which was integrated into the chromosomal element (SCCmec) of methicillin-sensitive *S. aureus*(Dinescu *et al.*, 2021).

Growth of *S.aureus* resistance through the development of a genomic island named Staphylococcus cassette chromosome (SCCmec) which transport resistant methicillin factor *mec*A results in beta-lactams (Bitrus *et al*., 2018). Reported a relatively high incidence of community associated methicillin resistant *S. aureus* (CA-MRSA) with SCCmec among healthy carrier patients as in the case with penicillin, methicillin resistance *S. aureus* were identified among individuals in the community and more recently in livestock (Bosch *et al*., 2015, Lakhundi *et al*., 2018).

1.7. Methicillin-resistant *Staphylococcus aureus* and *mec*A gene

Methicillin-resistant *Staphylococcus aureus* (MRSA) was first reported in United Kingdom in 1961, just after the description of methicillin. Methicillin was designed to be resistant to β-Lactamase, but Methicillin-Resistant *S. aureus* (MRSA) strains were discovered shortly after methicillin was introduced into clinical practice. MRSA belongs to the most dangerous bacteria, causing nearly half of all antibiotic-resistant organism-related deaths. The advent of MRSA, which causes infections of the sinuses and ears, is caused by different risk factors. Widespread use of broad-spectrum antibiotics and prior nose operations are the main contributing factors (El-Bouseary *et al*., 2018). MRSA could induce severe infectious diseases in human including; pyogenic endocarditis, suppurative pneumonia, otitis media, osteomyelitis, pyogenic infections of the skin, soft tissues, and septic arthritis. The emergence of multidrug-resistant virulent MRSA strains is a remarkable public health problem (Gajdács *et al*., 2019).

There are many kinds of antibiotics that target key bacterial processes, such as cell wall synthesis, translation, transcription, and DNA synthesis, and that can be used to treat staphylococcal infections (Assis *et al*., 2017). Methicillin-resistant *S. aureus* (MRSA) attracted global attention in the 1960s (Fri *et al*., 2020), and its antibiotic resistance occurs via several mechanisms. Generally, the β-lactam mechanisms of resistance of MRSA strains support cross-resistance to all β-lactam antibiotics .The key mechanism for resistance is the enzyme-catalyzed modification and ultimate destruction of the antibiotic, causing its dynamic efflux from cells and antibiotic target alteration(Gonzalez-Bello,2017).

MRSA strains carry a unique and transmissible genetic component known as Staphylococcal cassette chromosome mec (SCCmec) that harbors the mecA gene at the 30 end of a chromosomal open reading frame named orfX. It encodes a penicillin-binding protein (PBP2a) with a reduced affinity for beta-lactam antibiotics. Consequently, these strains are resistant to all beta-lactam antibiotics, with the exception of fifth-generation cephalosporins (Abdelwahab *et al*., 2023).

Although MRSA began as a hospital-acquired infection, it can be found in all communities and livestock. The terms HA-MRSA (healthcare-associated or hospital-acquired MRSA), CA-MRSA (community-associated MRSA) and LA-MRSA (livestock-associated) reflect the MRSA infections in a variety of hosts (Haysom *et al* ., 2018). CA-MRSA is genetically different from HA- MRSA by possessing a small type of SCCmec, and the frequent production of Panton-Valentine leukocidin, and cytotoxin. CA-MRSA strains are restricted people outside the health care practice and are usually cause mild infections such as skin and soft tissue infections. However, recent epidemic-molecular investigations reported that CA-MRSA

could affect several patients within health care settings (Alaklobi *et al*., 2015).

1.7.1. Penicillin Binding Proteins (PBPs)

Penicillin Binding Proteins (PBPs) The antibacterial activity of β-lactam antibiotics results from their covalently binding to the active sites of penicillin-binding proteins (PBP). PBPs are enzymes that catalyze the cross-linking reactions between peptidoglycan polymers, one of the final steps in the bacterial cell wall assembly. Therefore, β-lactam antibiotics are potent inhibitors of cell wall synthesis (Sauvage and Terrak, 2016). Susceptible strains of *S. aureus* produce four or five PBPs: PBPs 1, 2, 3, 3', and 4 with approximate molecular weights of 85,000, 80,000, 75,000, 70,000, and 45,000 kilo Dalton, respectively (Costa *et al*., 2018)

Methicillin resistance is associated with the production of a novel PBP that is not present in susceptible Staphylococci. Resistant strains of S. aureus produce an additional 78-kilodalton PBP, termed PBP2a or PBP2', that has a low binding affinity for β-lactam antibiotics (Costa *et al*., 2018). Methicillin-resistant strains of coagulase-negative Staphylococci also produce PBP2a (Ferrer-González *et al*., 2017). In contrast to other Staphylococcal PBPs, which generally bind β-lactam antibiotics at low concentrations, PBP2a binds β-lactam antibiotics only at relatively high concentrations (Sauvage and Terrak, 2016). Penicillin-binding proteins (PBPs) are membrane-bound enzymes that catalyze the transpeptidation reaction which is necessary for cross-linkage of peptidoglycan chains (Zhong and Zhou, 2014).

Beta-lactam antibiotics produce a bactericidal effect by binding of penicillin-binding protein PBP to β-lactam inhibiting the membrane-bound enzymes responsible for catalyzing vital stages in the biosynthesis of the cell wall. While, in methicillin-susceptible strains, have a high affinity for most

βlactam antimicrobials, PBP2a has a low affinity for binding β-lactams. In methicillin-resistance strains, the essential function of PBP is undertaken by PBP2a to maintain the survival of the bacterium in the presence of antimicrobials. This is achieved by the antibiotic binding directly and covalently to one or more of the penicillin-sensitive enzymes called penicillin-binding proteins (PBPs) (Fishovitz *et al*., 2014).

The peptidoglycan component of the bacterial cell wall provided an excellent selective target for the antibiotics. The presence of mutations in the penicillin-binding domain of penicillin-binding proteins (PBPs) resulted in decreased affinity to β-lactam antibiotics. This mechanism of resistance had arisen in several clinically important Gram-negative and Gram-positive bacteria (Rani and Yadav, 2014).

1.7.1.1. Penicillin-Binding Protein 2a (PBP2a)

The PBP2a belongs to the group of high molecular mass (75 KDa) family of PBPs and consists of a transpeptidase domain and a non-penicillin binding domain of the unknown function (Fishovitz *et al*., 2014).

PBP2a appears to be a rather poorly active enzyme compared to other native PBPs that synthesize highly cross-linked peptidoglycan (Salah *et al*., 2017). In some strains, PBP2a is inducible by β-lactam antibiotics and its production differs according to growth conditions. Inducibility of PBP2a may be controlled by regulatory genes present on plasmids encoding penicillinase production (Ferrer-González *et al*., 2017). Penicillinase-negative strains may be still inducible for PBP2a, possibly due to a chromosomal location of the penicillinase regulatory genes. Inducibility and differences in amounts of PBP2a produced might appear to account for the heterogeneous expression of resistance (Sauvage and Terrak, 2016).

In addition, the resistance of β-lactam antibiotics was increased like Oxacillin/Methicillin, Ampicillin, Amoxicillin, Amoxicillin/ Clavulanic acid, and Cephalosporins by the presence of the PBP2a (Kolář *et al*., 2010).

Resistance is conferred by the *mecA* gene, which codes an altered penicillin-binding protein (PBP2a or PBP2') that has a lower affinity for binding β-lactams (penicillins, cephalosporins, and carbapenems). This allows resistance to all β-lactam antibiotics and obviates their clinical use during MRSA infections. As such, the glycopeptide vancomycin is often deployed against MRSA, the strains that produce PBP2a are resistant to all β-lactams (Kim *et al*., 2012, Grema *et al*., 2015).

1.7.2. Staphylococcal Cassette Chromosome (SCC)

SCCmec is mainly characterized by four factors: (i) carriage of mecA in a mec gene complex, (ii) carriage of a ccr gene(s) (ccrAB and/or ccrC) in the ccr gene complex, (iii) integration at a specific site in the staphylococcal chromosome, designated as the integration site sequence (ISS) for SCC, which serves as a target for ccr-mediated recombination, and (iv) the presence of flanking direct repeat sequences containing the ISS (Uehara, 2022)

Five classes of mec gene complex (A - E) have been identified with many subclasses in various types of MRSA isolates. Class A mec, B mec and C mec are the most common types(Yamaguchi *et al*., 2020). The ccr gene complex encodes DNA recombinase enzymes that catalyze the mobility of the SCCmec cassette by its excision or insertion into several integration sites. Three types of ccr have been reported (ccrA, ccrB, and ccrC) with multiple allotypes for each gene forming eight complexes (Turlej *et al*., 2011).

Five mec gene and eight ccr gene complexes combination are essential in SCCmec nomenclature and assignment of new types. Availability of complete sequences from different MRSA isolates facilitated the assignment of thirteen (I-XIII) SCCmec types (McClure *et al*., 2020). SCCmec I, SCCmec II, and SCCmec III carry mecB and ccr1, mecA and ccr2, and mecA and ccr3 gene complexes, respectively. SCCmec IV is the smallest element that has a unique combination of mecB and ccr2 gene complexes. SCCmec V, SCCmec VI, SCCmec VII, and SCCmec VIII carry mecC2 and ccr5, mecB and ccr4, mecC1 and ccr5, and mecA and ccr4 gene complexes, respectively. SCCmec IX, SCCmec X, SCCmec XI, SCCmec XII, and SCCmec XIII carries mecC2 and ccr1, mecC1 and ccr7, mecE and ccr8, mecC2 and ccr5(C2) and mecA and ccr5(C2) gene complexes, respectively (Liu *et al*., 2016).

SCCmec elements are highly diverse. According to the International Working Group on the Classification of Staphylococcal Cassette Chromosome Elements (IWG-SCC), there are currently 13 recognized SCCmec types (I–XIII), based on the combinations of five mec complexes (A, B, C1, C2 and E) and nine ccr gene complexes (types 1–9) (Noriko *et al* .,2019).

On the basis of SCCmec elements, MRSA strains are classified as hospital acquired(HA), community-acquired (CA), and livestock-associated (LA) infections HA-MRSA isolates contain mainly type I, II, and III SCCmec elements, while CA-MRSA and LA-MRSA contains SCCmec IV to XIII (Peechanika *et al*., 2021).

1.7.3. Hospital acquired and Community Acquired MRSA

community acquired methicillin resistant *S. aureus* (CA-MRSA) has also came into view which showed the β-lactam resistance akin to

the hospital acquired strains. CA-MRSA which is obtained in the outpatient settings or isolated within 48 hours of hospital admission occurs in a person without a history of prior MRSA infection or colonization (Sadaf *et al.*,2020).

Hospital acquired MRSA (HA-MRSA) is prevalent in nearly all healthcare facilities and constitutes a huge infectious disease burden in the world. Health care associated MRSA cases are defined as patients with MRSA infection identified after 48 hours of admission to a hospital with a history of hospitalization, surgery, dialysis, residence in a long-term health care facility, a permanent indwelling catheter or percutaneous medical device present at the time of culture or a known positive culture for MRSA prior to the study period. It is resistant to many antibiotic classes, and often they are resistant to the common beta lactam and non-beta lactam antibiotics (Abiye and Alem, 2021). Hospital-acquired MRSA (HA-MRSA) infections often lead to ventilator associated pneumonias, intravenous catheter associated infections or surgical wound infections, while community-acquired MRSA (CA-MRSA) infections commonly cause skin and soft tissue infection, sometimes are invasive and life threatening

To better understand the differences between hospital-acquired -MRSA and community-acquired-MRSA and their degree of resistance, an analysis of their genetic and structural configuration must be explored. It is these resistance genes, virulence factors, and toxins that aid in further explaining the resistance *S. aureus* expresses against most traditional therapeutic agents, thus contributing to the increased morbidity and mortality of patients . This resistance makes *S. aureus* one of the leading causes of nosocomial infections (Nicholas *et al* .,2020).

In the late 1990s, a phenotypically and genotypically distinct highly virulent MRSA clone emerged as community acquired/associated MRSA

(CA-MRSA) causing skin and soft tissue infection, and severe haemorrhagic pneumonia in children and young adults without any predisposing conditions It usually carries smaller staphylococcal cassette chromosome mec (SCCmec) elements e.g. IV, V that do not contain other resistance genes and many clones spread independently worldwide. They produce Panton Valentine Leukocidin toxin (PVL) which is responsible for both skin infection and severe haemorrhagic necrotizing pneumonia through tissue necrosis and abscess formation. Many studies have shown significant association of *pvl* gene with CA-MRSA isolates compared with HA-MRSA isolates despite its controversial significance (Samaranayake, 2019).

1.8.Skin Infections

SSTIs are caused by microbial invasion of the layers of skin and underlying soft tissues. SSTIs have variable clinical presentations, etiology and severity. Infections may occur at sites where the skin barrier has been breached, such as a wound or surgical site infection. However, infections may also appear without apparent breach of the skin barrier, such as folliculitis occurring at hair follicles, or furuncles and carbuncles forming at pores. The involvement of deeper layers such as the dermis and/or subcutaneous tissues leads to cellulitis, with the involvement of yet deeper tissues, such as underlying muscle leading to fasciitis. SSTIs are common and can affect all age groups, however, certain conditions such as trauma, immunosuppression, certain skin conditions and drug use may predispose an individual to SSTIs. *S. aureus* is capable of causing infections at all mentioned sites in the skin and in some instances outbreaks of *S. aureus* SSTIs can occur. These are mainly seen in cases where there is close body contact, in groups such as prisoners, athletes and soldiers. CA-MRSA strains from the lineage USA300 are the most common cause of skin infections and 97% of all MRSA SSTI cases were caused by this lineage. Many *S. aureus*

SSTIs are self-limiting, however, complicated SSTIs can occur, and this often leads to the formation of a large abscess. Abscesses can form in the dermis, epidermis and subcutaneous tissue and function primarily to contain the pathogen, preventing the spread of infection to adjacent healthy tissue. Although abscess formation is part of the body's defense mechanism, they can cause significant pathology and lead to benign or malignant obstruction in tissues. They can rupture, releasing bacteria into the surrounding tissue and local inflammation at the site of the abscess can lead to painful swelling for the patient. An abscess begins as an acute localised inflammatory response to the invading bacteria. The abscess forms and becomes a collection of pus composed of live and necrotic neutrophils, tissue debris and live bacteria, encased in a fibrous capsule. Severe SSTIs may also lead to dermonecrosis of adjacent skin tissue (Otto *et al*., 2010, Kobayashi *et al*., 2015 , Lacey *et al*., 2016).

1.8.1.Skin and soft tissue infections (SSTI) Infection caused By MRSA carried *pvl* gene

Skin and soft tissue infections (SSTIs) represent severe forms of infectious diseases that involve deeper soft tissues, responsible for significant risk of relapse, prolonged hospitalization, and death . Although many hospitals have adopted specific measures to reduce the emergence of adverse events and increase the effectiveness of surgical procedures, SSTIs remain a challenging and costly problem .*S. aureus* is the most common cause of SSTIs worldwide. In addition, methicillin-resistant *S. aureus* (MRSA) is increasingly frequent in postoperative infection and responsible for a significant increase in the risk of death and hospital readmission compared to uninfected surgical patients (Sivori *et al*.,2022).

Skin and soft-tissue infections (SSTI) caused by Panton-Valentine leukocidin-producing *S. aureus* (PVL-SA) represent a significant burden for patients. The prevalence of CA-MRSA or susceptible *S. aureus* (MRSA/ MSSA)-producing PVL varies worldwide (Saeed *et al*., 2018).The hospitalizations and medical burden of SSTIs have increased nationwide since the emergence of community-acquired methicillin-resistant *S. aureus* (CA-MRSA). CA-MRSA strains are genetically different from the hospital-acquired MRSA (HA-MRSA) strains. They have been recognized as a novel pathogen group. The CA-MRSA strains are usually characterized by limited antibiotic resistance (except to β-lactams). They possess different exotoxin gene profiles (e.g., Panton-Valentine leucocidin (PVL) genes) and carry the type IV staphylococcal cassette chromosome (SCCmec IV). Presence of PVL has some major clinical therapeutic impacts, such as the association with deep-seated abscess, multiple lesions, recurring SSTI episodes, multiple antibiotic resistances and outbreaks of SSTIs(Yueh *et al*., 2022).

Panton Valentine leukocidin (PVL) is a virulence factor which is associated with *S. aureus* strains causing SSTI and severe forms of community acquired pneumonia (Gillet *et al*., 2021). PVL consists of two distinct components which form polymeric pores in the membranes of white blood cells which leads to cell death. This might explain the higher risk of complicated SSTI infection associated with PVL positive *S. aureus* strains(Senok *et al*., 2021). For SSTI, the presence of *pvl* has major clinical therapeutic implications as clinical and epidemiological data suggest that *pvl* is associated with deep-seated abscesses, multiple lesions, recurring SSTI episodes, multiple antimicrobial resistances and outbreaks of SSTI . Acquisition of *pvl* in *S. aureus* and MRSA is associated with community-acquired infections and is often found in returning travellers suffering from

SSTI. Systematic data on the prevalence of PVL in Central Europe are scarce but it is estimated to be very low (Sabrina *et al*., 2019).

The typical medical history shows recent first-time recurrent purulent skin abscesses of varying degrees of severity and without predilection site. Many cases show rapid dynamics of abscess development. It predominantly affects young, healthy patients with no pre-existing diseases. Differentially, PVL-SA can be distinguished, for example, from acne inversa, which tends to occur in the genital or axillary areas, or acne vulgaris, which has usually been present for a longer period of time (Saeed *et al*., 2018).

Infections of the skin are the most common form of *S. aureus* infection. This can manifest in different ways, including small benign boils, impetigo, cellulitis, folliculitis, and more severe, invasive soft-tissue infections (Tong *et al*., 2015).

2.1. Materials

2.1.1. Laboratory Instruments and Equipments

The main scientific apparatus, and technical instruments with disposable materials respectively, those were employed during the course of this study listed down in Tables (2-1) .

Table (2-1): Laboratory instruments and equipments that used in present study.

Equipments/Instruments	Company/ Origin
Autoclave	Fanem/brazil.
Centrifuge	Gemmy/Taiwan
Collection swab with media	Memmert/China
Deep freezer	Revco/USA
Disposable gloves	Great glove (Malaysia)
Disposable (Pteri Dish, Syringe, Plane and gel tu plastic cap)	Citro/China
Eppendorf tubes	Eppendorf/Germany
Gel electrophoresis apparatus	Cleaver/USA
Hood	Memmert (Germany)
Micropipettes	Dragon / USA

Parafilm	BDH/England
Refrigerator	Concord/ Lebnan
Standard wire loop (1µL)	Himedia (India)
Vortex mixer	Griffin/Germany

2.1.2 Culture Media

The main biological materials utilized in this study was listed in Table(2-2)

Table (2-2) The culture media used in the study

Medium	**Company/ Origin**	**USA**
Mannitol Salt agar(MSA)	Himedia (India)	Selective medium to disting bacteria *S.aureus*
Blood base agar(BA)	Himedia (India)	To check the susceptibility of bact to hemolysin production
MeReSa Agar Base	Himedia (India)	Is recommended for Isolation,selection, identification of MERSA
Nutrient agar(NA),Nutrient broth(NB) Brain heart infusion broth (B	Himedia (India) Himedia (India)	A general medium for isolate and cultivation

2.1.3. Chemical and Biological Materials

Chemicals and biological materials utilized in this study were listed in Table (2-3)

Table (2-3): Chemicals and Biological Materials utilized in this study

Materials	Company/ Origin
Blood	Blood bank
Plasma	Blood bank
Agarose	Bioneer (Korea)
Ethanol (70%), Ethanol (99%)	GCC /England
Glycerol	Merck England
DNA ladder (100bp)	Geneaid/Uk
ethidium bromide, Loading dye	Promega/USA

2.1.4. Kits for bacteriological studies

Kits for bacteriological studies utilized in this study were listed in Table (2-4)

Table(2-4): Bacteriological Kits

Kit	Components of the kit	Company/Origin
VITEK 2 system	card cassette, card filler mechanism, cassette loading processing mechanism, card sealer, bar code reader, cassette carousel and incubator, in addition to transmittance optics, waste processing, instruments control electronics and firm ware	BioMerieux /Franc

2.1.5 Antibiotic Powders

Antibiotic powder used in the present study in Table (2-5)

Table(2-5): Antibiotic powder

Antibiotic	Company/Origin	Concentration
Clindamicin	U.A.E /Julphar	500mg

2.1.6. DNA Polymerase Chain Reaction Materials

2.1.6.1 DNA Extraction Kits Content

DNA extraction kits content used in this study as in Table (2-6)

Table (2-6): DNA extraction kits and PCR Master Mix

The name of the kit	Ingredient	Company/Origin
Bacterial DNA extraction kit	RBC Lysis Buffer, FABG Buffer FATG Buffer, W1 Buffer, Wash Buffer (concentrated) Elution Buffer, FABG Column 2 ml Collection Tube	Favorgen/ Europe
G2 Green Master Mix	Reaction Buffer (pH 8.5), 400µM dATP, 400µ dGTP, 400µM dCTP, 400µM dTTP and 3mM MgCl2	Promega/ (USA)
Tranzol UP	Tranzol UP,RNA Dissolving Solution	iNtRON/korea
One step qPCR master mix,2x	One step qPCR master mix,2x,forward primer,10x,reverse primers,10x,RNA tamplate CXR reference Dye,30µM , MgCl2, RT	Promega/ (USA)

2.1.7 Primers

As in Table (2-7) the primers used in this study from (Bioneer, Korea)

Table (2-7): Primers used in present study

Primer	Primer Sequence (5'_3')	Product size (bp)	Reference
*mec*A-F	GTG GAA TTG GCC AATACA GG	1339	Weller,1999
*mec*A-R	TGA GTT CTG CAG TAC CGG AT		
SCC*mec* I-F	GCTTTAAAGAGTGTCGTTACAGG	613	Zhang *et al*., 2005
SCC*mec* I-R	GTTCTCTCATAGTATGACGTCC		
SCC*mec* II-F	CGTTGAAGATGATGAAGCG	398	Zhang *et al*., 2005
SCC*mec* II-R	CGAAATCAATGGTTAATGGACC		
SCC*mec* III-F	CCATATTGTGTACGATGCG	280	Zhang *et al*., 2005
SCC*mec* III-R	CCTTAGTTGTCGTAACAGATCG		
SCC*mec* IV-F	TTTGAATGCCCTCCATGAATAAA AT	450	Okuma *et al*.,2002
SCC*mec* IV-R	AGAAAAGATAGAAGTTCGAAAG A		
SCC*mec* V-F	GAACATTGTTACTTAAATGAGCG	325	Zhang *et al*., 2005
SCC*mec*	TGAAAGTTGTACCCTTGACACC		

V-R			
Luk-*pvl*- F	ATCATTAGGTAAAATGTCTGGAC ATGATCCA	433	Lina *et al*., 1999
Luk-*pvl*- R	GCATCAASTGTATTGGATAGCAA AAGC		

2.1.8. Laboratory Prepared Media

2.1.8.1.Sterilization

All culture media and most of the solutions used in this study were sterilized with a device autoclave at a temperature of 121 °C and a pressure of 15 pounds / inch for 15 minutes, while the glassware was sterilised in an electric oven at a temperature of 180 °C for an hour (MacFaddin, 2000,Greenwood and Irving, 2012).

2.1.8.2 Mannitol Salt Agar Medium:

Mannitol salt agar was prepared according to manufacturer by dissolving 111 gm mannitol salt agar base in 1000 ml D.W. The medium was autoclaved at 121ºC for 15 min.

2.1.8.3 Blood Agar Medium

Blood agar medium was prepared according to manufacturer by dissolving 40 gm blood agar base in 1000 ml D.W. The medium was autoclaved at 121ºC for 15 min, cold to 50 ºC and 5% of fresh human blood was added.

2.1.8.4 MeReSa Agar Base

MeReSa agar base was prepared according to manufacturer by dissolving 40 gm MeReSa agar base in 500 ml D.W. The medium was autoclaved at 121ºC for 15 min, cold to 50 ºC and aseptically add sterile rehydrated contents of 1 vial of MeReSa selective supplement (FD229) and Cefoxitin supplement (FD259) both in combination for more selectively , mix well and pour into sterile petri plates.

2.1.8.5 Nutrient Agar Medium

Nutrient agar medium was prepared according to the method suggested by the manufacturing company. It was used for the cultivation of the bacterial isolates when necessary.

2.1.8.6 Nutrient Broth

This medium was used to grow and preserve the bacterial isolates supplemented with 15% glycerol.

2.1.8.7 Brain Heart Infusion Broth

This medium was prepared by dissolving 37gm of medium in 100 ml distilled water. This medium used for the cultivation of *Staphylococcus aureus* (MacFaddin, 2000).

2.1.8.8 Maintenance Medium

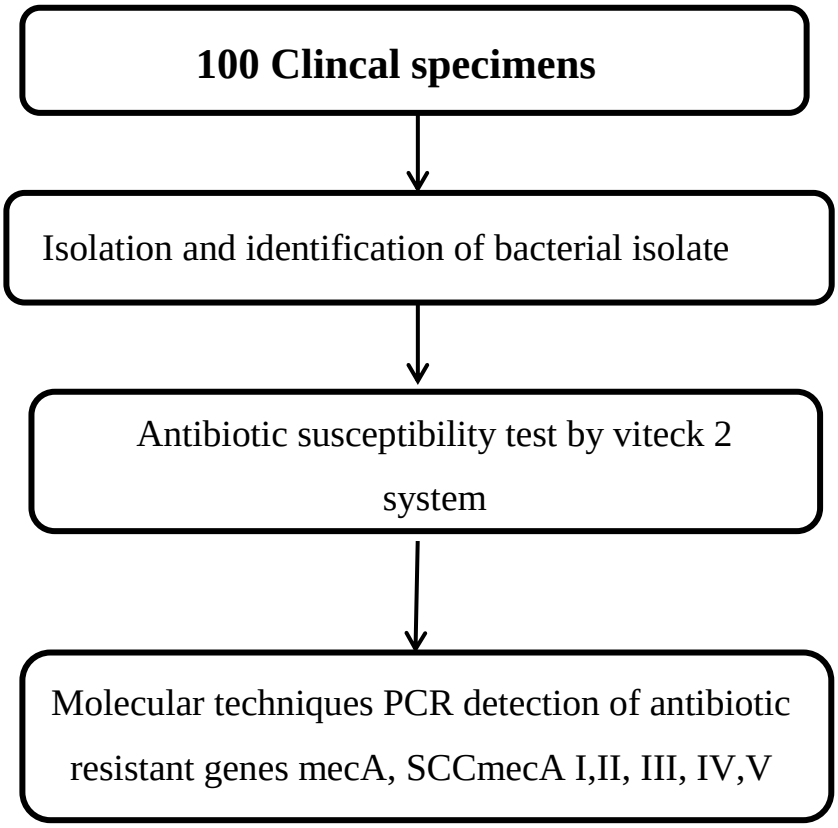
The medium consisted of brain heart infusion broth as a basal medium, supplemented with 15% glycerol, after autoclaving at 121oC for 15 minutes and cooling to 45oC. It was distributed in 5 ml sterile test tube. This medium was used to preserve the bacterial isolates at -20 C for long term storage (Collee *et al*., 1996).

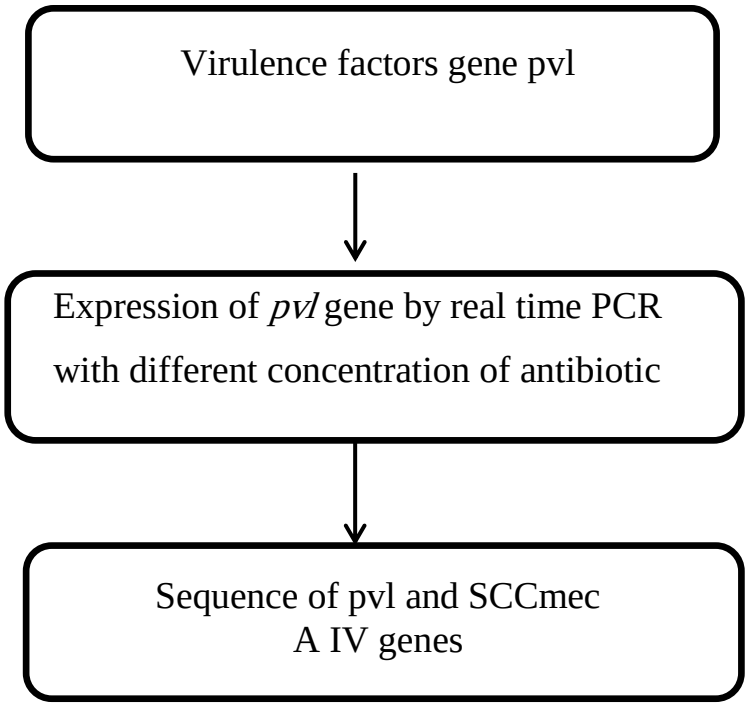
2.2 Patients

This study included 100 patients suffering from different skin infections (burn, wound, impetigo, boil acne, abuses, folliculitis, infected atopic dermatitis, secondary infection) who admitted to two health centers in Al-Hilla city, These patients were admitted to different hospital wards including Intensive Care Unit, Burns. In addition to swabs taken from private clinics during a period extending from July 2022 to the November 2022.

2.3 Collection of specimens:

The proper specimens collected from patients for bacteriological analysis were collected in proper way to avoid any possible contamination. Swabs for culture were placed in transport medium and then taken to the laboratory the swab was inoculated on blood agar, Mannitol salt agar , then incubated at 37C for 24hrs.





2.4 Laboratory diagnosis:

2.4.1. Colonial morphology and microscopic examination:

A single colony was taken from each primary positive culture and its identification was depended on the morphological properties (Colony size, shape, color and nature of pigments, translucency, edge, elevation and texture). Bacterial smear stained with Gram stain was used to check the morphological properties of bacterial cells.

2.5 Biochemical Tests

2.5.1. Catalase

This test was performed by transferring a 24-hour-old bacterial colony to a slide clear glass with drops of 3% hydrogen peroxide H_2O_2 solution. The immediate release of gas bubbles indicates a positive test as a result of the release of O_2 (Prescott *et al.*, 2002).

2.5.2 Oxidase

The assay was carried out by transferring 24-hour old colony onto a filter paper saturated with. reagent Tetramethyl - P-Phenylenediamine Dihydrochloride oxidase at a concentration of 1% using a wooden stick, the positive test is a purple color within 10 seconds (Prescott *et al.*, 2002).

2.5.3 Coagulase

The method of Benson, (2001) was followed with some modifications. Several colonies of bacterial growth were transferred with a loop to a tube containing 5 ml of brain heart infusion broth. The tube was covered to prevent evaporation and incubated at 37°C in the incubator overnight .Then the tube mixed and centrifuged, 0.5 ml of the supernatant withdrawn and mixed with 0.5 ml of human plasma, then incubated in the water bath at 37°C for several hours. If the plasma coagulates, the organism is coagulase-positive. Some coagulations occurred in 30 minutes or several hours later. Any degree of coagulation, from a loose clot suspended in plasma to a solid immovable clot, was considered to be a positive result, even if it takes 24 hours to occur.

2.5.4 Hemolysis test

Blood agar medium was streaked with a pure culture of bacterial isolate to be tested and incubated at 37Cº for 24-48 hrs. The appearance of a clear zone surrounding the colony is an indicator of β- hemolysis while the greenish zone is an indicator of α- hemolysis (Forbes *et al*., 2007).

2.6. Phenotyping Assays

2.6.1. Determination of Minimum Inhibitory Concentration

2.6.1.1 VITEK diagnostic system:

The automated VITEK system (bio-Merieux SA, France) including VITEK DensiCHEK standard, VITEK cassette and VITEK BCL card was

used for identification of Staphylococcus spp. according to the manufacturer's instructions. The card consisted of 64 plastic wells.

In this system, the phenotypic identification of bacterial species based on the findings of 43 colouri metric substrates. Substrates measure various metabolic activities such as acidification, alkalinization, enzyme hydrolysis, and growth in the presence of inhibitory substances (Pincus, 2006).

According to the manufacturer instruction:

1. Preparation of the implant: The isolates under study were developed on the nutrient agar medium and incubated at a degree of 37°C for 24 hours, after which stick is used to a sterile swab or applicator stick is used to transfer a sufficient number of colonies of pure culture and to suspend the microorganism in 3.0 mL of sterile saline in a clear glass test tube to make a suspension (only for diagnostic tubes).

2. The density was measured by a Densichek device whose density was in the range (0.5-0.63).

3. Put the test tube containing microorganism suspension in a special rack (cassette) and the identification card is placed in the neighboring slot while inserting the transfer tube into the corresponding suspension tube.

4. The filled cassette was transferred to avitk2 device to identification the bacteria through 64 biochemical test.

5. The results were obtained after an incubation period of approximately 18hrs. of placing the sample in the device.

2.7. Molecular Methods

2.7.1. Preparation of Molecular Materials

2.7.1.1. Preparation of 1X TBE Buffer

The preparation of 1X TBE buffer was performed by dilution of a concentrated 10X TBE buffer, this dilution was accomplished as 1:9 (v/v); 1 volume of 10X TBE: 9 volumes of distilled water. This solution was used to prepare agarose gel and as a transmission buffer in electrophoresis process. (Sambrook and Russel, 2001).

2.7.1.2. Preparation of Agarose Gel

The agarose gel was prepared according to the method of Sambrook and Rusell (2001) by adding 1-1.5gm agarose to 100ml of 1x TBE buffer. The solution was heated to boiling (using water bath) until all the gel particles dissolved. The solution was allowed to cool down within 50-60oC, and mixed with 0.5µg/ml ethidium bromide (Sambrook and Russel, 2001).

2.7.1.3. Ethedium Bromide

Prepared by dissolving 0.25 g from ethidium bromide in 50 ml D.W to get a final concentration of 0.5 mg/ ml (Sambrook and Russel ,2001).

2.8. Genomic Bacterial DNA Extraction

Chromosomal DNA was extracted from clinical isolates. One colony of each isolate cultured on solid medium was inoculated into 5 ml of BHI (Brain Heart Infusion) and grown overnight at 37ºC. From these isolate cultures, DNA was purified from bacterial cells using Genomic DNA Mini kit supplemented by the manufacturing company. Chromosomal DNAs obtained were used as templates for all PCR experiments. The PCR reactions were carried out in a Thermal Cycler. Before PCR assay, DNA profile were performed by using bacterial DNA and loading buffer without thermal cycling conditions, and according to the following steps :

1. transferred of appropriate number of bacterial cell(up to 1×10^9) to 1.5 ml micro centrifuge tube, centrifuged for 1minute at 14-18,000 g and the supernatant was discarded.
2. An aliquot of 200 µl of lysozyme buffer (20mg/ml lysozyme,20Mm Tris-Hcl,2Mm EDTA, 1% TritonX-100, Ph 8.0)and resuspend the pellet by Vortex or pipetting.
3. Incubate at room tempreture for 10 minutes. During incubation, the tube was inverted every 3 minutes.
4. Add 200 µl of FABG Buffer to the sample and vortex for 5 seconds.
5. Incubate for 10 minutes at 70C or until the sample lysate is clear. During incubation, the tube was inverted every 3 minutes.

6. Preheat required Elution Buffer in a 70C water bath.
7. Added 200 µl ethanol (96-100%) to the sample and vortex for 10sec. pipette the sample to mix well if there any precipitate formed .
8. Place a FABG Column to a Collection tube . transfer the sample mixture carefully to FABG Column . centrifuge at speed 14,000 rpm or 18,000g- for 1 minutes. Discard the collection tube and place the FABG Column to a new collection tube.
9. Add 400 µl of W1 Buffer to the FABG Column and centrifuge at speed 14,000 rpm or 18,000g- for 30sec. Discard the collection tube and place the FABG Column to a new collection tube.
10. Add 600 µl of Wash Buffer to the FABG Column and centrifuge at speed 14,000 rpm or 18,000g- for 30sec. Discard the flow through and place the FABG Column back to the collection tube .
11. Centrifuge at speed 14,000 rpm or 18,000g- for 3 minutes to dry the column.
12. Place the dry FABG Column to anew 1.5 ml micro centrifuge tube.
13. Add 100 µl of preheated elution buffer to the membrane center of FABG Column .
14. Incubate the FABG Column at 37c for 10 min. in incubator.
15. Centrifuge for 1 minte at full speed14,000 rpm or 18,000g to elute the DNA.
16. Store the DNA fragment at 4 C or -20 C.

2.8.1. Determination DNA Concentration and Purity

The extracted genomic DNA was checked by using Nanodrop spectrophotometer, which measured DNA concentration (mg/µL) and check the DNA purity by reading the absorbance at (260/280 nm) as following steps:

1. After opening up the nanodrop software, chosen the appropriate application (Nucleic acid, DNA).

2. A dry paper-wipe was taken and clean the measurement pedestals several times. Then carefully pipet 2µl of ddH2O onto the surface of the lower measurement pedestals for blank the system.

3.The sampling arm was lowered and clicking OK to initialized the Nanodrop, then cleaning off the pedestals and 1µl of extracted DNA carefully pipet onto the surface of the lower measurement pedestals ,then check the concentration and purity of extracted DNA.

2.8.2. Polymerase Chain Reaction Protocols

The DNA extract of *S. aureus* isolates were subjected to different genes by monoplex PCR. The protocols used depending on manufacturer's instruction. All PCR components were assembled in PCR tube and mixed on ice bag under sterile conditions as in Table (2-8).

Table (2-8): Protocols of PCR reaction mixture volumes

PCR reaction mixture	Promega protocol(final volume 25µl)
Go Taq G2 Master mix, 2X	12.5µl
Primer forward (10µM)	2.5 µl
Primer reverse (10µM)	2.5 µl
DNA template	5 µl
Nuclease free water	2.5 µl

2.8.3. Total PCR Thermo cycling Conditions

The PCR tubes were placed on the PCR machine and the right PCR cycling program parameters conditions were installed as in Table (2-9).

Table (2-9): Programs of PCR thermo cycling conditions

Gene	**Temperature (C)/ Time** **Cycling condition**					**cycle**
	Initial denaturation	**denaturation**	**annealing**	**Extension**	**Final Extension**	
*mec*A	95/5min	94/1min	58/1min	72/1min	72/10min	35
Luk-pvl	95/5 min	94/30 sec	55/30 sec	72/1	72/10 min	35
SCCmec I	95/3 min	95/30 sec	50/45 sec	72/ 45 sec	72/ 1 min	40
SCCmec II	95/3 min	95/30 sec	50/45 sec	72/ 45 sec	72/ 1 min	40
SCCmec III	95/3 min	95/30 sec	50/45 sec	72/ 45 sec	72/ 1 min	40
SCCmec IV	94/5 min	94/1 min	55/1 min	72/1.5 min	72/1.5 min	35
SCCmec V	95/3 min	95/30sec	50/45sec	72/ 45 sec	72/ 1 min	40

2.8.4. PCR Products Investigation

Successful PCR amplification was confirmed by agarose gel electrophoresis by visualization against UV light (Sambrook and Russell, 2001).

Agarose gel was prepared according to (2.7.1.2). Then the comb was fixed at one end of the tray for making wells used for loading DNA sample. The agarose was poured gently into the tray, and allowed to solidify at room temperature for 30 min . The comb was then removed gently from the tray. The tray was fixed in an electrophoresis amplicon was transferred into each well of agarose gel, and in one well we put the 5µl DNA ladder.

The electric current was allowed to pass at 70 volts for 50min. UV trans-illuminator was used 280 nm for the observation of DNA bands, and the gel was photographed using digital camera.

2.6.1.4 RNA Extraction

2.6.1.4.1. Total RNA Mini Kit

Total RNA were extracted from samples by using (Tranzol UP reagent kit) and done according to the manual procedure of company. The Genomic RNA extracted kit for bacterial cell component show in Table (2- 10).

Table (2-10): RNA extraction kit for bacterial cell contents

Component	**Volume Final**
Tranzol UP	100 ml
RNA Dissolving Solution	15 ml

Protocols:

1. Transfer suspension cell including culture dish to a Micro centrifuge tube ,then centrifuged at 8000xg for 2 minutes at 2-8C° , discard the supernatant10×7 cells
2. Pipetting up and down until no visible precipitates are present in the lysate.
3. Incubate at room temperature for 5 minutes

4. Add 200 µl of chloroform per ml Transzol Up , then shake the tube vigorously by hand for 30 seconds and incubate at room temperature for 3 minutes .

5. Centrifuge the sample at 10,000xg for 15 minutes at 2-8°C. The mixture separates into a lower pink organic phase, an interphase, and a colorless upper aqueous phase which contains the RNA. The volume of the aqueous upper phase is approximately 50 % volume of TransZol Up reagent

6. Transfer the colorless, upper phase containing the RNA to a fresh RNase - free tube , then add 500 µl of isopropanol for per ml TransZol Up used . Mix thoroughly by inverting tube and incubate at room temperature for 10 minutes .

7. Centrifuge the sample at 10,000xg for 10 minutes at 2-8°C , then discard the supernatant and the colloidal precipitate can be seen at the wall and the bottom of the tube .

8. Add 1 ml of 75 % ethanol (prepared with DEPC - treated water), vortexing vigorously (add at least 1 ml of 75 % ethanol for 1 ml of TransZol Up used).

9. Centrifuge the sample at 7,500xg for 5 minutes at 2-8°.

10. Discard the supernatant. Air - dry the RNA pellet (for about 5 minutes) .

11. RNA pellet is dissolved in 50-100 µl of dissolving solution .

12. Incubate at 55-60°C for 10 minute. For long-term storage, store the purified RNA at -70 °C.

2.6.1.4.2. qRT-PCR for Detection *PVL* gene Expression

RNA was used as a template for qRT-PCR to detect the expression *pvl* gene. A pair of specific primer and primer for housekeeping gene were used for the amplification of a fragment gene shown in Table(2-11).

Table (2-11): The Sequence of Primer that was Used in the Present Study for Detection *pvl* gene Expression

Genes		Primer sequence(5' ----- 3')	size	Reference
PVL	F	ACACACTATGGCAATAGTTATTT		Shariati *et al*.,2016
	R	AAAGCAATGCAATTGATGTA		
gyrB-1P1		AATTCTAATACGACTCACTATAGGGCTCCATCCACATCGGCAT	189	
gyrB-2P2		CGCAGGCGATTTTACCATTA		Chen *et al*.,2012
gyrB-MB		5′-CAL Fluor Red 610-cgcgaATCACAGCATTTGGTACAGGAtcgcg-BHQ-2-3′		

2.6.1.4.3.PCR Master Mix Preparation and Condition

PCR master mix *PVL* gene was prepared by using (Go Taq®One Step RT-qPCR) and this master mix done according to company instructions as following Table(2-12) and the condition (2-13).

Table (2-12): Contents of the qRT -PCR reaction mixture with their volumes

PCR master mix	final volume 20µl
One step qPCR master mix,2x	10µl
Forward primer,10x	2 µl
Reverse primers,10x	2 µl
RNA tamplate	3.7 µl
CXR reference Dye,30µM	0.3 µl
MgCl2	1.6 µl
RT Mix for 1-step	0.4 µl

Table (2-13):Thermo cycles condition of *pvl* gene

Stage	Temperature (C)/ Time Cycling condition	Cycle
Reverse transcription	37C/15 min.	1
RT inactivation/Hot start activation	95C/10min.	1
Denaturation	95C/10seconds	45
Annealing	53 C /30 seconds	45
Extension	72 C /30 seconds	45

CHAPTER THREE

RESULTS

AND

DISCUSSION

3.1. Isolation and Identification of *Staphylococcus. aureus*

In this investigation, a total of 100 clinical specimen were collected , 24 *S. aureus* isolates (24%) were recovered, of which 7 isolates (28%) from burns, 10 isolates (33%) from Impetigo, 5 (23%) from wound, 2(16%) from boil, 0(0.00%) for each of acne, abscess, secondary infection, infected atopic dermatitis, folliculitis (Table3-1) .

Table (3-1): The percentage of *Staphylococcus aureus* isolated from different skin infections

Type of specimen	No. of specimens	No. of *S. aureus* isolates	Percentage(%)
Burns	25	7	28%
Impetigo	30	10	33%
Wounds	21	5	23%
Boil	12	2	16%
Acne	2	0	0.00%
Abscess	2	0	0.00%
Secondary infection	2	0	0.00%
Infected atopic dermatitis	3	0	0.00%
Folliculitis	3	0	0.00%
Total number	100	24	100%

The prevalence of *S. aureus* according to the specimen source revealed that Impetigo recorded the highest number of isolates reached 10 isolates(33%), which was less than the results obtained by Alsterholm *et al*.,

(2010). who found that *S. aureus* was isolated in a rate of (68%), recent local studies have investigated the isolation of *S. aureus* from different clinical samples. Kareem *et al*. (2015) isolated *S. aureus* from Medical City in Baghdad and the highest source of isolation was from the skin (51.35%) then other samples, another previous local study by Al-Hasnawy, (2012). recorded that a high percentage (69.6%) of isolation rate were identified as *S. aureus* was detected in SSTIs.

Impetigo is a contagious, superficial bacterial infection of the skin that most frequently occurs in children 2–5 years of age, infection generally occurs in warm, humid conditions, and risk factors include poor hygiene, crowding, poverty, and scabies Baddour, (2019).Children experience the greatest disease burden, with incidence decreasing with increasing age Bowen *et al*., (2015). Diabetes and other underlying systemic conditions increase susceptibility (Hartman-Adams *et al*., 2014).

S. aureus, an opportunistic Gram-positive pathogen, is a common cause of SSTIs, ranging from the benign (e.g., impetigo and uncomplicated cellulitis) to the immediately life-threatening Esposito *et al*.,(2019); fonso *et al*., (2021). Among *S. aureus* strains, methicillin-resistant *S. aureus* (MRSA) isolates are of particular concern because they can also exhibit concomitant resistance to many commonly used antibiotics.

S. aureus expresses several factors that facilitate skin colonization and infection. These include various toxins and immune evasion factors, and a large array of protein and non protein factors that enable host colonization during infection (Cheung *et al*., 2021).

The skin is the largest organ of the body and with the underlying fat layers, fascia, and muscle, represents the majority of the tissue in the body. *S. aureus* is the most common causative pathogen of the SSTIs particularly in purulent

infections such as furuncles, carbuncles, cutaneous abscesses, and impetigo (Krishna and Miller, 2012; Tong *et al*.,2015)

In relation to the burn, the isolation rate (28%), which is adjacent to results done by Kareem *et al*., (2015), who found that the isolation rate of *S. aureus* from burn infections was (33.6%), on the other hand the result was more than the results obtained by Al-Hassnawi, (2012) and Salman, (2022), who found that (11%), (4.3%) of *S. aureus* isolates were responsible for burn infection respectively. The high percentage of *S. aureus* isolates in burn infections was due to the impairment of skin barrier in those patients and frequent scrubbing, debridement and manipulation of the burn site. Burns is a thermal injury of the skin, although electrical and chemical injuries may also result in burns . Thermal injury destroys the physical skin barrier that normally prevents invasion of microorganisms. During the first weeks following thermal trauma, the affected sites are colonized with bacteria . Following colonization, these organisms of the surface start to penetrate the burn escher to available extent and viable sub eschar tissues become invaded . It is now estimated that about 75% of the mortality following burn injuries is related to infections. The pattern of infection differs from hospital to hospital; the varied bacterial flora of infected wound may change considerably during the healing pertiod . When a hole is created on the skin, microorganisms, usually the opportunistic organisms, invade the holes and multiply leading to a delay in the healing process and finally infectious condition (Ibed and Hamim, 2014)

In the present study, it was found that out of 21, 5 (23%) isolates were recovered from wound specimens , this result was less than the results obtained by Kahsay *et al*., (2014) and Sapkota *et al*., (2019) who found that (39.7%), (78.95%)of *S. aureus* isolates were responsible of wounds infection respectively. Wound infection on the skin surface easily in colonization by a wide variety of organisms. Some studies suggest several different kinds of

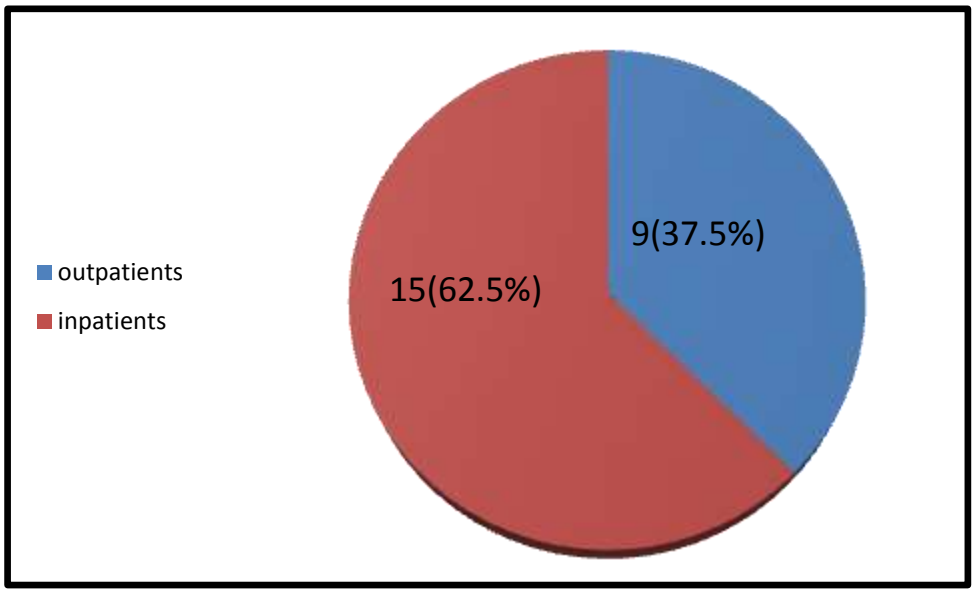
bacteria isolated from patients living in areas with different geographical. Microorganisms that cause inflammation is a group of pyogenic bacteria. Skin infections are most often caused by bacteria. Gram positive bacteria are the most common causes of skin infection is hemolytic Streptococcus and *Staphylococcus aureus* (Ekawati *et al*., 2020).

In relation to the isolation rate of *S. aureus* from boils infection, it was found that the lowest number of isolates 2(16%) was isolated from boils infection, this result was disagreement with those obtained from Hasan and Ismael, (2018) who found that (38.67% %), *S. aureus* isolates were isolated from boils infection, This low percentage was due to numbers of samples, different climatic conditions, personal hygiene, severity of infection.

Boils are deep inflammation of hair follicles, leading to accumulation of pus and dead tissues. Boils appear as red swollen nodules in many parts of the body caused by *S. aureus* El-Gilany *et al*., (2009) and especially Methicillin - resistant *S. aureus* (MRSA), the most serious pathogen of the skin and soft tissue. This disease is often recurrent and may spread among family members, where some healthy people are considered as carriers of *S. aureus* Ibler and Kromann ,(2014) .No isolates were recovered from the other clinical specimens (Table 3-1).

3.2. Distribution of the *S. aureus* according to the origin of isolates

In the current study out of 24 *S. aureus* isolates 9 isolates 37.5% recovered from outpatients and 15 isolate 62.5% from inpatients Figure(3-1).

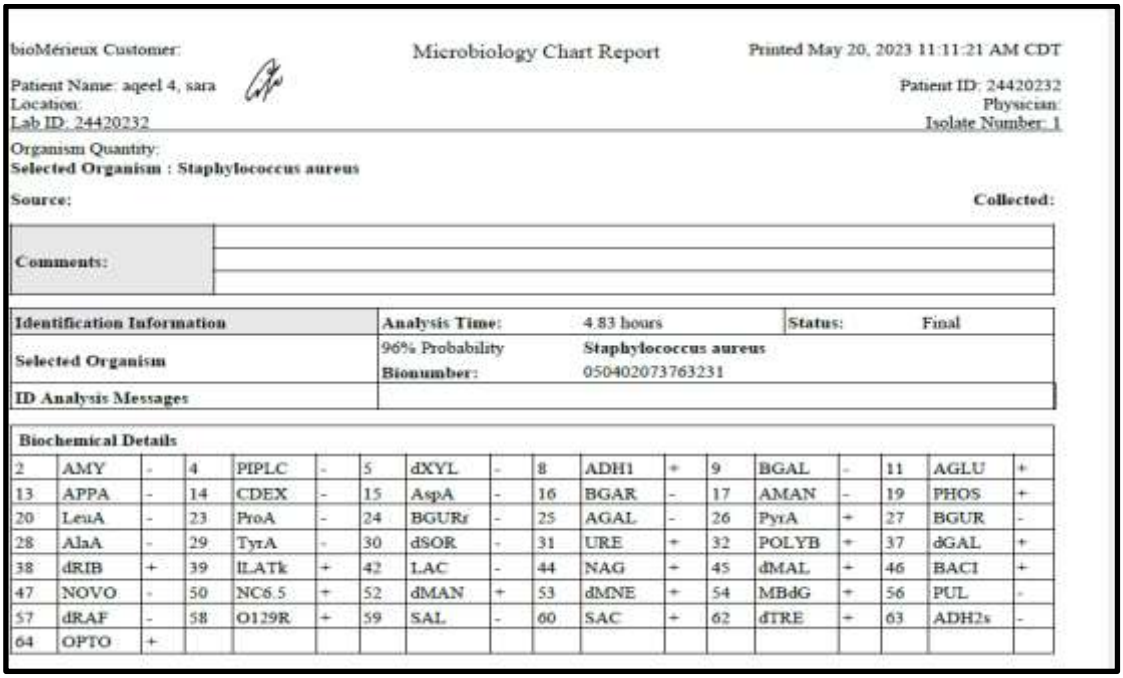


Figure(3-1) Distribution of the *S.aureus* according to the origin of isolates

These results were in agreement with the result of Abdel-Maksoud *et al*., (2016) who found the incidence of *S. aureus* was higher among medical staff (71%) than that found in the community (29%), but disagreement with result obtained by Mandour *et al*., (2023) who found that the distribution of *S. aureus* was (48.4%) and (51.6%) among medical staff and community people respectively

3.3. Identification of *S. aureus* by VITEK system

VITEK 2 compact systems was used for precise and accurate identification of isolates related to the species of *S. aureus* isolates which was previously identified by conventional biochemical tests (Figure 3-2).



bioMérieux Customer: Microbiology Chart Report Printed May 20, 2023 11:11:21 AM CDT

Patient Name: aqeel 4, sara Patient ID: 24420232
Location: Physician:
Lab ID: 24420232 Isolate Number: 1

Organism Quantity:
Selected Organism : Staphylococcus aureus

Source: Collected:

Comments:	

Identification Information	Analysis Time:	4.83 hours	Status:	Final
Selected Organism	96% Probability	Staphylococcus aureus		
	Bionumber:	050402073763231		
ID Analysis Messages				

Biochemical Details

2	AMY	-	4	PIPLC	-	5	dXYL	-	8	ADH1	+	9	BGAL	-	11	AGLU	+
13	APPA	-	14	CDEX	-	15	AspA	-	16	BGAR	-	17	AMAN	-	19	PHOS	+
20	LeuA	-	23	ProA	-	24	BGURr	-	25	AGAL	-	26	PyrA	+	27	BGUR	-
28	AlaA	-	29	TyrA	-	30	dSOR	-	31	URE	+	32	POLYB	+	37	dGAL	+
38	dRIB	+	39	ILATk	+	42	LAC	-	44	NAG	+	45	dMAL	+	46	BACI	+
47	NOVO	-	50	NC6.5	+	52	dMAN	+	53	dMNE	+	54	MBdG	+	56	PUL	-
57	dRAF	-	58	O129R	+	59	SAL	-	60	SAC	+	62	dTRE	+	63	ADH2s	-
64	OPTO	+															

Figure (3-2): VITEK2 compact system for identification of *S. aureus.*

3.4. Antibiotic susceptibility profile of *S. aureus*

3.4.1.Phenotypic detection of MRSA

The MRSA isolates were identified according to their phenotypic characteristics (resistance to cefoxtine screen and oxacillin) by VITEK system, The results shown in Table(3-2) indicate the MRSA was fully resistance to oxacillin, cefoxtin.

Table(3-2): Numbers and percentages of Methicillin resistant *S. aureus* isolates using cefoxitin and oxacillin screening test

Susceptibility cefoxitin and oxacillin	No. of isolates	%
Resistance	24	100%
Sensitive	0	0.00%
Total	24	100%

The previous studies showed the same resistance results at 100% according to Al-Azawi , (2013), the results of the current study also close to the results obtained by Aziz and Hassan, (2019) who found that 99(93.4%) isolats were determined as MRSA by oxacillin antibiotic and 93(87.7%) were determined as MRSA by cefoxitin antibiotic.

Oxacillin can be considered a representative antibiotic for susceptibility test to all β-lactams (Loftus *et al*., 2017). Cefoxitin also used for MRSA detection, because it is a powerful influence of mecA gene that shows to be less affected than oxacillin by isolates have the ability to produce penicillinase excessively (Wu *et al*., 2016).

Antibiotic susceptibility tests were studied to 21 antibiotics using Viteck system, and Table(3-3) showed percentages of resistant and sensitive bacterial isolates to antibiotics used.

Table (3-3) Percentage of resistance and sensitivity to antibiotics

Antibitic	Resistant	Sensitive	intermediate
Benzylpenicillin	100%	0.00%	0.00%
Amoxicillin/Clavulanic Acid	100%	0.00%	0.00%
Ceftriaxone	100%	0.00%	0.00%
Meropenem	100%	0.00%	0.00%
Amikacin	30%	/ *	0.00%
Gentamicin	35%	65%	0.00%
Ciprofloxacin	15%	85%	0.00%
Levofloxacin	15%	80%	/**
Moxifloxacin	15%	80%	5%
Erythromycin	70%	25%	5%

Clindamicin	65%	35%	0.00%
Lincomycin	65%	10%	/ ***
Linezolid	0.00%	100%	0.00%
Teicoplanin	0.00%	100%	0.00%
Vancomycin	0.00%	95%	/ ****
Doxycycline	75%	20%	/ *****
Tetracycline	80%	20%	0.00%
Tigecycline	0.00%	100%	0.00
Fusidic Acid	60%	40%	0.00%
Rifampicin	20%	80%	0.00%
Trimethoprim/ Sulfamethoxazole	0.00%	100%	0.00%

*err 70%, ** err 5%, ***err 25% , **** err 5%, *****err 5%

The results revealed that all bacterial isolates showed high resistance (100%) to Benzylpenicillin and this result was very close to what was found in a study Akanbi *et al.*, (2017) as bacteria were resistant to penicillin by 96.7% and It is also slightly higher than the percentage obtained in the study Bastidas *et al.*, (2019) which was 93.5%, the reason for this increased resistance may be due to its irregular and continuous use, which enhances the resistance of the stimulating bacteria, as well as the production of MRSA bacteria the hydrolyzed penicillinase enzyme.The isolates expressed high resistance against the third generation of cephalosporins represented by 100% for Ceftriaxone.

MRSA isolates showed the high rates of resistance toward Meropenem 100% , this result is close with the findings of the study Mahmood *et al*., (2019) showed highly resistance of MRSA against Meropenem (93%). our results were contrary to what the researchers found in the study Al-Hassnawi

et al., (2012) (11.3%) , Abd-Alamer and Al-Khozai, (2016) 20%, and Hasan and Ismael, (2018) which reported did not find any isolates was resistance to Meropenem (0%).

The resistance to the antibiotic Gentamicin was 35%،and this result was more than the result obtained by Al-Ubaidy, (2006) who found that isolates had resistance rate (19%).

For the antibiotic Ciprofloxacin the resistance rate was 15%, where the results we obtained differ from what was found by the researcher Mohamed *et al*., (2020), where the resistance to the antibiotics Ciprofloxacin and Norfloxacin is 25% and 0%, respectively. It is also close to the study Salman, (2022), and its percentage was 12% .However the results we obtained are less than what the researcher Kwoji *et al.*, (2017)concluded, where the resistance rate to the antibiotics Ciprofloxacin and Norfloxacin, is 44% and 20%, respectively. As for the antibiotic Levofloxacin, the results we obtained are 15%, and this is less to the percentage obtained in the study Preeja *et al*., (2021) where the antibiotic resistance rate is 41.2%.

As for the resistance to the antibiotic Moxifloxacin, where the results we obtained are 15%, and this percentage is lower than the percentage obtained in the study Alseqely *et al.*, (2021) where the rate of resistance to the antibiotic is 64%.

As for macrolides antibiotics, the rate of resistance to Erythromicin, was 75% as it is very close to what the researchers found in the study obtained by Al-Khudheiri, (2008) and Schmitz et al., (2000) 81.5%, 93%, respectively, who isolated erythromycin resistance *S. aureus* from hospital infections in Najaf and European countries. But more than the result obtained by Hashemzadeh *et al*., (2021) Where bacteria resisted this antibacterial by 58%.

As for the antibiotic Clindamycin, it recorded a resistance rate of 65%, and this result is consistent with what was reached Khodabandeh *et al*., (2019) where the percentage was 68.8% but lower of study Assefa, (2022) where the percentage was 19.8%.

For the antibiotic Lincomycin the resistance rate was 65%, where the results we obtained differ from what was found by the researcher Yıldız *et al*., (2014) where the resistance to the antibiotics Lincomycin (42.3%).

MRSA isolates showed the lowest rates of resistance toward Linezolid with sensitivity rates reach up to 100% this result was in accordance with result obtained by Lee *et al*., (2020) who found high sensitivity rate (100%).

A degree of resistance toward teicoplanin 0.00% and this differs somewhat from the percentage obtained in the study El-Baghdady *et al*., (2020)where the antibiotic resistance rate is 71.4%.

MRSA isolates showed the lowest rates of resistance toward Vancomycin with sensitivity rates reach up to 95% this result was in accordance with result obtained by other researchers, in Iraq Al-Ghazi, (1998), Al-Khudhieri, (2008) who found high sensitivity rate (100%) to vancomycin in Nassirya and Najaf respectively. this disagree with local studies Al-azawi, (2014) who found that the resistant rate toward vancomycin was(37.5%).

As for the Tetracyclines antibiotics represented by Doxycycline and Tetracycline The isolates were resistant to Doxycycline by 75% and Tetracycline by 80% the percentage of resistance to Doxycycline was lower than what was found in a study Fri et al., (2020) ,Gandhi et al., (2020), where the resistance rate was 30% the percentage of resistance to tetracycline was very close to what was found by the researcher Al-Hasnawy et al., (2019) as the percentage was 80.9% S. aureus resists Tetracyclines through two

mechanisms: active influx by genes encoded by plasmid, and ribosomal protection by transposon or chromosomally encoded genes(Graber, 2021).

MRSA isolates showed the lowest rates of resistance toward Tigecycline with sensitivity rates reach up to 100% this result was in accordance with result obtained by other researchers Tsouklidis *et al*., (2020) who found high sensitivity rate (100%) to Tigecycline

As for the antibiotic Fusidic Acid, the isolates showed a resistance rate of 60%, and thus it was higher than the percentage reached by researchers in the study Yıldız *et al.,* (2014) , where it reached 8.1%.

As for the antibiotic Rifampin, the isolates showed a resistance rate of 20%, and thus it was higher than The percentage reached by researchers in the study Al-Hasnawy *et al.*, (2019) in Iraq, where it reached 9.5%, and on the contrary in the study Bai *et al.*, (2021), where the rate of sensitivity was 25% to antibiotic.

And finally MRSA isolates showed the lowest rates of resistance toward Trimethoprim/Sulfamethoxazole with sensitivity rates reach up to 100 .Where the results we obtained are similar to what was found in the study Kwoji *et al*., (2017), where the resistance to the antibiotics (TMP) and (SXT) is 11%.

3.5. Molecular Confirmation of MRSA by *mec*A Gene Using PCR

*mec*A was analyzed by PCR for all *S. aureus* isolates (24). Among all isolated the results were showed (19) isolates is MRSA (79.16%), (5) isolates was found MSSA (20.83%) as show in Table (3-4).

Table (3-4):- Percentage of *Mec*A gene in MRSA isolates

No. of isolate	***Mec*A gene**
1	-ve
2	-ve
3	+ve
4	+ve
5	+ve
6	+ve
7	+ve
8	+ve
9	+ve
10	+ve
11	+ve
12	-ve
13	+ve
14	+ve
15	+ve
16	+ve
17	-ve
18	+ve
19	+ve

20	+ve
21	-ve
22	+ve
23	+ve
24	+ve

Figure (3-3) illustrates positive results in the Amplification plot. Although more than one isolate was phenotypically resistant to cefoxitin, it did not show amplification of *mec*A that might carry another gene, such as *mec*C, instead of *mec*A Deplano *et al*., (2014). In contrast to this result, *mec*A was reported among cefoxitin- sensitive strains Mottola *et al*., (2016). This variation between the phenotypic and genotypic methods may be related to culture settings, temperature, configuration of culture medium, size of inocula, time of incubation, and manual skill of medical staff (Kavitha *et al*., 2017).

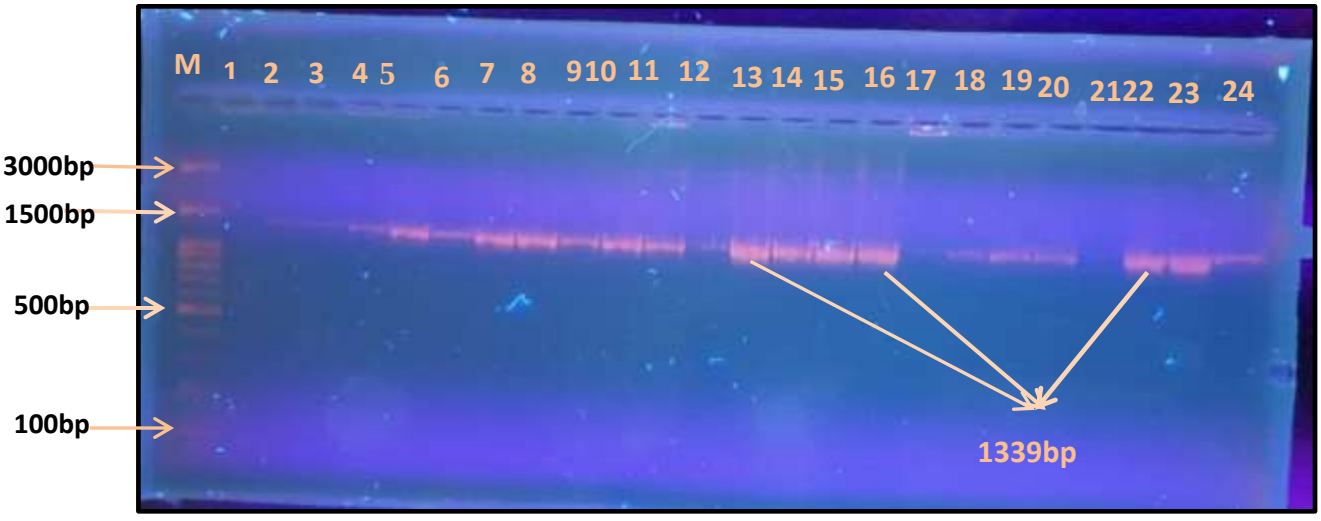


Figure (3-3): Agarose gel electrophoresis (1% agarose, 50V/cm, for 1hr) for *mec*A gene (amplified size 1339bp) compared with (100 bp) DNA ladder. lanes (3-11), (13-16),(18,19,20,22,23,24) gave positive *mec*A gene, while lane1,2, 12,17,21represent negative result.

The current study were close with result obtained by Ibed and Hamim, (2014) and Kali *et al*., (2014) who demonstrated *mec*A prevalence in MRSA isolates (75.5%) (90.1%) respectively, while other studies found low percentage of *mec* A gene such as Abulreesh *et al*., (2017), Ibrahim *et al*., (2017) and Sherfi and Badri, (2018) reported the percentage were 44%, 55.5% and 42% respectively.

Interestingly, *mec*A gene encodes PBP2a leading to confer resistance against all β-lactam antibiotics even though the semi-synthetic penicillins. This type of resistance is due to the low affinity of PBP2a towards β-lactam antibiotics, leading to normal biosynthesis of cell wall when the native PBPs are inactivated. The *mec*A gene is located within a large mobile genetic element known as the staphylococcal chromosomal cassette mec (SCCmec). Various SCCmec types are likely found in both community associated and hospital-acquired MRSA (Otter and French, 2010; Peacock and Paterson, 2015).

The SCCmec carry *mec*A (pbp2a) gene which encodes the alternative supplementary target protein PBP2a with low affinity for βlactams including cephalosporins, carbapenems and β-lactamase inhibitor combinations. Therefore, it is capable of substituting the biosynthetic functions of the normal PBPs even in the presence of the β-lactams, thereby preventing cell lysis. PBP2a appears to be a rather poorly active enzyme comparing to other native PBPs that synthesize highly cross-linked peptidoglycan. In addition SCC can contain genes of resistance for non-βlactam agents causing multi-antibiotics resistant strains that were found to be hospital associated (Iyer *et al*., 2014; Srisuknimit, 2019).

3.6.Molecular characterization of CA-MRSA and HA-MRSA by SCCmecA IV and *pvl* gene

3.6.1. Identification of Staphylococcal Cassette Chromosome (SCCmec) Types using PCR

Among all MRSA isolates(19), 13(68.42%) isolates belonged to SCCmec type IV and no one isolate belonged to SCCmec type V (0.0%) as show in Table (3-5) Figure(3-4), SCCmec type IV is the smallest structural type among the SCCmec types and believed to be the most mobile version that is associated with CA-MRSA infections Milheiriço *et al*., (2007). These isolates identified as CA-MRSA when carrying the SCCmec types IV or V. 12 isolates (63.15%) belonged to SCCmec type II and identified as HA-MRSA. Figure (3-5). Both SCCmec type I and SCCmec type III were not detected among isolates.

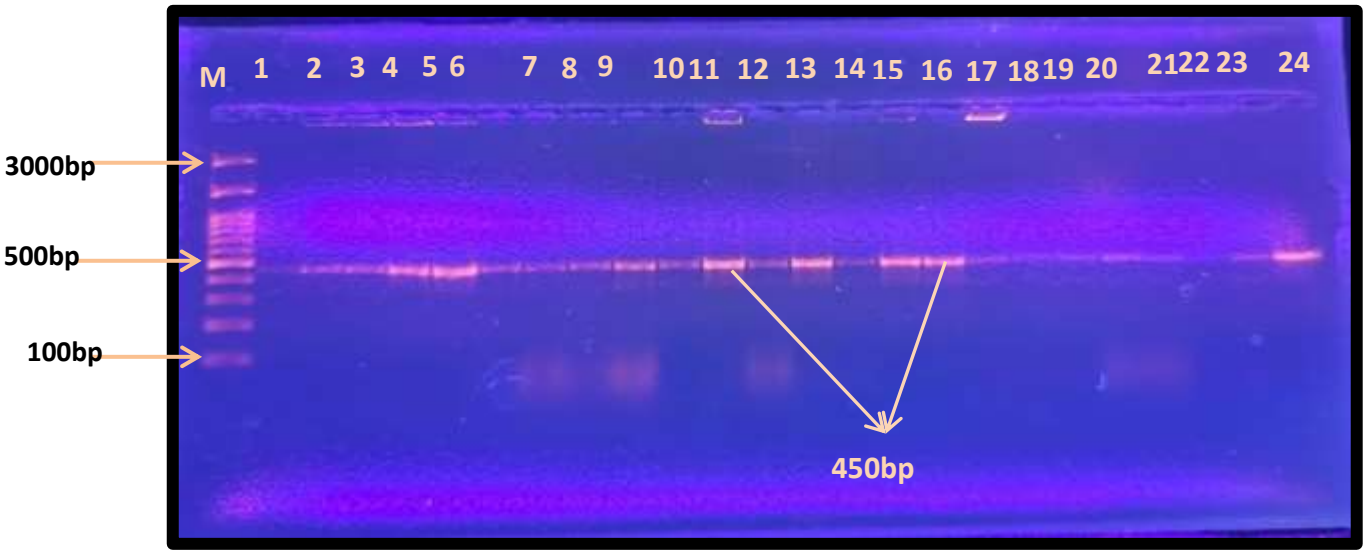
The results we obtained are less than the result obtained by Al-Hassnawi, (2012) and Hadyeh *et al*., (2019) which finding that the percentage of SCCmec type IV (95%), (86.6%) respectively, In a recent study conducted in Gaza by Al Laham and his colleagues, similar results were obtained with a high frequency of the SCCmec type IV, which accounted for (79.3%) of MRSA isolates and Abd El Hamid and Bendary, (2015) detected SCCmec type IV in 48% MRSA isolates in their study that was conducted in Egypt, in Iran. Yu *et al*.,(2015) found SCCmec type IV as the most frequent cassette amongst 128 *S. aureus* isolates . In addition to SCCmec type IV, this study also found the percentage of SCCmec type II in MRSA isolates was 12 isolates (63.15%), the result of this study have high frequency of the SCCmec type II in contrast to the result obtained by Humaryanto *et al*., (2020) who found that the percentage of SCCmec type II in MRSA isolate was (7.14%).

The SCCmec elements allow to classify the MRSA strains into HA and CA Naorem *et al*., (2020) A change was observed in the distribution of the SCCmec elements in our hospital. Type II decreased, while type IV was detected more frequently in recent years. In some regions of the world, there has been a decrease in cassettes I, II and III, historically associated with HA infections, and an increase in cassettes IV and V (associated with CA infections). This exchange has been widely described in the Iran Tajik *et al* .,(2019) and South Africa Singh-Moodley *et al* .,(2019). Currently, the detection of SCCmec elements and their classification is not sufficient to determine the best treatment, since the search for virulence factors is also important.

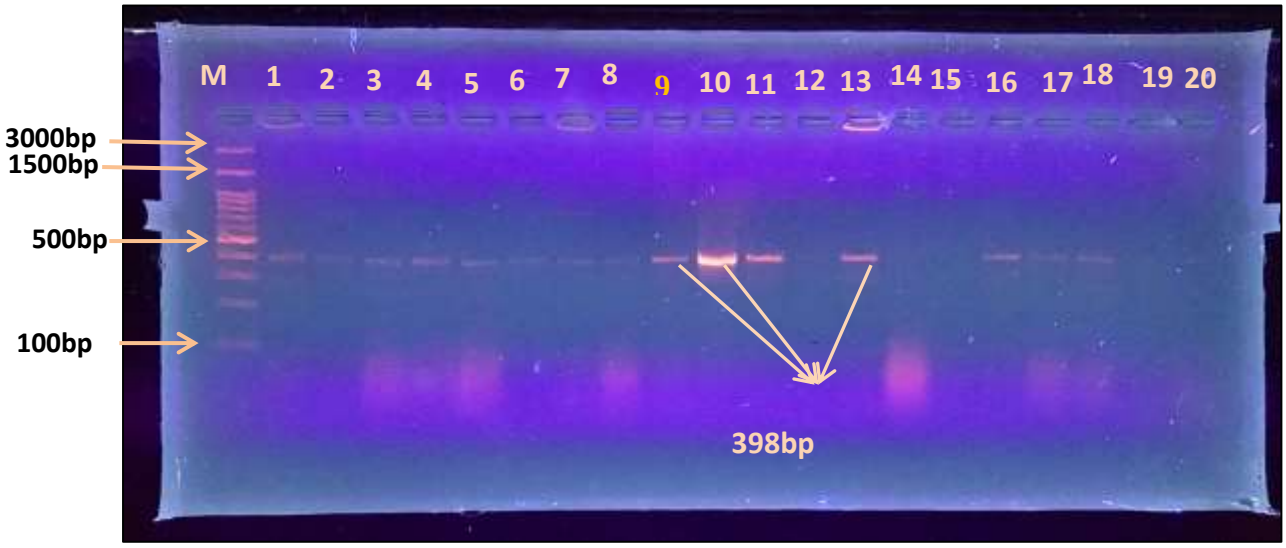
Table (3-5):- Percentage of SCCmec type II and SCCmec type IV in MRSA isolates.

No. of isolate	***Mec*A**	**SCCmec type II**	**SCCmec type IV**
1	-ve	-ve	-ve
2	-ve	-ve	-ve
3	+ve	+ve	+ve
4	+ve	+ve	+ve
5	+ve	+ve	+ve
6	+ve	+ve	+ve
7	+ve	+ve	+ve
8	+ve	+ve	+ve
9	+ve	+ve	+ve

10	+ve	+ve	+ve
11	+ve	+ve	-ve
12	-ve	+ve	-ve
13	+ve	+ve	+ve
14	+ve	+ve	+ve
15	+ve	+ve	-ve
16	+ve	-ve	+ve
17	-ve	+ve	+ve
18	+ve	-ve	+ve
19	+ve	-ve	-ve
20	+ve	-ve	-ve
21	-ve	-ve	-ve
22	+ve	-ve	-ve
23	+ve	-ve	-ve
24	+ve	-ve	+ve



Figure(3-4):- 1 % Agarose gel electrophoresis (50V/cm, for 1hr) for SCCmec type IV gene (450bp) compared with (100 bp) DNA ladder. lanes (2-13), (15,16,17,20,23,24) gave positive result, lane (1,14,18,19,21,22)represent negative result.



Figure(3-5):- 1 % Agarose gel electrophoresis (50V/cm, for 1hr) for SCCmec type II (398bp) compared with (100 bp) DNA ladder. Lanes(1-11), (13,16,17,18,) gave positive result, lane (12,14,15,19.20)represent negative result.

3.6.2.Detection of Extracellular Proteins Panton-Valentine Leukicidin (*pvl*) gene

Polymerase chain reaction was used to determined the bi-component leukocidin *pvl* gene in MRSA isolates was 12(63.15%) as show in Table (3-6), (Figure 3-6).

Table (3-6):- Percentage of *PVL* gene in MRSA isolates

No. of isolate	**MecA**	**Luk-pvl**
1	-ve	+ve
2	-ve	-ve
3	+ve	-ve
4	+ve	+ve
5	+ve	+ve
6	+ve	+ve
7	+ve	+ve
8	+ve	+ve
9	+ve	+ve
10	+ve	+ve
11	+ve	-ve
12	-ve	+ve
13	+ve	+ve
14	+ve	-ve

15	+ve	+ve
16	+ve	+ve
17	-ve	+ve
18	+ve	+ve
19	+ve	-ve
20	+ve	-ve
21	-ve	-ve
22	+ve	-ve
23	+ve	-ve
24	+ve	+ve

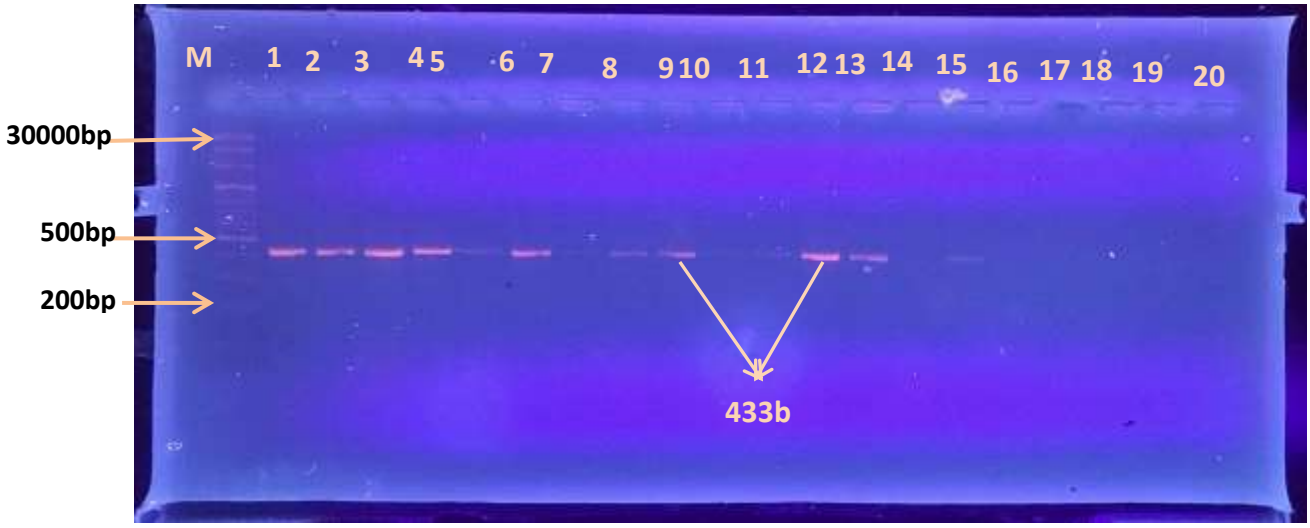


Figure (3-6):- Agarose gel electrophoresis (1% agarose, 50V/cm, for 1hr) for *pvl* gene (amplified size 433bp) compared with (100 bp) DNA ladder.lanes(1,2,,3,4, 6, 8,9,12, 13,15) represent positive for Pvl gene and lane (5,7,10,11,14,16,17,18,19,20) represent negative for *pvl* gene.

The current study reported that MRSA harbored *pvl* gene were detected in 12(63.15%) of MRSA isolates. In contrast Samsudin *et al*., (2021) who showed that MRSA harbored *pvl* gene (4.4%) of total MRSA isolates. This percentage is also higher than that found by Mandour *et al*., (2023) who showed that MRSA harbored *pvl* gene (30.52%) of total MRSA isolates, and close the result obtained by Darboe *et al*.,(2023) the percentage of *pvl* gene was (61.4%).

Leukocidin is a pore-forming cytotoxin, the presence of *luk*- pvl is associated with increased virulence of certain strains Pany *et al*., (2022).

3.7.*pvl* Expression by Quantitative -Real Time PCR

In this study q-Real Time-polymerase chain reaction (qRT-PCR) was used to determine the effect of antibiotic (Clindamycin) on the expression of *PVL* gene. Table(3-4) showed the effect of clindamycin in (4 isolate) of MRSA. this table showed the activity of different concentrations (10 µg/ml ,5 µg/ml, 1 µg/ml, 0.5 µg/ml) of clindamycin on the expression of PVL gene.

Table(3-7): Effect of different concentrations of clindamycin on the level of *pvl* gene expression.

control	No. of isolate	House keeping gene gyrB average	*pvl* Average Ct	dCt	ddct	Fold change	Average fold change
Control (without antibiotic)	1	20.35	23.07	2.72	-2.148	4.431	1.00
	2	25.66	34.12	8.46	3.593	0.083	
	3	29	34.49	5.49	0.623	0.650	

	4	29.55	32.35	2.8	-2.068	4.192	
10 µg/ml	1	20.11	26.78	6.67	1.803	0.287	0.24
	2	24.22	31.63	7.41	2.543	0.172	
	3	19.21	25.27	6.06	1.193	0.438	
	4	14.58	22.12	7.54	2.673	0.157	
5 µg/ml	1	31	37.8	6.8	1.933	0.262	0.35
	2	26	32.43	6.43	1.563	0.339	
	3	15	20.99	5.99	1.123	0.459	
	4	23.14	29.5	6.36	1.493	0.355	
1 µg/ml	1	25.12	30.49	5.37	0.502	0.706	0.73
	2	18.23	23.66	5.43	0.562	0.677	
	3	17.93	23.66	5.73	0.862	0.550	
	4	16.22	21.01	4.79	-0.077	1.055	
0.5 µg/ml	1	15.01	19.72	4.71	-0.158	1.115	0.84
	2	31	36.22	5.22	0.352	0.783	
	3	15	20	5	0.133	0.912	
	4	16.46	22.01	5.55	0.683	0.623	

RNA was extracted to study the gene expression of *pvl* gene using real time PCR(Relative gene expression, in this method the level of expression *pvl* gene in tested samples as well as control samples normalize with house keeping gene for test sample as show in figure (3-7).

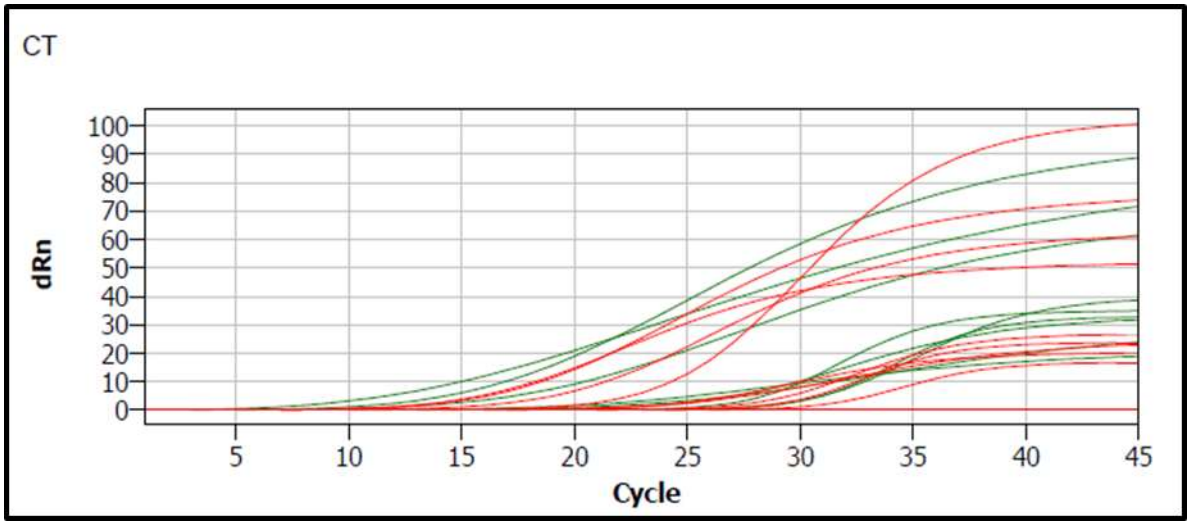


Figure (3-7): The real time amplification plots of *pvl* gene expression in MRSA (red target gene) , green house keeping gene (gyrB).

The current study showed that the different concentrations (10 µg/ml ,5 µg/ml, 1 µg/ml, 0.5 µg/ml) of clindamycin reduced the level of gene expression of PVL(lukS-pv and lukF-pv) genes (0.24, 0.35, 0.73, 0.84) respectively compared to the control group (1.000) that was not treated with antibiotic , it mean a concentration-dependent decrease, Figure (3-8).

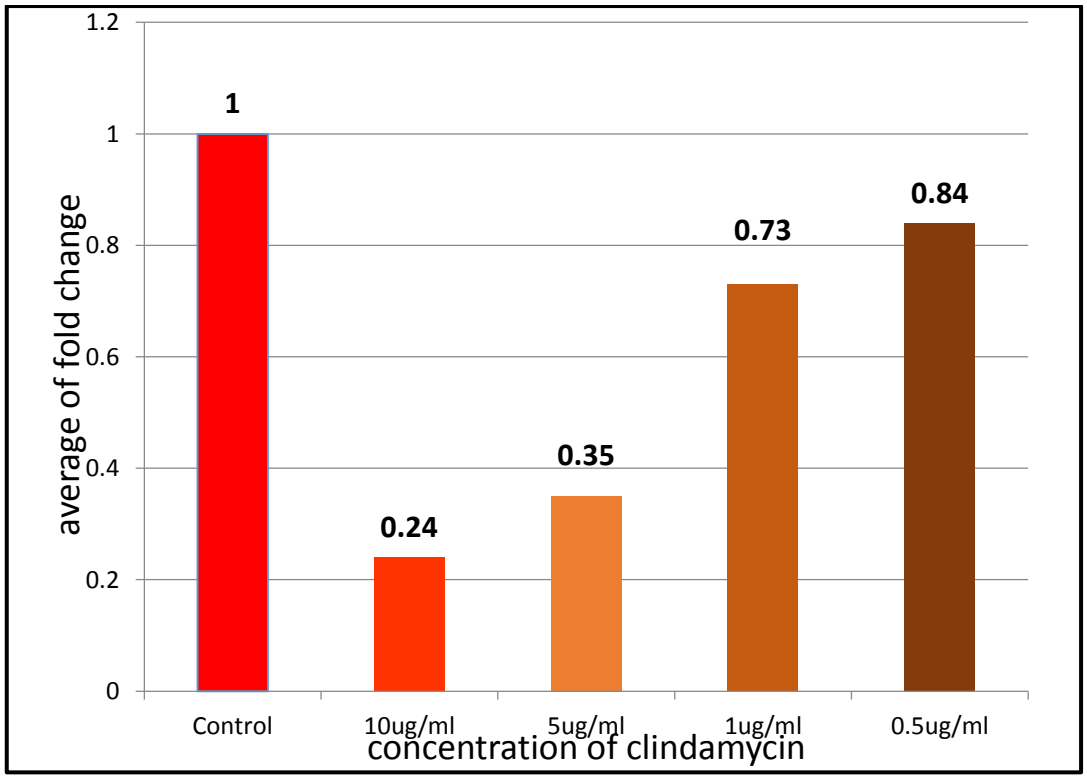


Figure (3-8): The level of *pvl* expression in regard to different concentration of clindamycin

A study proposed by Hodille *et al*., (2018) found that the sub-MICs of clindamycin significantly decreased PVL release compared to that of the growth control (without antibiotics) . Other study by Otto *et al*., (2013) found that Clindamycin, tested on the four susceptible strains, decrease in the *pvl* mRNA level from 2.20-fold to 11.76-fold depending on the strain, study of Yi, (2011). Show that the Clindamycin does not show a significant increase in PVL expression at all phases.

Several case reports of necrotizing pneumonia and staphylococcal toxic shock highlight clindamycin's efficacy when used as an substance for the treatment of *S. aureus* related toxin infections Novick *et al*.,(2003).The underlying mechanism of clindamycin's anti-toxin effect is linked to the ribosome-blocking action in which the transcription of exoprotein and SaeRS global regulator system, are reduced (Hodille *et al*., 2017).

The present study revealed a decrease in the mRNA level upon clindamycin treatment, suggesting a possible inhibitory effect of clindamycin on the transcription of virulence factor genes. Current results were consistent with previous reports by Herbert *et al*., (2001) they demonstrated that sub inhibitory concentration of clindamycin essentially blocked nearly all exoproteins production, including protein A, alpha-hemolysin and serine protease at the transcriptional level. They suggested that this inhibitory effect is due to the inhibition of the synthesis of one or more regulatory proteins that regulate the transcription of these exoprotein genes .Compared with our result, we have also found that clindamycin exhibits an inhibitory effect on PVL expression at the stationary phase in the *in vitro* study, suggesting that PVL may also be under the same regulation as other exoproteins. They also showed that although sub inhibitory concentration of clindamycin inhibited the expression of exoproteins, the production of certain surface protein, such

as coagulase and fibronectin binding protein B was stimulated (Herbert *et al*., 2001).

3.8. Sequence Analysis of *pvl* and SCC*mec* A IV genes of CA-MRSA Isolates

The results of PCR were subjected to DNA sequencing and analyzed to confirm the nucleotide sequences and close relations with other world strains, the test castoff to approve was using NCBI-Blast-query nucleotide -online, was a program and it produced the exact percentage of identity results by further world strains and extended from,(98-99%). Genotypic variations in *pvl* and SCCmec A IV within (10) isolates figure (3-9), (3-10) were studied for the first time in Iraq in the present study. CA-MRSA evolutionary genetic relationships were examined by comparative sequence analysis using NCBI. In this study, sequence analysis of (10) isolates for pvl and SCCmec A IV gene was carried out as shown in (Appendix).

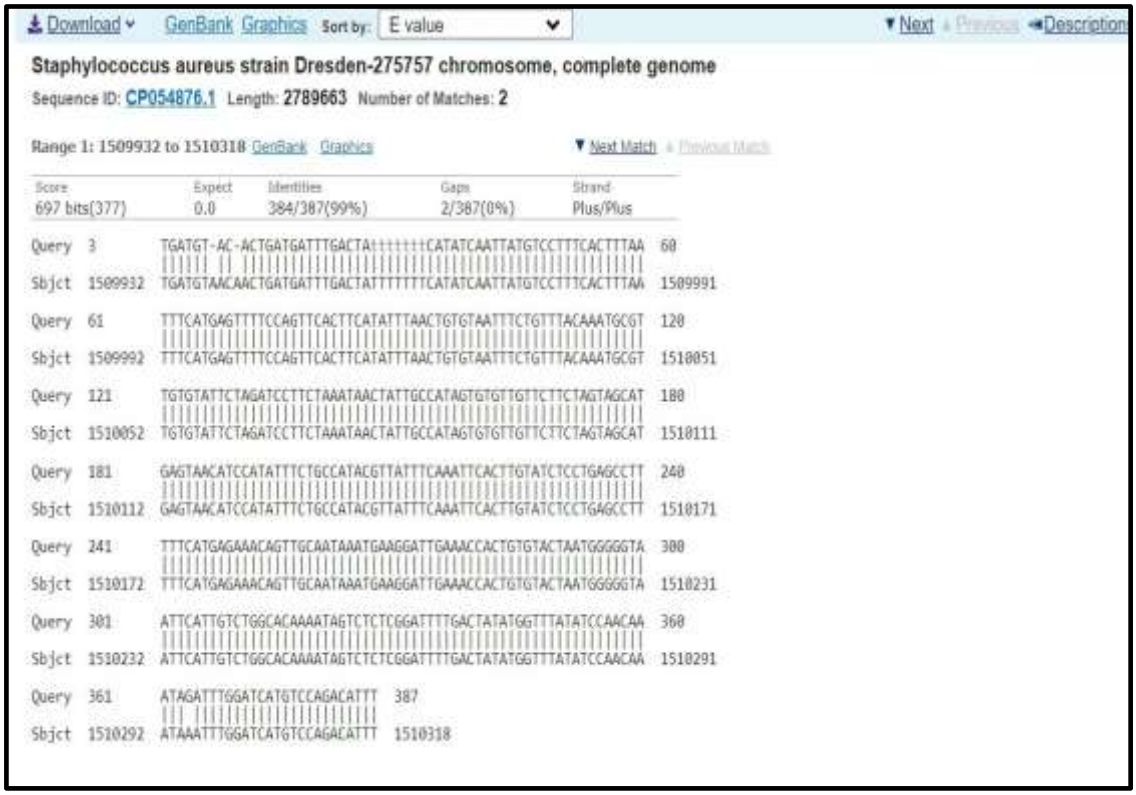


Figure (3-9): Pairwise Sequence Alignment Analysis of *pvl* Gene Partial Sequence For Local *S. aureus* (Dresden-275757) With NCBI of *S.aureus*. (Isolates NCBI BLAST Online). Pairwise Sequence Alignment Analysis was Constructed Using the Clustalw Alignment Tool in (MEGA 6.0 Version). That Showed the Nucleotide Alignment Similarity As (99%) With Different *S.aureus*. Isolates.

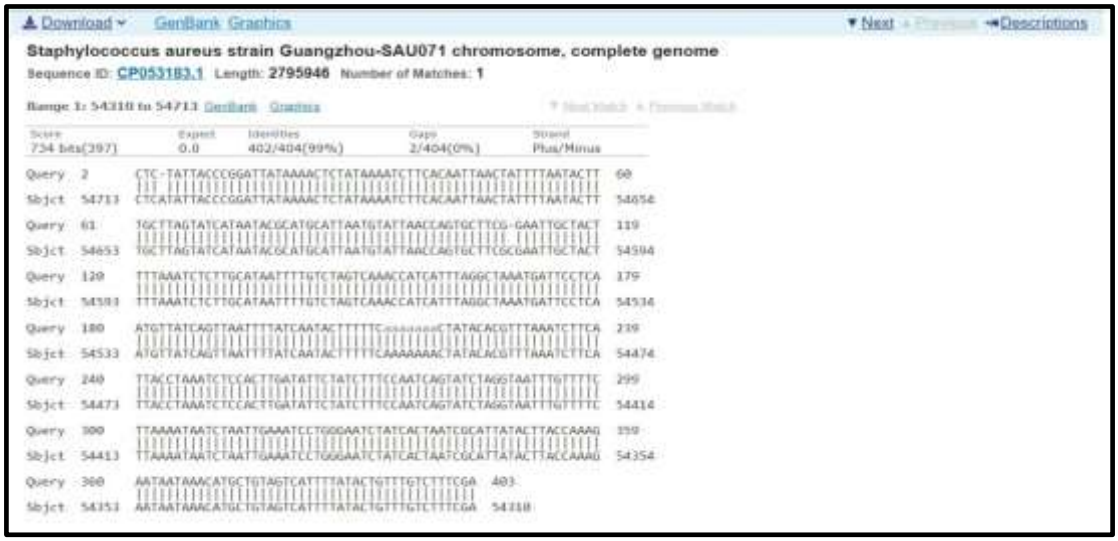


Figure (3-10): Pairwise Sequence Alignment Analysis of *SCCmec* A IV Gene Partial Sequence For Local *S. aureus* (Guangzhou-SAU071). With NCBI of *S. aureus*. Isolates. Pairwise Sequence Alignment Analysis was Constructed Using Clustalw Alignment Tool in (MEGA 6.0 Version) was used. the Nucleotide Alignment Similarity As (99%) With Different *S.aureus*. Isolates.

Conclusions:

1. The results of current study have been showed high *S. aureus* isolates percentage in a burn followed by impetigo, wound, and boil, respectively. In contrast, there was no bacterial isolates in acne, Secondary infection and Infected atopic dermatitis.

2. There were high percentage of MRSA isolates that exhibit obvious level of resistance against β-Lactam antibiotics used, maximum resistance against (Benzylpenicillin, Ceftriaxone, meropenem) while the lowest rates of resistance toward (Linezolid, Teicoplanin, Vancomycin, Tigecycline).

3. According to current study CA-MRSA was more than HA-MRSA indicating the spread of MRSA isolate in the community.

4. Molecular technique found to be the most reliable method in discrimination between CA-MRSA and HA-MRSA depending on SCCmec II-IV and *pvl* genes.

5. Quantitative real time pcr was excellent tool for estimation the level of expression for *pvl* gene and clindamycin in different concentration was effective in reducing the level of *pvl* gene indicating that this antibiotic could be used as a good choice for treatment of staphylococcal skin infection through minimizing release of *pvl* and reduce the severity of disease and improve treatment outcome in CA-MRSA infections.

6. In the present study confirmation of CA-MRSA pcr product was conducted by sequencing of SCCmec II-IV and *pvl* genes .

Recommendations:

1. Spreading health awareness among citizens about the use of antibiotics without a prescription in order to prevent the emergence of resistant bacterial isolate.
2. Study the *pvl* gene expression in other staphylococcal infection like necrotizing pneumonia and septic arthritis.
3. Understanding the mechanism of expression of other leukotoxins such as *luk* ED toxin gene.
4. Using other technique to assess the *pvl* concentration in different type specimen.
5. Study other genetic markers for CA-MRSA.

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