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Molecular analysis of *mecA* and *PVL* Virulence Factors Genes in Methicillin-resistant *Staphylococcus aureus* Clinical Isolates from Baghdad Hospitals

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Abstract

Background: Methicillin-resistant Staphylococcus aureus (MRSA) represents a significant public health concern due to its role as a prevalent pathogen responsible for severe and potentially life-threatening infections. **Aim:** This study aims to assess the prevalence of the *mecA* and *pvl* genes among MRSA isolates obtained from multiple hospitals in Baghdad, Iraq. **Methods:** A total of 110 clinical specimens were collected from patients aged between <1 and 80 years, encompassing various infection types, including burns, bloodstream infections, ear infections, nasal infections, urinary tract infections, and wounds. Identification of *S. aureus* was performed using biochemical methods and the VITEK 2 system. Polymerase chain reaction (PCR) technique was employed to detect key virulence genes, specifically *mecA* and *pvl*. **Results:** The genotypic investigation results revealed that an overall MRSA prevalence of 74%, with *mecA* and *pvl* genes present in 100% of the *S. aureus* isolates analyzed (20 isolates). These results underscore the significance of mecA in the molecular identification of *S. aureus* and highlight its role in distinguishing MRSA strains. Notably, the highest resistance rates were observed against benzylpenicillin, erythromycin, oxacillin, and clindamycin. **Conclusion:** The study concludes that a substantial percentage of *S. aureus* isolates in the cohort were derived from UI infections, followed by impetigo, wounds, and boils respectively. Furthermore, A higher percentage of MRSA isolates contain the *mecA* and *pvl* genes, indicating a concerning trend in resistance patterns within Baghdad hospitals.

Keywords: MRSA, *mecA*, PVL, antibiotic resistant, cefoxitin, Inducible Clindamycin(iCL).
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Introduction

Staphylococcus aureusis an opportunistic pathogen. It is most common in skin and soft tissue. This microbe can cause more diseases as a burn inflammation and tonsillitis through the production of virulence factors (1). Nearly all strains of *S. aureus* shown sensitivity to penicillin throughout that year. Since 1940, around 95% of the strains have been reported Penicillin resistant. The beta-lactamase enzyme that degrades beta-lactam ring and mutation in Penicillin-binding proteins (PBPs) are recognized for their role in penicillin activity opposition(2).Methicillin-resistant *Staphylococcus aureus* (MRSA) is a variant of *S. aureus* that has developed resistance to β-lactam medicines, including penicillins and cephalosporins. MRSA strains are adaptable and important hospital-acquired pathogens, frequently responsible for postsurgical wound infections predominantly originating from healthcare settings, first noted in 1961(3).

MRSA infections constitute 20–80% of all nosocomial *S. aureus* infections in numerous global centers and result in heightened mortality, morbidity, prolonged hospital stays, and increased expenses. The World Health Organization (WHO) has indicated that 64% of patients infected with MRSA had a higher likelihood of mortality compared to those without MRSA infection (4). Methicillin resistance developed because of the acquisition of a mobile genetic element known as staphylococcal cassette chromosome mec (SCCmec), which contains the *mecA* resistance gene that encodes a penicillin-binding protein variant (PBP2a) with a lower affinity for β-lactam antibiotics (5) Many of the virulence components they produce are encoded in plasmids, transposons, prophages, and pathogenicity islands(6).This extraordinary adaptability is largely a result of these virulence factors(7).Exoproteins like enterotoxins, exfoliatins, toxic shock syndrome toxin, and Panton–Valentine leucocidin [PVL]) are among these factors, as are cell surface substances like protein A, fibronectin-binding protein, collagen-binding protein, and clumping factor, as well as extracellular protein toxins that increase pathogenicity(8).It's intriguing that some of these toxins were found more frequently in methicillin-resistant *S. aureus* (MRSA) infections than in cases of non-MRSA(9). The rise and proliferation of multidrug-resistant *S. aureus* bacteria pose a global danger to the therapeutic management of staphylococcal infections. So, in these regards it is crucial to study the frequency of *mecA* and *pvl* gene amongst the pathogenic population isolates of Methicillin Resistant *Staphylococcus aureus* (MRSA) in Baghdad hospitals.

Materials and methods

Sample collection

This study included 110 patients (aged <1 to 80 years) suffering from burn and ear infections, Furuncle, Urinary issues, and Wounds. These patients were admitted to two teaching hospitals (Al-Yarmouk Teaching Hospital and Al-Kadhimiya Teaching Hospital in Baghdad), during the period from January 2023 to April 2024. This study characterized all bacterial isolates of *S. aureus* using morphological, microscopic, biochemical assays, and the Vitek II system.

Isolation, and Identification of *S. aureus* by: A. Culture Characteristics

The culture media sterilization was done by autoclave at 121 ° C, under 1bar pressure for 15-20 min. Identification of *S. aureus* were done by Brain heart infusion (BHI) broth medium, blood agar, and mannitol Salt Agar (MSA) was used for the activation of bacterial isolates, and A loopful of inoculum from nutrient broth was streak on MSA and incubated for 48 hours at 37˚C(10).

B. Microscopic Identification

Microscopic examination give bacterial shape, organization, and reaction to Gram staining, distinguishing between Gram-positive (purple) and Gram-negative (red) bacteria (11)

C. Biochemical Characteristics

The strains were identified and characterized by morphological and biochemical tests, these tests were Oxidase , Catalase, Coagulase, Indole, Methyl red , and Citrate (12).

Antibiotic susceptibility Test (AST)

The AST card for the VITEK-2 system is an automated testing methodology that is based on the MIC technique that was

disclosed by MacLowry and Marsh and Gerlach. The Vitek 2 AST-GP67 card, manufactured by bioMe´rieux in France, was utilized in accordance with the guidelines provided by the manufacturer. To provide a brief overview, three to five colonies of a culture of *S. aureus* that had been growing for 18 to 24 hours were injected in a solution of 0.45% sodium chloride and then adjusted to a concentration that was equivalent to 0.5 to 0.63 McFarland standards, then loaded into the Vitek 2 system together with the card. To determine whether or not the Vitek 2 cards included inducible clindamycin resistance, two wells were utilized. The MIC values for each type of antibiotic on the card were calculated at the conclusion of the incubation cycle (11). Based on the Vitek 2 system, the median time to final susceptibility reporting was 7 hours, with a range of 6 hours and 15 minutes to 12 hours and 30 minutes(13).

Antibiotics susceptibility test

The sensitivity test was carried out using the disc spread method according to the Kirby-Bauer method on MH (Mueller-Hinton agar), which was prepared (according to the manufacturer). Two antibiotics are used as gentamicin and trimethoprim.

Minimum Inhibitory Concentration

The minimum inhibitory concentration (MIC) is defined as the lowest concentration of an antimicrobial agent that can limit observable bacterial growth without causing cell death. The broth microdilution method utilizing a 96-well polystyrene plate is the most suitable technique for determining MIC values. This method was employed for quantitative assessment to investigate the in vitro antimicrobial efficacy of Gebtmycin and Trimethoprim against bacterial isolates, as outlined by (14), with some modifications.

Preparation of antimicrobial agents

A stock solution was made in 10 ml plain tubes by dissolving gentamicin and trimethoprim powder in distilled water to achieve a final concentration of 128,512 µg/ml, and subsequently filtered using a 0.22 µm Millipore filter. Two-fold serial dilutions were performed from the stock solution to achieve concentration ranges from 512, 128 µg/ml to 2, 1 µg/ml using Muller-Hinton broth (MHB) on a 96-well plate.

Genotypic detection of MRSA isolates

- **DNA Extraction**

Bacterial DNA was extracted using the FavorPrep Total DNA Mini Extraction Kit (FAVORGEN, Korea), following the manufacturer's protocols. A conventional PCR assay was conducted to amplify the *mecA* and *PVL* genes of MRSA using the GoTaq® Green Master Mix kit (Promega, USA), as detailed in Table (1). Primer stock solutions were prepared from lyophilized primers obtained from Macrogen (Korea), and the specific primers for *mecA* and *PVL* genes utilized in this study are provided in Table (2).

The PCR results were analyzed via electrophoresis on a 1% agarose gel stained with ethidium bromide (Carl Roth, Germany), utilizing a 100 bp DNA ladder (TransGen, China) as a reference to compare the sizes of the DNA fragments. Visualization of the agarose gel was performed using a UV transilluminator.

Table (1): Components of PCR reaction with their volume

Component	volume
Master mix	12.5µl
Forward primer	10 picomols/µl (1 µl)
Reverse primer	10 picomols/µl (1 µl)
DNA	1.5µl
Nuclease free water	9µl
Final volume	25 µL

Table (2): Primers sequences to detection *mecA, PVL genes.*

Primer	Sequence	Primer sequence 5'- - 3'	Tm (°C)	GC (%)	Amplicon size (bp)	Ref.
mecA	**F**	**TGAGTTGAACCTGGTGAAGTT**	**58.9**	**43**	**855**	**(16)**
	R	**TGGTATGTGGAAGTTAGATTGG**	**56.6**	**41**		
PVL	**F**	**GCCAGACAATGAATTACCCCCA**	**62.8**	**50**	**199**	**Primer study design**
	R	**TGCGTTGTGTATTCTAGATCCTT**	**56.9**	**39**		

Table (3): The PCR optimum condition program of detection *mecA, PVL genes.*

No.	Phase	Temperture (°C)	Time	No. of cycle
1-	**Initial Denaturation**	**95°C**	**5 min**	**1 cycle**
2-	**Denaturation -2**	**95°C**	**30 Sec**	**35cycle**
3-	**Annealing**	**57°C**	**40 Sec**	
4-	**Extension-1**	**72°C**	**30 Sec**	
5-	**Extension -2**	**72°C**	**5 min.**	**1 cycle**

Results

From One hundred ten swaps were taken from different clinical samples highly percentage of isolated were 6 blood samples (5.45%), 24 burn samples (21.82), 4 ear samples (3.64), 6 furuncle samples (5.45%), 48 urine samples (43.64%), and 22 wound samples (20%).as shown in table (4). Samples were distributed according to age groups, where the higher percentage of infection were in the age group (21-30) years was (30.7%), while the lowest in age group (51-60) that were (4.6%). As shown in Table (5).

Table (4): The number and percentage of *S. aureus* isolates from different types of specimens

Type of specimens	No.	Percentage
Blood swabs	6	5.45
Burn swabs	24	21.82
Ear infection swabs	4	3.64
Furuncle swabs	6	5.45
Urine from UTI patients	48	43.64
Wound swabs	22	20
Total	100	100%

Table (5): Distribution of isolated samples according to age groups

Age ranges	No.	%
10-20	**17**	**26.1**
21-30	**20**	**30.7**
31-40	**16**	**24.6**
41-50	**9**	**13.8**
51-60	**3**	**4.6**

Diagnosis of samples

To diagnose the bacterial isolates, a set of tests were conducted. The diagnosis was confirmed using the VITEK 2 device. Out of a total of 110 clinical samples studied, 27 samples Showed positive results for *S. aureus* when cultured on Petri dishes, which represents a percentage of (25%) of the total samples, while the percentage of samples that gave other bacterial isolated results was (83%). As shown in table (6).

Table (6): Numbers and percentages of diagnosed samples

Types of bacteria	No.	%
S.aureus	27	25
Others	83	75

as figure ([1]A, B, C, D, E) *S. aureus* colonies on blood agar plates were large, round, creamy and three isolates only produced-golden yellow colonies, 8 (53%) isolates were exhibited β hemolysis. On Mannitol Salt agar, they produced small colonies and the media turn yellow as it can grow at high

concentrations of salt, ferment Mannitol, and form an acid which changes the indicator color of the media from red to yellow. *S.aureus* tested positive for the catalase test, as evidenced by the formation of oxygen gas bubbles on the glass slide or agar plate, indicating the bacteria's capacity to manufacture the catalase enzyme, which decomposes hydrogen peroxide into water and oxygen. Additionally, positive results were obtained for the Methyl Red, Voges Proskauer, and urease tests. Notably, they exhibited a negative result for indole

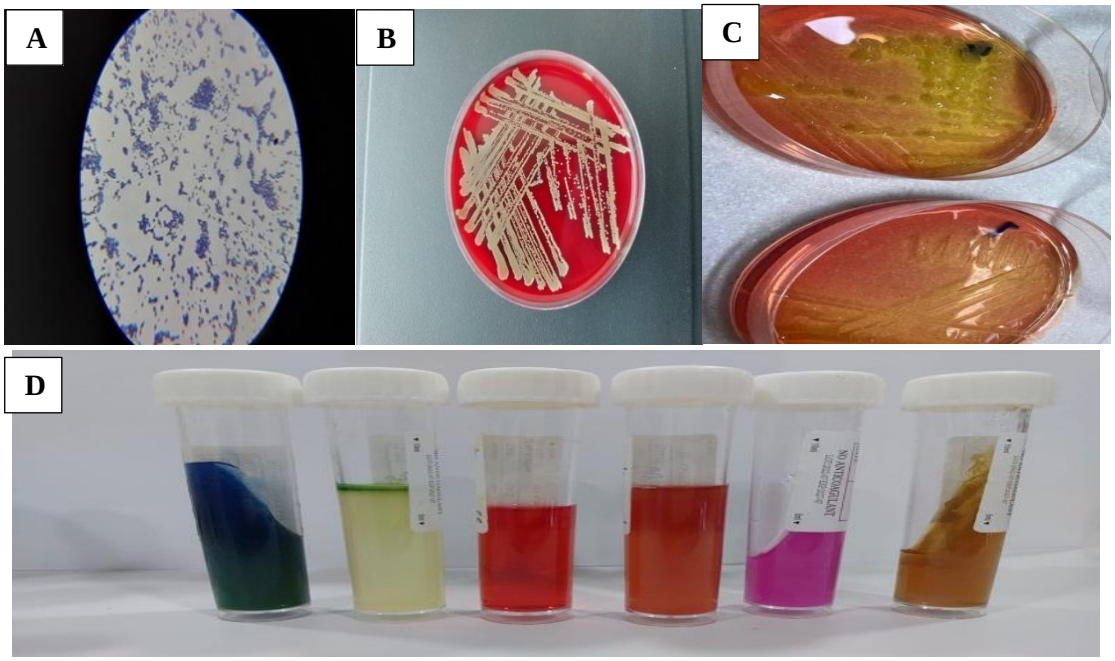

Figure 1: (A): Microscopic examination of *S.aureus* after performing the dying process with a gram stain, that appears small, cocai-shaped, Gram-positive (blue color) bacterial cells.(B): *s.aureus* grown on Blood agar at 37°C for 24 hrs. (C): Colonies of *S. aureuss* bacteria on Mannitol salt agar at 37°C for 24hr. (D): Figure: The results of some biochemical tests of *Staphylococcus aureus*;(from left - right), , citrate utilization(+) due to Simon citrate agar color changed from green to blue , indole test (-),,, urease test (+) , KIA (K/K) methyl red test (+),Vogas Proskauer test (+), urase test (+).

The present study differentiated methicillin resistant ***S. aureuss*** (MRSA) from non -methicillin resistant ***S. aureuss*** (MSSA) by VITEK test depending on cefoxitin and iCR. A total of 20 samples of MRSA, 18 (90%) and 9(45) were positive for cefoxitin and iCR respectively, while 2(10%) and 11(55%) negative for cefoxitin and iCR respectively. Detection of inducible clindamycin resistance (iCR) by double disk diffusion method. as shown in Table (7).

Table (7): Percentage of isolated MRSA depending on cefoxitin and iCR

Antibiotics	Positive		Negative	
	No.	%	No.	%
Cefoxitin	18	90	2	10
iCR	9	45	11	55

Determination of Minimum Inhibitory Concentrations (MIC):

The concentration of tri+gent 1/0.25**µ** /ml was inhibiting the growth isolates in percent of 100%(8/8). Observing that

elevated MIC values are required solely to slow the growth of *S. aureus* isolates, rather than eradicate them, indicates that these bacteria possess a significant resistance capability, potentially leading to infections in the individuals involved in the current investigation. Concentration of tri 8 **µl** /ml was inhibited the growth isolates in percent of 75 % (6/8), and 1.5 mg/ml in percent 25% (2/8). The concentration 1 µl /ml was inhibited the growth isolates in percent of 62.5 %(5/8), and 0.5 µl /ml in percent 37.5% (3/8). As shown in table (8) and figures (2).

No.	MIC of antibiotic Gentamicine	MBC of antibiotic Gentamicine	MIC of antibiotic Trimethoprim	MBC of antibiotic Trimethoprim	MIC of antibiotic Mix(Tri+Gent)	MBC of antibiotic Mix(Tri+Gent)
1	0.5	1	4	8	1/0.25	2/0.5
2	0.5	1	4	8	1/0.25	2/0.5
3	0.5	1	8	16	1/0.25	2/0.5
4	1	2	8	16	1/0.25	2/0.5
5	1	2	8	16	1/0.25	2/0.5
6	1	2	8	16	1/0.25	2/0.5
7	1	2	8	16	1/0.25	2/0.5
8	1	2	8	16	1/0.25	2/0.5

Table (8) MIC and MBC of Gentamicine, Trimethoprim and Mix both of them

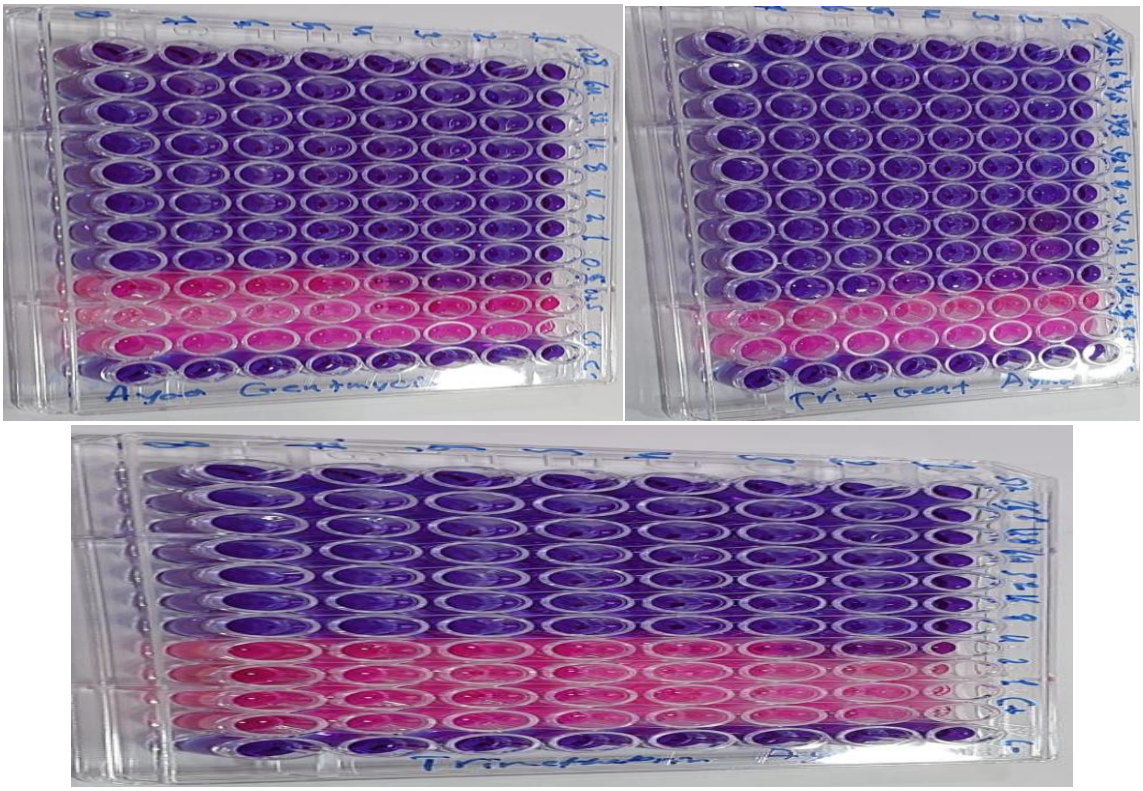

Figure (2): Results of Broth microdilution method to determination minimum inhibitory concentrations (MIC) values of *tri* for Staphylococcus aureus. (C-)" Negative control (only broth), (C+)" Positive control (only bacteria, broth),. Wells with Blue color had no or inhibited growth, but, Wells with red color with growth.

Antibiotic susceptibility testing of MRSA

Using the NCCLs guide, 20 MRSA isolates were tested for susceptibility to17 types of antibiotics by Kirby-Bauer disk

diffusion susceptibility test. All the isolates of MRSA species were 100% resistant to benzylpenicillin,95 % resistant to erythromycin and 90% resistant to both oxacillin and clind. While moderate resistant to Vancomycin and Tetracycline that were (60.75%). In contrast MRSA were highly sensitive for TIG, Linezolid, trimethoprim–sulfamethoxazole, tobramycin, Levofloxacin, and Moxifloxacin. As shown in table (9).

Table (9) Antimicrobial susceptibility results and of 20 MRSA isolates

Antibiotics	**Resistant (No. &%)**	**I (No. &%)**	**Sensitive**
	R	**I**	**S**
benzylpenicillin.	20(100%)	0(0%)	0(0%)
oxacillin	18(90%)	0(0%)	2(10%)
Gentamicin 10mg	4(20%)	0(0%)	16(80%)
tobramycin	3(15%)	0(0%)	17(85%)
Levofloxacin	4(20%)	0(0%)	16(80%)
Moxifloxacin	4(20%)	0(0%)	16(80%)
Erythromycin	19(95%)	0(0%)	1(5%)
Clindamycin	18(90%)	0(0%)	2(10%)
Linezolid 30mg	1(5%)	0(0%)	19(95%)
Teicoplanin 30mg	7(35%)	0(0%)	13(65%)
Vancomycin 30mg	12(60%)	0(0%)	8(40%)
Tetracycline	15(75%)	0(0%)	5(25%)
nitro	0(0%)	4(20%)	16(80%)
Fusidic Acid	16(80%)	0(0%)	4(20%)
Rifampicin 5mg	6(30%)	0(0%)	14(70%)
trimethoprim–sulfamethoxazole (25 mg	2(10%)	0(0%)	18(90%)
Tigcycline 15mg	1(5%)	0(0%)	19(95%)

Detection of the *mecA* and *PVL* genes by conventional polymerase chain reaction

In our study, polymerase chain reaction (PCR) results demonstrated the presence of both *mecA* and *PVL* genes in all 20 isolates. The amplified products for *mecA* and *PVL* were observed at 855 bp and 199 bp, respectively, as illustrated in Figures (3) and (4). Validation of the detected genes was performed via electrophoresis on a 1% agarose gel, run at 70 volts for 1.5 hours, and stained with ethidium bromide. Visualization was achieved using a ultraviolet (UV) transilluminator. All MRSA isolates were conclusively identified using gene-specific primers for *mecA* and *pvl,* with all exhibiting positive results

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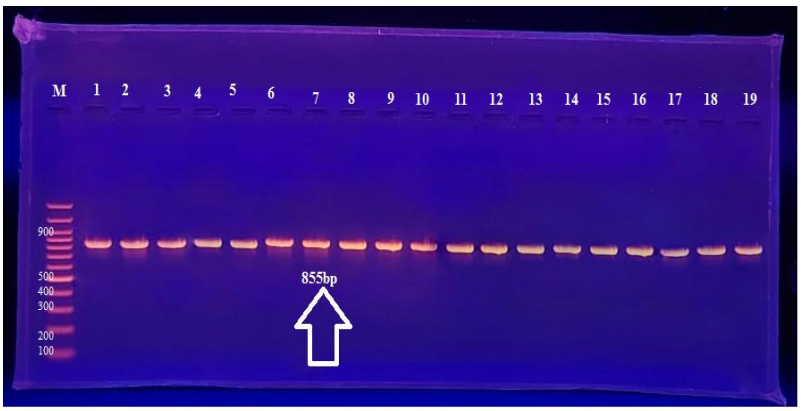

Figure (7): Detection of *mecA* (855bp) by PCR for *S. aureus MRSA* isolates, which was migrated in an agarose gel at a concentration of (1) % at a voltage difference of (70) volts for (60) minute.

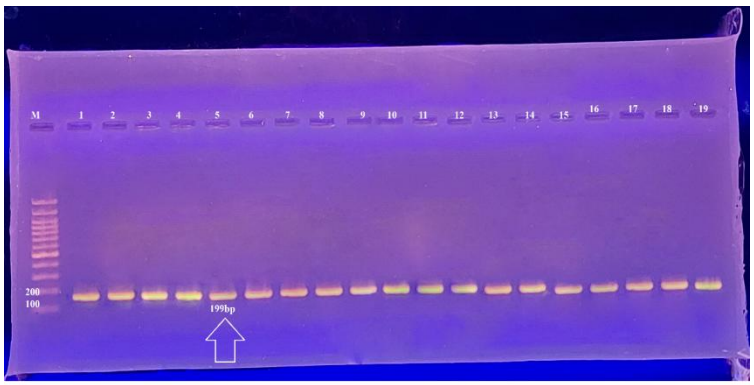

Figure (7): Detection of *pvl* (199bp) by PCR for *S. aureus MRSA* isolates, which was migrated in an agaros gel at a concentration of (1) % at a voltage difference of (70) volts for (60) minute.

Discussion

In recent decades, methicillin-resistant *Staphylococcus aureus* (MRSA) has proliferated globally, becoming a significant pathogen due to its ability to acquire antibiotic resistance and pathogenic factors. Since the emergence of MRSA in 1961, infections caused by S. aureus strains resistant not only to methicillin but also to other β-lactams have increased, leading to treatment failures and higher case fatality rates(16). The resistance is attributed to the staphylococcal cassette chromosome mec (SCCmec) genes(17). The current study identified a heightened incidence of infections among individuals aged 21 to 30 years, likely due to increased activity and environmental exposure. This finding aligns with previous research (18) indicating a similar prevalence in this age group.

The rising incidence of staphylococcal infections and the evolving antimicrobial resistance landscape have generated interest in using clindamycin for treatment. Regular assessment of resistance patterns and susceptibility testing is recommended to inform antibiotic therapy.(19). Since 2004, CLSI guidelines have recognized cefoxitin as a surrogate marker for methicillin resistance(18). Clindamycin is favored for treating skin and soft tissue infections due to its favorable pharmacokinetic profile. The VITEK systems from bioMérieux are widely used in clinical microbiology for species identification and antimicrobial susceptibility testing, utilizing a robust knowledge base to analyze AST results(19). This allows the system to identify over 2,300 phenotypic antimicrobial resistances (20). The analysis revealed a higher prevalence of

inducible and constitutive resistance in MRSA compared to methicillin-sensitive S. aureus (MSSA). High resistance rates to common β-lactam antibiotics, such as benzylpenicillin, oxacillin, and cefoxitin, highlight the overuse of these drugs in Iraqi healthcare. Most bacterial isolates exhibited resistance to multiple antibiotics, complicating treatment, particularly for MRSA strains that have also developed resistance to tetracyclines, aminoglycosides, macrolides, glycopeptides, and lincosamides.(21). Aminoglycosides remain effective against various staphylococcal infections and are often used in combination with β-lactams and glycopeptides(22) . All *S. aureus* isolates demonstrated high sensitivity to linezolid and tigecycline, regarded as effective options against MRSA. Cefoxitin has been shown to induce the *mecA* gene more effectively than methicillin and oxacillin, contributing to its increased sensitivity in the Kirby-Bauer disk diffusion method(23). Studies indicate that cefoxitin is more specific and sensitive than oxacillin. Notably, research by Alsalami *et al*. (24) found that 93.1% of *S. aureus* isolates were resistant to cefoxitin, with 100% resistance to both benzylpenicillin and oxacillin. Methicillin-resistant Staphylococcus aureus (MRSA) has developed resistance to β-lactam antibiotics through the acquisition of the *mecA* gene, which encodes penicillin-binding protein 2a (PBP2a). This protein exhibits significantly reduced affinity for β-lactam antibiotics, thereby conferring resistance (25).

The detection of *mecA* by polymerase chain reaction (PCR) is regarded as the gold standard for identifying methicillin resistance (26). Our findings align with those of Al-Oubaidy and Al-Jubori (27), who reported a 100% prevalence of *mecA* in *S. aureus* isolates. However, these results contrast with the findings of Ibed and Hamim (28), as well as Kareem et al., (29) who documented *mecA* prevalence rates of 75.5% and 82.43%, respectively. Panton-Valentine leukocidin (PVL) is a critical characteristic of community-associated MRSA (CA-MRSA) and serves as a clinically relevant virulence marker for this pathogen (30). Recent years have seen an increase in nosocomial transmissions and outbreaks of PVL-positive MRSA in Europe, indicating that the distinction between hospital-associated and community-associated MRSA strains has become increasingly indistinct. There is a well-established correlation between the presence of PVL and the severity of infections; the high rate of *PVL* gene detection in this study may be attributed to the prevalence of supportive infections, such as boils and abscesses. The current study's findings, which indicate a 100% prevalence of the *PVL* gene, surpass those of previous study (31) that reported *PVL* gene detection in 58.33% of MRSA isolates. PVL is associated with increased virulence in certain S. aureus strains and is implicated in the development of necrotic lesions affecting the skin and mucosa, including conditions such as necrotizing hemorrhagic pneumonia, which are particularly challenging to treat. Therefore, the early detection of the *pvl* gene in *S. aureus* is crucial for effective management of infections and for evaluating treatment outcomes.

Conclusion

A significant prevalence of methicillin-resistant Staphylococcus aureus (MRSA) was observed in various clinical samples, with multidrug resistance (MDR) identified at a notable frequency among resistant isolates. Antibiotic resistance remains a critical and escalating public health concern in Iraq and other countries, highlighting the necessity for comprehensive reviews of local studies. The epidemiology of antibiotic resistance

among regional bacteria, including MRSA, is not well understood. This report provides data on MRSA recurrence in Baghdad, revealing a high prevalence of isolates containing the *mecA* and *PVL* genes. These findings can guide future studies focused on infection control and the optimal use of antimicrobial agents in Iraq. While acknowledging the challenges in implementing these recommendations, particularly in the long term, the report suggests a preliminary evaluation of existing infection control measures. More effective strategies should include screening for MRSA carriers, isolating or cohorting patients, managing healthcare workers in clusters, and ensuring continuous environmental decontamination. Additionally, investment in laboratory facilities and training for staff should be prioritized to reduce the spread of this pathogen.

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