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*Original Article*

# Phenotypic and Genotypic Characteristics in MDR MRSA from Clinical Sources

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## ABSTRACT

*Staphylococcus aureus* is a principal pathogen responsible for conditions such as pneumonia, osteomyelitis, septicemia, soft-tissue infections, and other skin infections. Its pathogenicity stems from various virulence factors, some among which are acquired like antibiotic resistance. One hundred and thirty pathogenic samples were collected from patients with suspected symptoms at the Ramadi Hospitals in Iraq. Upon culturing samples, a total of 116 bacterial isolates were collected, which were subjected to biochemical and microbiological characterization using standard procedures validated by the OXDD, FOXDD, and VITEK 2 Compact Systems. From these, the 20 isolates detected were selected for further investigation. Initially, 16S rRNA gene amplification was performed to identify the organism at molecular level. Biofilm production of all 20 isolates was assessed using 96-well microtiter plate and tube method, Congo Red Agar (CRA) and Modified Congo Red Agar (MCRA). Its production varied across isolates, which were grouped as strong (3, 15%), moderate (8, 40%), and weak (9, 45%). Interestingly, all 20 isolates exhibited MDR to beta-lactam, tetracycline, aminoglycoside, lincosamide, macrolide, fluoroquinolone, and sulfonamide antibiotics, with 100% resistance to penicillin and macrolide antibiotics. The existence of virulence genes such as *mecA*, *icaA*, *femA*, and *clfA*, were assessed through PCR. Similarly, phenotypic traits to assess their virulence factors showed positive for hemolysis of blood, deoxyribonuclease, urease, gelatinase, lecithinase, and protease. This study provides a comprehensive characterization of MRSA in Ramadi city, Iraq, indicating high levels of multidrug resistance, with virulence factors, and a high propensity to form biofilms. These findings highlight the need for highly early diagnostic and effective treatment strategies to combat this pathogenic bacterium.



*Keywords: MRSA, MDR, VITEK 2, 16S rRNA, Biofilm, Virulence factors.*

## 1 Introduction

*Staphylococcus aureus* is a gram-positive, widespread bacterium that causes human health hazards and plays a vital role in causing diseases like skin infections, inflammation, wounds, burns, bloodstream infections, pneumonia, and urinary tract infections [1]. The bacteria developed resistance to methicillin several decades ago, from which a group of MRSA emerged [2]. Such strains were characterized by the existence of the *mecA* gene, which encodes for penicillin-binding protein 2a (PBP2a), that leads to reduced binding of methicillin and other relevant antibiotics. *mecA* gene is located on genomic islands known as Staphylococcal Cassette Chromosome (SCCmec) elements, which play a critical role in the acquisition and transfer of resistance [3,4]. The rapid spread of Methicillin-resistant *Staphylococcus aureus* has resulted in the acquisition of resistance to a broad spectrum of antibiotics, such as tigecycline, ceftaroline, and daptomycin.

The complexity of treatment options lies in MRSA's ability to acquire resistance to multiple drugs, that makes infection management more complicated [4].

Biofilm production protects bacteria from drugs and the host's humoral immune response, leading to chronic bacterial infections [5,6]. Such biofilm formation is one of the factors contributing to pathogenicity of MRSA. Intracellular polysaccharide binding that creates biofilms is mediated by the ica operon, which includes the *icaA* gene, and is essential in the synthesis of the polysaccharide intercellular adhesin (PIA). The expression of genes associated with biofilm formation differs substantially among strains [7].

Of late, the *femA* gene serves as a molecular marker for *S. aureus* identification, as it plays an essential role in adding glycine

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bridges for peptidoglycan synthesis, a process critical for bacterial cell wall formation [8]. Another gene, *clfA*, is responsible for encoding clumping factor A, a protein that helps the accumulation of *Staphylococcus aureus* in blood plasma. This protein binds exactly to fibrinogen, a key element of the blood clotting cascade. Due to its role in bacterial adhesion and virulence, *clfA* has been determined as a potential target for the development of a vaccine [9]. This work investigates multidrug-resistant methicillin-resistant *Staphylococcus aureus* (MRSA) from suspected symptomatic patients of Ramadi, Iraq. The study addresses both phenotypic and genetic levels basis which provides basic data on virulence factors and antimicrobial resistance patterns.

## 2 Materials and Methods

### 2.1 Collection of Samples and Identification

The study was carried out with due approval of the Institutional Human Ethics Committee, where patients with skin infections, inflammation, wounds, burns, bloodstream infections, pneumonia, and urinary tract infections served as the source of specimens. Samples were collected from both genders and across all age groups at Al-Ramadi Teaching Hospital and Al-Ramadi Teaching Hospital for Women and Children, Iraq. Clinical samples include urine, burn swabs, wound swabs, blood, ear swabs, pus abscesses, nasal swabs, and sputum. All the samples were cultivated on Mannitol salt agar (Accumix/India) to screen them for *S. aureus*. Those bacterial strains thus isolated were subjected to VITEK 2 Compact system for automatic organism identification.

### 2.2 Vitek-2 Compact system

The VITEK 2 Compact system (bioMérieux) is a fully automated instrument used for rapid identification and antimicrobial susceptibility testing (AST) of bacteria, using Advanced Colorimetric technique that monitors metabolic changes via fluorescence and turbidity.

It employs miniaturization technology, sealed 64-microwell ID/AST cards, such as ID for gram-negative or positive bacteria, and AST for susceptibility, inoculated with a standard bacterial suspension 0.5 McFarland, providing species-level identification and minimum inhibition concentration (MICs) for most gram-positive/negative within 3-18 hours, with >90% precision [10].

### 2.3 Phenotypic Detection of MRSA

To identify MRSA, all isolates were subjected to oxacillin (1 µg) and cefoxitin (30 µg) using the disk diffusion method. The suspension of the Overnight bacterial culture was adjusted to a 0.5 McFarland standard suspension, and a lawn culture was formed on a Mueller-Hinton agar plate onto which cefoxitin and oxacillin disks was placed. The plate was then incubated at 37 °C for 18-24 hours to measure the zone of clearance [11].

### 2.4 Extraction of bacterial DNA

Bacterial genomic DNA was extracted from suspected MRSA isolates using the Geneaid Genomic DNA extraction Kit (South Korea), following the manufacturer's instructions, and resolved in agarose gel (1% ) [12]. Purity (260/280) and concentration (260 nm) were determined using Nanodrop spectrometry [13].

### 2.5 Molecular confirmation of MRSA isolates using 16S rRNA gene analysis

For the uniplex-PCR reaction, the master mix was prepared in a total volume of 25 µl, composed of 12.5 µl OneTaq® Quick-Load® 2X master mix with standard buffer,1 µl forward primer (5′-CACCTTCCGATACGGCTACC-3′), and 1µl reverse primer (5′-GTTGACTGCCGGTGACAAAC-3′) [14], 4 µl template DNA, and 6.5 µl DNAse-RNAse-free D.D.W. The 16S rRNA gene of suspected isolates was amplified using conventional PCR thermocycling.

An initial denaturation at 95°C (5 min), followed by 30 cycles of denaturation at 95°C for 53 s, annealing at 59°C for 40 s, extension at 72°C for 40-60 s, and final extension at 72°C for 7 min was performed to ensure full synthesis of all amplicons. The reaction was thereafter maintained at 4°C until further examination [15].

Amplified PCR products were resolved on 1.0 % agarose gel and visualized under a UV transilluminator.

### 2.6 Antibiotic susceptibility

These identified MRSA isolates underwent antimicrobial susceptibility testing by the Kirby–Bauer disk diffusion method on Mueller-Hinton agar following CLSI disk diffusion guidelines [16]. A 0.5 McFarland standard of the bacterial suspension was prepared, and it was cultured on MHA plates. For each strain, antibiotic disks were placed on MHA plates, and the plates were incubated overnight at 37°C for 18–24 h with a range of tested antibiotics: penicillin (P 10 µg), ampicillin (AM 10 µg), erythromycin (ERY 15 µg), clindamycin (DA 2 µg), gentamicin ( CN 10 µg), tetracycline (TET 30 µg), levofloxacin (LV 5 µg ), trimethoprim/sulfamethoxazole (SXT 5 µg), Amikacin (AK 30 µg), Ciprofloxacin (CIP 5 µg), Ceftriaxone (CRO 30 µg), and Amoxicillin/ Clavulanate (AMC 30 µg).

Following incubation, inhibition-zone diameters were recorded in millimeters and interpreted based on CLSI breakpoints as susceptible, intermediate, or resistant. *Staphylococcus aureus* ATCC 25923 was used as a positive control in disk diffusion for reference purposes. Multidrug resistance was ascertained based on non-susceptibility to at least one antimicrobial agent in three or more antimicrobial classes. Thus, all the isolates were grouped as resistant, intermediate, and susceptible categories.

## 2.7 Phenotypic Detection of Virulence Factors

### 2.7.1 Biofilm formation

#### *2.7.1.1 96-well microtiter plate assay*

Evaluation of MRSA biofilm formation was performed using a 96-well microtiter plate assay with slight modifications. 180 µL of brain heart infusion broth was added to each well, and then 20 µL of an overnight bacterial culture was added to each well. Control wells contained 200 µL of uninoculated brain heart infusion broth. During incubation, the microtiter plates were covered with parafilm to prevent evaporation and drying out of the wells.

The wells were washed two to three times with phosphate-buffered saline (PBS, pH 7.0) after incubation in order to remove non-adherent bacterial cells. Attached cells were then fixed with 200 µL of methanol for 10–15 min. The wells were dried at room temperature after fixation and then stained with 200 µL of 0.1% crystal violet for 15 min. Unbound dye was washed out by washing the wells three times with PBS and left briefly for drying at room temperature. The bound crystal violet was solubilized with 200 µL of 95% Ethanol per well. The optical density of each well was measured at 630 nm using an ELISA microplate reader in triplicate [17]. The mean optical density (OD) of the negative control plus three standard deviations was defined as the cut-off OD (ODc). Biofilm production was scored as a non-biofilm producer when OD ≤ ODc, a weak biofilm producer when 2ODc < OD < 4ODc, a moderate biofilm producer when 2ODc < OD < 4ODc, and a strong biofilm producer (SFIB) when OD> 4ODc.

#### *2.7.1.2 Tube method*

Qualitative biofilm formation by *Staphylococcus aureus* was determined using test tube method. Brain heart infusion broth (10 ml) was dispensed into sterile test tubes and inoculated with *S. aureus* colonies from culture plates incubated overnight. The tubes were incubated at 37 °C for 24–48 h.

After incubation, the broth was removed, and the tubes were rinsed with phosphate-buffered saline (PBS, pH 7.3) to discard non-adherent bacterial cells. Tubes were allowed to dry at room temperature and then stained with crystal violet (2.5mL). Tubes were gently rolled in a circular motion to ensure uniform distribution and incubated for 10 min. The excess stain was scraped away, and the tubes were inverted to dry.

Visual assessment of biofilm formation was performed based on the presence of a stained film or ring layered along the inner wall and bottom of the tube. Visible ring formation was considered a biofilm positive [18].

#### *2.7.1.3 Congo Red Agar (CRA) and Modified Congo Red Agar (MCRA)*

Congo Red Agar (CRA) was used as a qualitative approach to distinguish biofilm formation in Methicillin-resistant *Staphylococcus aureus* isolates. Culture medium was prepared by dissolving 37 g/L brain heart infusion broth, 50 g/L sucrose, and 10 g/L agar in 900 mL distilled water, adjusting the pH to 7.2, and autoclaving at 121°C for 15 minutes; a separate aqueous solution of 0.8 g/L Congo red was autoclaved and added after the base agar cooled to 55°C. Inoculated the Plates with bacterial suspensions were incubated at 37°C for 24–48 hours. Biofilm formation was identified by the observation of black, dry, crystalline colonies, while pink or red colonies indicated non-biofilm formation.

Modified Congo Red Agar (MCRA) was prepared to detect biofilm formation in Methicillin-resistant *Staphylococcus aureus* isolates, with some modifications such as reducing the concentration of Congo red (0.4 g/L), replacement of glucose (10 g/L) with sucrose, and utilization of blood agar base (40–52 g/L) rather than brain heart infusion agar. The material was dissolved in 1000 mL of distilled water, autoclaved at 121°C for 15 minutes, cooled to 55°C, dispensed into Petri dishes, inoculated with overnight bacterial culture, and incubated at 37°C for 24–48 hours. Biofilm formation was observed as black, dry, crystalline colonies, while pink or red bacterial colonies indicate non-biofilm formation [19].

### 2.7.2 Hemolysins production

Blood Agar plate (Accumix/India) was inoculated with a bacterial culture and then incubated at 37 °C for 24 hours to score the type of hemolysis as α partial, β complete, or γ no hemolysis [20].

### 2.7.3 Lecithinase production

To test for lecithinase, the bacterial culture was streaked onto egg yolk agar (HiMedia/India) and incubated at 37 °C for 24 hours [21].

### 2.7.4 Protease production

The bacterial culture inoculum was streaked on skim milk agar (HiMedia/India) and then incubated at 37 °C for 24 hours. An isolate is considered protease-producing if a clear zone appears around the bacterial colony, resulting from the hydrolysis of casein.

### 2.7.5 Urease production

Urea agar base (Christensen; HiMedia, India) was prepared according to the manufacturer's guidelines. The bacterial isolates were inoculated on the surface of the medium, incubated at 37 °C for 24-48 h to screen for urease-producing bacteria (UPB).The urease test tubes were examined for a color alteration from pale yellow to pink [22].

### 2.7.6 Deoxyribonuclease

The deoxyribonuclease test was performed by inoculating overnight bacterial culture onto DNase agar (HiMedia/India) and incubating it at 37 °C for 18-24 hours. Upon the addition of HCl to the surface of the plate, DNA hydrolysis was confirmed by the formation of a clear zone around the inoculum due to the dissolution of oligonucleotides [23].

### 2.8 Genotypic detection of virulence and resistance genes *mecA, icaA, femA,* and *clfA*

Extracts of genomic DNA were subjected to PCR amplification targeting the *mecA*, *icaA*, *femA*, and *clfA* genes in a 20 µl final reaction volume. Reaction mixtures included OneTaq® Quick-Load® 2X Master Mix with standard buffer (12.5 µl; Promega, Madison, WI, USA), 1 µl of 10 pmol/µl stock solution for forward and reverse primers (Table 1), each primer at 0.5 µM final concentration, 4 µl DNA template, and 1.5 µl of nuclease-free double-distilled water. Primers shown in Table 1 were custom-synthesized by Macrogen (South Korea).

PCR were performed with following thermal cycling parameters initial denaturation at 95°C for 5 min; 30 cycles of denaturation (95°C, 53 s), annealing gene-specific temperatures (57°C for *mecA*, 56°C for *clfA*, 55°C for *femA* and *icaA*; 40 s), and extension (72°C, 40 s); followed by final extension at 72°C for 7 min.

To detect the size of the amplicons, 5 µl of the 100 bp DNA ladder (Promega, USA) and PCR products were loaded onto a 1% agarose gel for electrophoresis.

**Table 1:** Primers selected for this work.

| Genes | Sequences | sizes (bp) | Reference |
|---|---|---|---|
| *mecA* | F: TCCAGATTACAACTTCACCAGG<br>R: CCACTTCATATCTTGTAACG | 162 bp | [24] |
| *IcaA* | F: ACACTTGCTGGCGCAGTCAA<br>R: TCTGGAACCAACATCCAACA | 188 bp | [25] |
| *FemA* | F: GAGGTCATTGCAGCTTGCTTAC<br>R: CTAGACCAGCATCTTCAGC | 988 bp | [26] |
| *clfA* | F: CGCCGGTAACTGGTGAAGCT<br>R: TGCTCTCATTCTAGGCGCACTT | 314 bp | [27] |

### 2.9 Sanger sequencing

Ten isolates were selected for Sanger sequencing to verify the PCR amplicons. The dideoxy chain-termination method was used to sequence DNA fragments. Sequencing was conducted via ABI 3730xl DNA Analyzer (Applied Biosystems, USA). The purified PCR products were sequenced to improve identification of the target gene. Sequencing was performed using the BigDye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems) according to the manufacturer's instructions [28].

### 2.10 Phylogenetic tree analysis of the isolates

To determine the genetic relationships among the 10 study isolates and reference strains, a phylogenetic tree was constructed using the UPGMA method in MEGA11.

## 3 Results

### 3.1 Distribution of MDR MRSA in different clinical specimens

All the specimen samples were cultured on Blood agar, MacConkey agar, and Mannitol salt agar, of which 116 (89%) isolates were obtained. All of them were characterized through the VITEK-2 Compact system, which resulted in 69 (59.5%) Gram-negative and 47 (40.5%) Gram-positive.

All Gram positives were further analyzed, which yielded 17 other bacteria, with 30 *S. aureus*. Further analysis evidenced the presence of 20 MRSA, which were taken for further study (Table 2).

**Table 2:** Distribution of MRSA isolates.

| Specimen Source | No of Isolates | Other bacterial species | | *S. aureus* | | MRSA% |
|---|---|---|---|---|---|---|
| | | Gram negative | Gram positive | Others | MRSA | |
| Urine | 22 | 11 | 2 | 1 | 8 | 40 % |
| Burn swab | 14 | 10 | 2 | 2 | 0 | 0 |
| Wound swab | 18 | 9 | 4 | 2 | 3 | 15 % |
| Pus abscess | 14 | 7 | 2 | 1 | 4 | 20 % |
| Ear swab | 8 | 6 | 1 | 0 | 1 | 5 % |
| Blood | 17 | 12 | 2 | 1 | 2 | 10 % |
| Sputum | 9 | 7 | 2 | 0 | 0 | 0 |

| | | | | | | |
|---|---|---|---|---|---|---|
| ENT | 5 | 3 | 1 | 1 | 0 | 0 |
| Nasal swab | 9 | 4 | 1 | 2 | 2 | 10 % |
| Total | 116 | 69 | 17 | 10 | 20 | 100% |

## 3.2 Molecular identification of MRSA isolates using 16S rRNA gene amplification

All 20 MRSA isolates were subjected to molecular analysis using the 16S rRNA gene. Species-specific primers for *Staphylococcus aureus* were used for PCR amplification (Figure 1). Partial 16S rRNA gene PCR yielded a clear band of approximately 372 bp in all clinical isolates tested, confirming the taxonomic affiliation. Random sequencing of 10 isolates, viz., (BU4, BU7, BU12, BU15, BU21, BU22, BU39, BU58, BU6, and BU66), confirmed the species identity, which was deposited in the public database (Table 3).

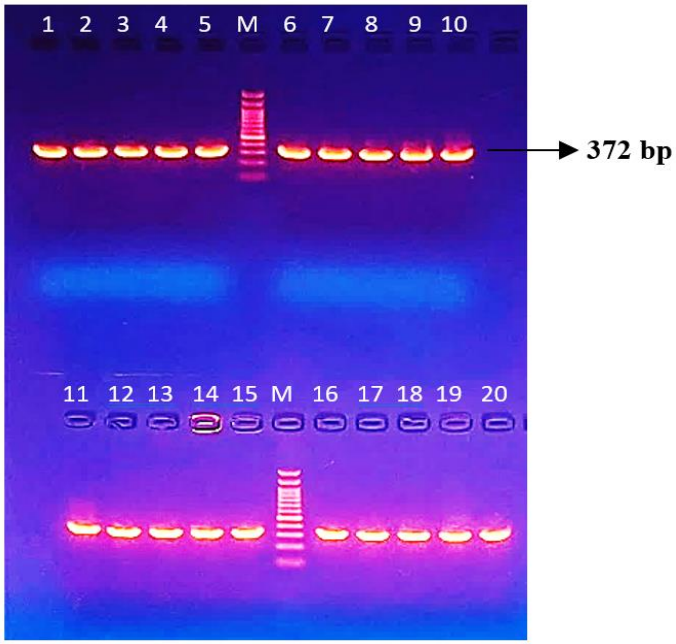


**Figure 1:** PCR amplification of the 16S rRNA gene, 372 bp from the bacterial strains from clinical sample. M: Marker (100-1500 bp ladder); Track 1: BU2; Track 2: BU4; Track 3: BU7; Track 4: BU9; Track 5: BU12; Track 6: BU15; Track 7: BU16; Track 8: BU19; Track 9: BU21; Track 10: BU22; Track 11: BU28; Track 12: BU30; Track 13: BU33; Track 14: BU39; Track 15: BU55;Track16: BU57; Track 17: BU58; Track 18: BU61; Track 19: BU64;Track 20: BU66.

**Table 3:** MRSA isolates with corresponding accession numbers.

| Isolate ID | Accession number |
|---|---|
| BU4 | PV173439 |
| BU7 | PV173440 |
| BU12 | PV173441 |
| BU15 | PV173442 |
| BU21 | PV173443 |
| BU22 | PV173444 |
| BU39 | PV173445 |
| BU58 | PV173446 |
| BU61 | PV173447 |
| BU66 | PV173448 |

## 3.3 Phenotypic detection of MRSA

Phenotypic detection analysis was performed on all selected isolates against specific antibiotics, which showed resistance to oxacillin and cefoxitin (Figure 2).

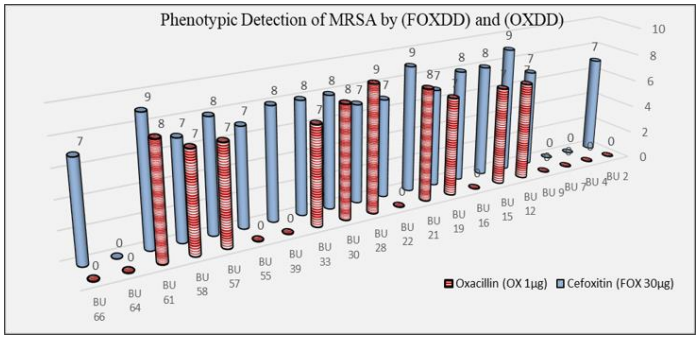


**Figure 2:** MRSA detection through Oxacillin and Cefoxitin.

## 3.4 Antibiotic susceptibility

The antibiotic susceptibility results showed that all 20 MRSA isolates were resistant to penicillin, ampicillin, and erythromycin. Resistance to clindamycin was detected in 7 isolates (35%), while 13 isolates (65%) were susceptible. Gentamicin resistance was observed in 2 isolates (10%), whereas 18 isolates (90%) were susceptible. Tetracycline resistance was found in 10 isolates (50%). For levofloxacin, 12 isolates (60%) were susceptible, 2 isolates (10%) were intermediate, and 6 isolates (30%) were resistant. Trimethoprim/sulfamethoxazole (SXT) showed susceptibility in 12 isolates (60%), intermediate response in 5 isolates (25%), and resistance in 3 isolates (15%). Most isolates were susceptible to amikacin, with 18 susceptible isolates (90%), 1 intermediate isolate (5%), and 1 resistant isolate (5%). Ciprofloxacin showed susceptibility in 8 isolates (40%), intermediate response in 3 isolates (15%), and resistance in 9 isolates (45%). Ceftriaxone showed susceptibility in 5 isolates (25%), intermediate response in 6 isolates (30%), and resistance in 9 isolates (45%). For amoxicillin/clavulanate, 9 isolates (45%) were susceptible, and 11 isolates (55%) were resistant, as shown in Figure 3. *Staphylococcus aureus* ATCC 25923 was used as a standard isolate was showed clear inhibition zones against all tested antibiotics, validating the reliability of the antibiotic susceptibility testing procedure (Table 4).

**Table 4:** Antimicrobial susceptibility interpretation of MRSA isolates according to CLSI.

| Antibiotics | Interpretation (R/I/S) | | | Isolate ID | | | | | | | | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | R | I | S | BU2 | BU4 | BU7 | BU9 | BU12 | BU15 | BU16 | BU19 | BU21 | BU22 | BU28 | BU30 | BU33 | BU39 | BU55 | BU57 | BU58 | BU61 | BU64 | BU66 | ATCC |
| P 10 µg | ≤28 | - | ≥29 | R | R | R | R | R | R | R | R | R | R | R | R | R | R | R | R | R | R | R | R | S |
| AM 10 µg | ≤28 | - | ≥29 | R | R | R | R | R | R | R | R | R | R | R | R | R | R | R | R | R | R | R | R | S |
| ERY 15 µg | ≤13 | 14-22 | ≥23 | R | R | R | R | R | R | R | R | R | R | R | R | R | R | R | R | R | R | R | R | S |
| DA 2 µg | ≤14 | 15-20 | ≥21 | S | S | R | S | R | R | S | S | S | S | R | R | R | S | S | S | S | S | S | R | S |
| CN 10 µg | ≤12 | 13-14 | ≥15 | R | S | S | S | S | S | S | S | S | S | S | S | S | S | S | R | S | S | S | S | S |
| TET 30 µg | ≤14 | 15-18 | ≥19 | S | S | S | R | R | R | S | S | S | R | S | R | R | R | S | S | R | S | R | R | S |
| LV 5 µg | ≤15 | 16-18 | ≥19 | R | S | R | S | S | I | R | S | S | S | S | R | I | S | S | R | S | S | S | R | S |
| SXT 5 µg | ≤10 | 11-15 | ≥16 | S | R | R | S | S | I | S | S | S | I | S | I | I | S | S | S | S | I | S | R | S |
| AK 30 µg | ≤14 | 15-16 | ≥17 | S | R | S | S | S | S | S | S | S | I | S | S | S | S | S | S | S | S | S | S | S |
| CIP 5 µg | ≤15 | 16-20 | ≥21 | I | R | R | S | R | R | R | R | S | S | S | R | R | S | S | I | S | S | I | R | S |
| CRO 30 µg | ≤13 | 14-20 | ≥21 | I | R | R | S | R | I | I | R | I | R | R | I | I | R | R | S | S | S | R | S | S |

| | | | | | | | | | | | | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| AMC 30 µg | ≤19 | - | ≥21 | S | R | R | S | R | S | R | S | S | R | R | S | R | R | R | S | R | S | R | S | S |

Note: R = resistant, I = intermediate, S = susceptible. ATCC 25923 was used as the standard quality-control isolate. Interpretation was based on the CLSI 2024 breakpoints provided in the source document. (P10µg: Penicillin, AM10µg: Ampicillin, ERY15 µg: Erythromycin, DA2µg: Clindamycin, CN10µg: Gentamicin, TET 30µg: Tetracycline, LV 5µg: Levofloxacin, SXT 5µg: Trimethoprim, AK 30 µg: Amikacin, CIP 5µg: Ciprofloxacin, CRO 30 µg: Ceftriaxone, and AMC 30 µg: Amoxicillin/ Clavulanate).

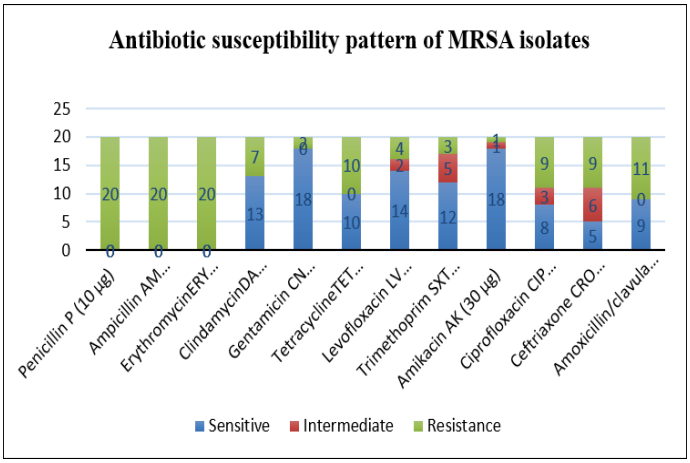


**Figure 3:** Antibiotic susceptibility pattern of MRSA isolates, which shows (sensitive blue), (intermediate red), and (resistant green).

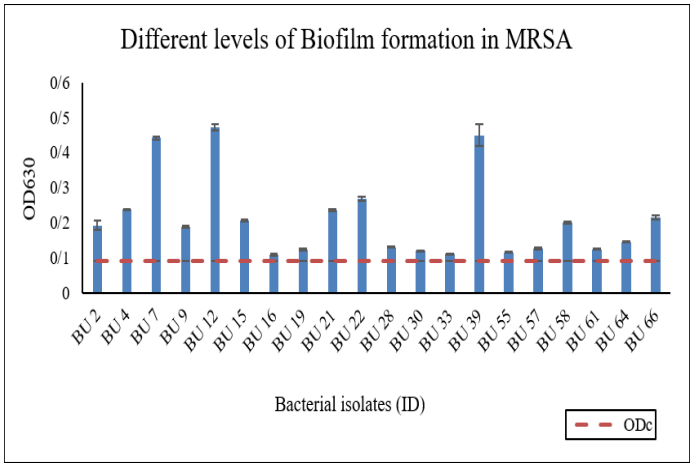


**Figure 4:** Different levels of biofilm formation among all 20 MRSA isolates.

## 3.5 Phenotypic detection of virulence factor

### 3.5.1 Biofilm formation among MRSA isolates

All 20 MRSA isolates were biofilm producers based on the cut-off values derived from the negative control, which had a mean OD630 of 0.089 and an ODc of 0.092. One-way ANOVA showed a significant difference in biofilm formation among MRSA isolates ($p < 0.001$). Nine isolates (45%) were weak biofilm producers, eight isolates (40%) were moderate biofilm producers, and three isolates (15%) were strong biofilm producers. The strongest biofilm formation was observed in BU12, BU39, and BU7, with mean OD630 values of 0.471, 0.449, and 0.441, respectively. (Figure 4).

In the tube method, the qualitative test also revealed that all isolates produce biofilms, as indicated by their adhesion to the tube wall and bottom observed after staining with crystal violet. In addition to Congo Red Agar and Modified Congo Red Agar, all 20 isolates had black, dry, and crystalline colonies.

### 3.5.2 Antibiotic Susceptibility Pattern in the Different Biofilm Producers

The antibiotic susceptibility patterns of all MRSA isolates were characterized for their biofilm-formation ability. Based on their biofilm formation, the studied isolates were grouped into Strong, Moderate, and Weak biofilm producers.

**Strong Biofilm formation**

MRSA strains classified as strong biofilm producers recorded higher resistance to several antibiotics, particularly ampicillin and penicillin. Resistance was also observed against erythromycin, tetracycline, and gentamicin. Furthermore, these strains exhibited moderate to low susceptibility to other antibiotics, such as levofloxacin and clindamycin, with distinct variation in susceptibility among the tested antibiotics.

**Moderate Biofilm Producers**

In this group, MRSA exhibiting moderate biofilm production showed lower resistance than strains with strong biofilm production; nevertheless, they still exhibited significant resistance to gentamicin and penicillin. Among this group, when compared with strains with strong biofilm production, their susceptibility rates were higher, indicating a moderate level of antibiotic susceptibility.

**Weak Biofilm Producers**

Methicillin-resistant *Staphylococcus aureus* (MRSA) strains with limited biofilm production capacity demonstrated greater susceptibility to a wide range of antibiotics. Specifically, these strains exhibited clear susceptibility to amikacin, trimethoprim, and clindamycin, as well as to other antibiotics, including ciprofloxacin and levofloxacin. These results suggest that strains

with weak biofilm-forming capabilities are more susceptible to antibiotic treatment compared to their robust counterparts.

The results indicate a clear correlation between biofilm formation and antibiotic resistance. It was observed that strains capable of forming strong biofilms exhibit higher resistance to various antibiotics, whereas strains producing weak biofilms demonstrate greater susceptibility to antibiotics. These findings underscore the significance of biofilm formation in the antibiotic resistance patterns of MRSA strains (Figure 5).

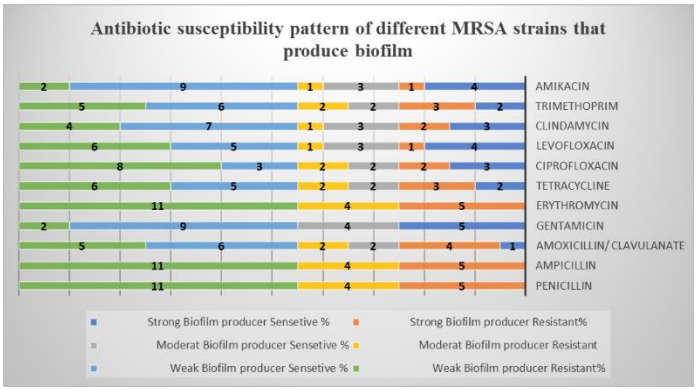


**Figure 5:** Antibiotic susceptibility pattern of MRSA strains that produce biofilm. Different.

### 3.5.3 Other Virulence Factors

Most MRSA isolates showed several virulence-associated enzymatic activities. These isolates confirmed hemolytic activity, the ability to lyse RBCs, and the production of lecithinase, protease, and urease. In addition, DNase activity was observed, reflecting their capacity to degrade extracellular DNA. Collectively, these phenotypic features highlight the pathogenic potential of the selected isolates, which indicate their ability to cause tissue damage, evade the immune response, and persist during infection.

### 3.6 Molecular detection of virulent genes *mecA*, *femA*, *clfA*, and *icaA*

The results of genetic detection for virulence factors showed that all 20 MRSA isolates possessed the genes *mecA*, *femA*, *icaA*, and *clfA* (Figure 6). The majority of lanes show a single clear band corresponding to the PCR product, confirming the presence of respective genes.

### 3.7 Phylogenetic tree analysis of the isolates

The Phylogenetic tree generated a distinctive clade of sequenced MRSA isolates, suggesting a potential common genetic origin among them. The clusters PV173439, PV173440, PV173441, PV173442, PV173445, PV173446, and PV173448 in the current study differed from the reference. While a more distinctive pattern was shown in the strains PV173443, PV173444, and PV173447, indicating an apparent genetic variation (Figure 7).

## 4 Discussion

Methicillin-resistant *Staphylococcus aureus* is one of the most common bacterial species that causes human disease, and the infection can be transmitted through communities as well as hospital environment [29]. Its clinical significance is related not only to methicillin resistance but also to the frequent association of multidrug resistance, biofilm production, and various virulence determinants, which together increase persistence, transmissibility, and treatment failure. Furthermore, people with immunodeficiencies, the elderly, and infants are more susceptible to infection with this organism [30]. In this study, MRSA was recovered from different clinical sources were investigated from 130 suspected patients. Overall, the incidence was recorded higher in urine samples than rest clinical samples [31].

Upon culturing, 116 isolates were obtained, of which 69 were Gram-negative. Among the rest, Gram-positive isolates, 30 were identified as *S. aureus*. Further antibiotic screening yielded 20 MRSA, which formed the base for the study.

OXDD and FOXDD results identified them MRSA, which was consistent with findings similar studies [10]. The broad resistance to penicillin, ampicillin, and erythromycin, together with variable resistance to tetracycline, ciprofloxacin, ceftriaxone, and amoxicillin/clavulanate, constitutes a clinically relevant multidrug-resistant phenotype [32]. Our findings align with previous research, indicating that the current isolates exhibit multiple forms of antibiotic resistance [33].

All isolates tested exhibited several phenotypic virulence traits, including hemolytic activity, protease, lecithinase, urease, and DNase production. These enzymes may enable tissue invasion, nutrient acquisition, immune evasion, and dissemination of the organism, thereby increasing their pathogenic potential. Such virulence-factor profiles have been reported in earlier studies of MRSA from clinical sources [34].

Biofilm formation was observed in all 20 isolates, which is also consistent with studies reported from Nepal (100%) [35] and Poland (99.2%) [36]. Although most isolates exhibit weak biofilm formation, some of them showed moderate or strong biofilm formation capacity, consistent with previous findings [37]. Biofilm formation is clinically important because it reduces antimicrobial penetration, promotes bacterial survival on tissues and medical devices, and facilitates chronic or recurrent infections.

Virulence of the organism is often reckoned by *mecA*, *icaA*, *femA*, and *clfA* genes. PCR detection of *mecA* confirmed that all 20 isolates possessed this gene. which encodes the altered penicillin-binding protein PBP2a, providing strong genotypic support for the MRSA phenotypic classification based on OXDD and FOXDD. This finding is in agreement with previous molecular studies that reported *mecA* gene as a reliable marker for MRSA identification [38]. The universal detection of *icaA* among the isolates further supports the biofilm-forming phenotype observed

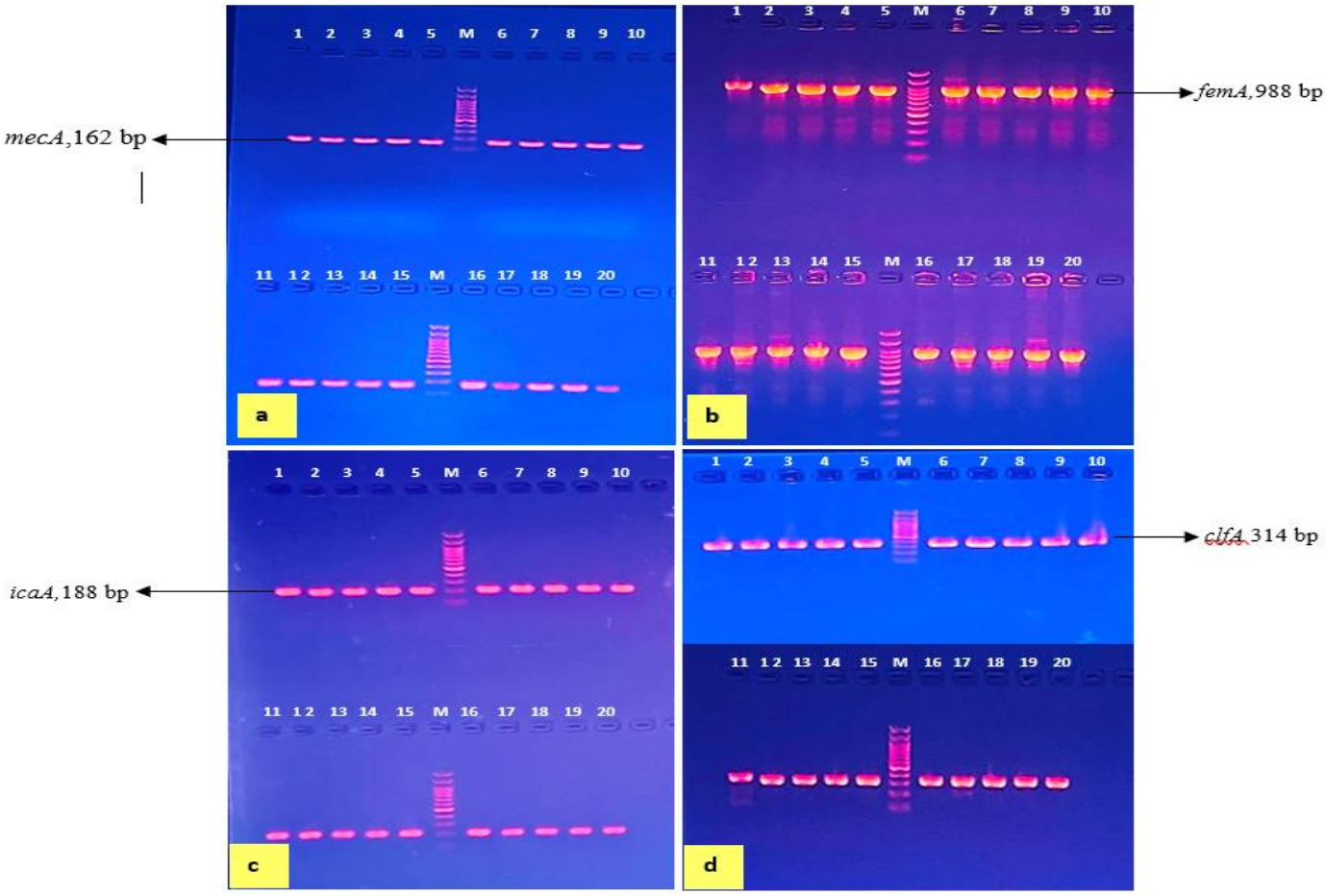


**Figure 6:** PCR amplification of the (a) *mecA*,162 bp (b) *femA*,988 bp (c) *icaA*,188 bp *and* (d) *clfA* 314 bp genes of MRSA.M: Marker (100-1500 bp ladder); Track 1: BU2; Track 2: BU4; Track 3: BU7; Track 4: BU9; Track 5: BU12; Track 6: BU15; Track 7: BU16; Track 8: BU19; Track 9: BU21; Track 10: BU22; Track 11: BU28; Track 12: BU30; Track 13: BU33; Track 14: BU39; Track 15: BU55; Track16: BU57; Track 17: BU58; Track 18: BU61; Track 19: BU64; Track 20: BU66.

in this study [39]. Similarly, *icaA* gene is involved in polysaccharide intercellular adhesin synthesis, and its presence may facilitate cell-to-cell adhesion and biofilm maturation. In addition, femA was detected in all isolates, supporting species confirmation that reflect conserved role of this gene in *S. aureus* cell-wall biosynthesis [40]. The *clfA* gene was also present in all tested isolates which is a fibrinogen-binding surface protein that promotes adhesion to host tissues and aid for colonization and virulence. Its detection in all isolates indicates a strong adhesive potential that partly explain ability of the bacteria to persist in diverse clinical infections. These results are in concurrence with studies that reported *clfA* gene among *S. aureus* isolated from bone, skin, joint, catheter-related, and soft-tissue infections [41,42].

Sequencing of the 16S rRNA gene confirmed the identity of the selected isolates as *Staphylococcus aureus* [43]. The phylogenetic analysis further indicated that several isolates clustered closely, suggesting their relatedness with other isolates that showed genetic divergence, indicating heterogeneity among circulating MRSA strains. Therefore, the combined phenotypic and genotypic findings demonstrate that MDR-MRSA isolates from clinical sources in Ramadi possess a substantial pathogenic profile characterized by antimicrobial resistance, biofilm production, enzymatic virulence, and key resistance through their adhesion genes. These findings reinforce the need for strict infection-control measures, routine molecular surveillance, and rationale in antibiotic use. Further studies using larger sampling and advanced typing methods is deemed essential to clarify transmission dynamics and guide targeted prevention strategies.

## Conclusion

This study presents the phenotypic and genetic description of multidrug-resistant methicillin-resistant *Staphylococcus aureus* (MRSA) isolated from diverse clinical sources in Ramadi, Iraq. Emphasizing urgent implications for infection control, the findings highlight the need for improved management, screening, and treatment strategies. Through VITEK 2 Compact characterization, all isolates were found to be resistant to cefoxitin and oxacillin, as determined by the FOXDD and OXDD methods, with considerable variability in antibiotic resistance. The MRSA isolates displayed diverse virulence factors, including hemolysis and the production of proteinases, lecithinases,

ureases, and DNases. All 20 isolates formed biofilms, exhibiting capacities ranging from strong to weak, and carried the *mecA*, *femA*, *clfA*, and *icaA* genes. These results underscore the need for extensive research towards developing faster treatment routines for patients, besides overall mitigation strategies for MRSA in the region.

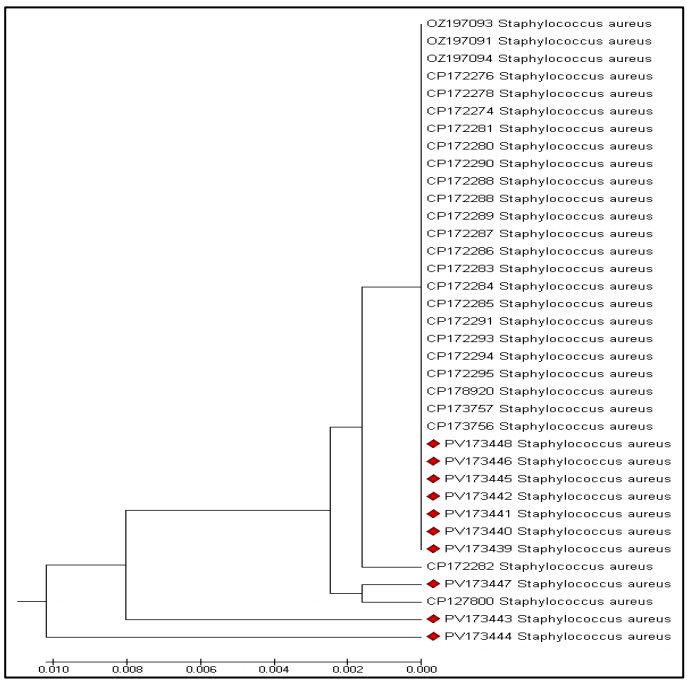


**Figure 7:** Phylogenetic analysis of the 16S rRNA gene amplified from select *Staphylococcus aureus* strains.

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## Authors' Contribution

FTH and SRP developed the research plan. FTH implemented the methodology, conducted the experiments, and processed the data. SRP supervised and managed the project, verifying the data. FTH wrote the manuscript, and SRP reviewed and edited it. The manuscript was read in its final form and approved for publication.

## Conflict Of Interest

The authors declare no conflict of interest.

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