Isolation, Molecular Identification, and Antimicrobial Susceptibility Testing of Staphylococcus aureus Isolates

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ABSTRACT

Staphylococcus aureus is a gram-positive pathogenic bacterium that can infect humans and animals. It has a high ability to develop resistance to several different antibiotics. In this study, 129 clinical specimens were collected from patients in three different hospitals in Baghdad during the period from October 13, 2020, to January 26, 2021. From all of these specimens, only 45 isolates showed cultural and biochemical characteristics that are similar to *S. aureus*. The identity of these isolates was further confirmed using specific primers for the *S. aureus* nuc gene. In addition, the susceptibility of these isolates to eleven virous antibiotics was studied. The results revealed that all *S. aureus* isolates demonstrated high resistance to cefoxitin, penicillin, and ceftazidime. On the other hand, the isolates showed virous susceptibility to the rest of the eight antibiotics. We also found that there were no antibiotics tested in this study that were able to kill all *S. aureus* isolates, and all isolates tested in this study were classified as MRSA.

Keywords: *S. aureus*; nuc gene; antibiotic; cefoxitin; penicillin; ceftazidime; MRSA

1. Introduction

Staphylococcus aureus appears to be a major cause of sickness and death in the world. A wide range of diseases exist, from simple skin infections to lethal pneumonia and sepsis. It generates a remarkable diversity of virulence factors. These contain plenty of toxins and immune evasion factors, as well as a multitude of protein and non-protein components that allow them to colonize and disseminate to various parts of the body during infection [1]. Bacteraemia caused by *S. aureus* has been found to kill more people than Human immunodeficiency virus, tuberculosis, and viral hepatitis combined [2]. Since the early 1940s, when penicillin resistance in *S. aureus* was first documented, *S. aureus* has gradually acquired resistance mechanisms, becoming resistant to nearly all current antibiotics [3].

Methicillin-resistant Staphylococcus aureus (MRSA) is one of the most successful modern pathogens, and *S. aureus* infections are particularly challenging due to recognizing resistance to antibiotics in *S. aureus* isolates. It can spread in both healthcare and community settings. MRSA epidemiology is primarily defined by the repeated emergence of epidemic strains due to its genetic diversity [4]. MRSA infections are associated with high mortality, morbidity, and hospital stay as compared to methicillin-sensitive *S. aureus* (MSSA) infections [5]. Treatment of *S. aureus* infections is difficult due to antibiotic resistance and a lack of effective vaccines that act against it [1].

S. aureus has a diverse set of virulence factors at its disposal (that are adhesive, damage the host-cell, and immune evasion molecules). They differ in their expression or specificity within clones, a variation mirrored in the wide range of infections caused by *S. aureus* [6]. Because several virulence genes are located within mobile genetic elements, their mixture varies significantly among clones and between strains that are genetically related. The potential of various virulence factors to be linked to specific types or levels of aggressiveness within *S. aureus* infections is still unclear. Perhaps this is due to the fact that several of these elements perform redundant or partially overlapped activities. Furthermore, since many virulence factors seem human-specific, they cannot be studied in experimental animals [7].

S. aureus virulence factors are frequently encoded in the pathogen's accessory-genome, which is distinct from the core-genome, which is primarily responsible for "housekeeping" activities. Mobile genetic elements (MGEs) such as transposons, plasmids, insertion sequences, pathogenicity islands and prophages, which carry antibiotic resistance determinants in addition to virulence factors, are found in the accessory-genome [8]. This study aimed at the isolation, molecular identification, and antimicrobial susceptibility testing of *S. aureus* from different clinical sources.

2. Methodology

2.1. Isolation and Identification of *S. aureus*

Using sterile swaps, clinical specimens were collected from blood, nasal, ear, wound, oral, pus, and urine infections. The specimens were taken from three different hospitals (Baghdad Teaching Hospital, Al Shaheed Ghazi Al Hariri Hospital, and Al-Imam Al-kadhimeen educational hospital) in Baghdad. The specimens were transferred directly to the laboratory. At 37°C for 24 hours, Mannitol salt agar (MSA) and blood agar media were used for the cultivation of *S. aureus*. The grown bacterial colonies were subjected to various biochemical tests (Catalase, Oxidase, and Coagulase Tests) to ensure their identity [9].

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2.2. Molecular Identification of S. aureus

2.2.1. Genomic DNA Extraction

The *S. aureus* genomic DNA was extracted using Presto ™ Mini gDNA Bacteria Kit (Geneaid, Taiwan) following the manufacture instructions.

2.2.2. PCR Experiments

A set of specific primers for *S. aureus* nuc gene forward 5'-GCATTGATGGTACGGTT-3'and revers 5'-AGCCAAGCCTTGACGAACTAAAGC-3' were used in this study [14]. The gene was amplified using Master Cycler gradient PCR (Techne, England) and GoTaq® G2 Green Master Mix (Promega, USA) in a total reaction volume of 50 µl. The PCR machine was programmed according to the following conditions (Table 1).

Table 1: PCR reaction conditions

Step	Temperature(°C)	Time	No. of cycles
Initial denaturation	95	2 min.	1
Denaturation	95	30s	35
Annealing	54		
Extension	72		
Final extension	72	5 min.	1
S=second min.= minute			

The PCR products were analyzed on an agarose gel (1%) and stained with ethidium bromide (0.5 µg/ml). From the PCR product, 5 µl was loaded into each well. Then, the electrophoresis chamber was connected to a power supply, and the gel was run at 5–7 V/cm for 80 min. When the run came to an end, the gels were transferred to the BIO-RAD Gel Imaging System to visualize and photograph the DNA bands.

2.2.3. DNA Sequencing

The PCR products of both nuc gene was sequenced by Macrogen company (in South Korea), and the result of the sequence data was processed by using Bio-Edit software. And then the blast of each sample sequence in the NCBI database to find the closest match that exists.

2.3. Antibiotic Sensitivity Test

The antibiotic sensitivity test was done following the disk diffusion method [10]. Using a sterile loop, 4-5 colonies were suspended in 2 mL of sterile normal saline. The bacterial suspension's turbidity was adjusted to a McFarland standard of 0.5. The bacteria were then inoculated into the mueller-hinton agar plate by using a sterile swab. The plates were left at room temperature for about 5 minutes. The following antibiotics were used: Cefoxitin (30µg), Ceftazidime (30µg), Penicillin G (10 U), Clindamycin (2µg), Erythromycin (15µg), Azithromycin (15µg), Ciprofloxacin (5µg), Tetracycline (30µg), Trimethoprim/ Sulfamethoxazole (1.25/23.75 µg), Gentamicin (10µg) and Rifampin (5µg). Using sterile forceps, the antibiotic discs were placed and fixed on the surface of the cultivated agar. Finally, the plates were inverted and incubated at 37°C for 18 h.

3. Results and Discussion

In this study, from 129 clinical specimens that were collected from different places in Baghdad and from different clinical sources during the period from 13/10/2020 to 26/1/2021, only 45 isolates (34.88%) were given the culture characteristics, biochemical and molecular tests that are specific to staphylococcus aureus. The distribution of staphylococcus aureus isolates in clinical samples based on sample source is shown in Table-4, which indicates the ability of *S. aureus* to cause diseases in most parts of the body.

Table 4: The distribution of *Staphylococcus aureus* isolates in different clinical sources.

Source	No. Isolates
Blood	10
Nasal	10
Ear	7
Wound	6
Oral	6
Pus	4
Urine	2
Total	45

3.1. Cultural Identification

The round and golden colons was recognized on Mannitol Salt Agar (MSA) as well as shift the color of media from light red to yellow, see figure-1A. on blood agar, it forms yellow, round, moderate-sized colonies surrounded by a clear zone, indicating the ability of bacteria to secrete β-hemolysin and complete lysis of the blood cells in the media, see figure-1B.

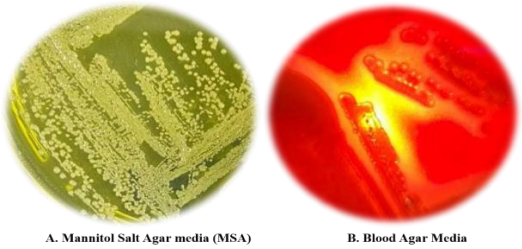

Figure 1: Growing of S. aureus colonies on MSA (Mannitol salt agar) and blood agar media after 24 hours of incubation on 37°C.

3.2. Biochemical Identification

The appearance of bubbles after the addition of hydrogen peroxide reagent to bacterial samples indicated that the catalase reaction was successful; see figure-2A, showing the breakdown of H_2O_2 to oxygen and water. The catalase enzyme's primary function is to prevent toxic levels of H_2O_2 from accumulating, which is used to distinguish the staphylococcus genus from the Streptococcus genus [11]. The coagulase positive reaction was given. After incubating the bacterial samples with plasma for 4 to 18 hours, a clotting formation was detected compared to the negative control (see figure-2B), which was used to differentiate *S. aureus* species from other Staphylococcus species [9]. Coagulation is caused by the coagulase enzyme, which converts fibrinogen to fibrin and causes clots to form in plasma or blood. It is thought to play a role in the creation of abscesses in host tissues as well as the pathogen's ability to induce deadly sepsis [12].

When adding the bacterial samples to the oxidase reagent absorber by filter paper, see figure-2C, a negative result

of the oxidase reaction was determined by no color change as compared to the positive control [9].

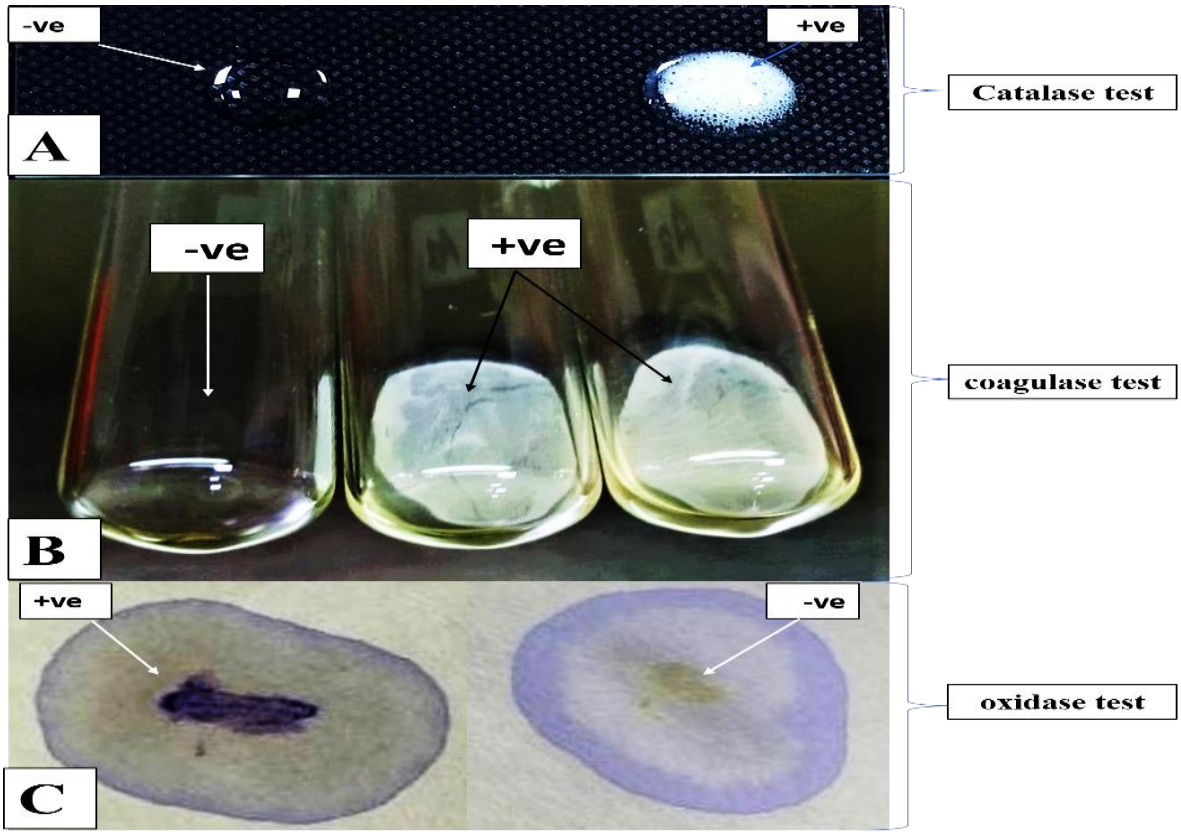

Figure 2: primary identification of S. aureus by three biochemical tests: A = catalase, B = coagulase and C = oxidase. -ve = negative and +ve = positive result.

3.3. Molecular Identification of Staphylococcus aureus (S. aureus)

S. aureus isolates were previously identified by culture characteristics (MSA and blood agar) and biochemical assays (catalase, coagulase, and oxidase tests). Furthermore, confirmation of *S. aureus* was done by molecular identification based on amplifying a conserved region in the nuc gene encoded for the thermo stable nuclease enzyme by using specific primers (nuc primers) for confirming the identity at the species level. Only *S. aureus* could be recognized by the nuc primer from other staphylococci [13, 14]. The PCR product of the nuc gene appears in the form of a single band of DNA, with a molecular size of about 279 bp [15] compared with the DNA ladder, see figure 3.

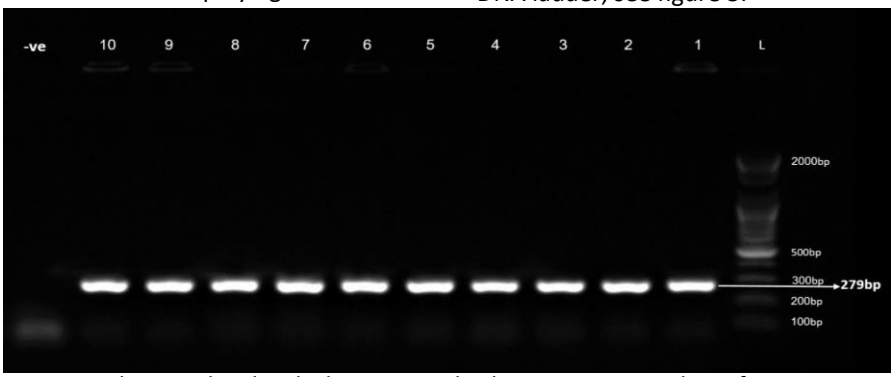

Figure 3: Agarose gel stained with Ethidium Bromide showing PCR products for nuc gene (279bp), the electrophoresis was run across 1% agarose gel at 70 volts for 90min. Line (L) for DNA ladder (100bp), Line (-ve) for Negative control.

3.3.1 DNA Sequencing and Data Analysis

The nuc gene PCR products for the forward and reverse strand were sequenced by Microgen Company (South Korea) to further corroborate the *S. aureus* identification The result of the PCR amplification size for the nuc gene in this investigation was 279bp. The nuc gene sequences were examined. Using the Bio-Edit program, cut out the distorted ends of the DNA strains and fill in the missing bases based on a comparison of forward and reverse strains. Following the processing of the data, the nuc gene sequences are compared to other sequences in the NCBI database using the BLASTn-database to determine their similarity to other sequences in the database. The blast of nuc gene sequences yielded results that were 99.64% to 100% identical to *S. aureus* species (Table-5 and Figure-4).

Table 5: The blast results of nuc gene sequences in NCBI-BLASTn database.

Query		Subject				Identities (%)
No. of isolates	S. L	Description	S. L	Start of alignment	End of alignment	
1	279	Staphylococcus aureus strain 277 genome	2749120	355576	355854	100%
2	279	Staphylococcus aureus strain 277 genome	2749120	355576	355854	100%
3	279	Staphylococcus aureus strain GHA4 chromosome	2809532	852386	852108	100%
4	279	Staphylococcus aureus strain GS426 genome	2673223	1795223	1795501	99.64%
5	279	Staphylococcus aureus strain 277 genome	2749120	355576	355854	100%
6	279	Staphylococcus aureus strain 277 genome	2749120	355576	355854	100%
7	279	Staphylococcus aureus strain 277 genome	2749120	355576	355854	100%
8	279	Staphylococcus aureus strain GHA4 chromosome	2809532	852386	852108	100%
9	279	Staphylococcus aureus strain 277 genome	2749120	355576	355854	100%
10	279	Staphylococcus aureus strain 277 genome	2749120	355576	355854	100%
Note: No.= Number, S. L = Sequence Length						

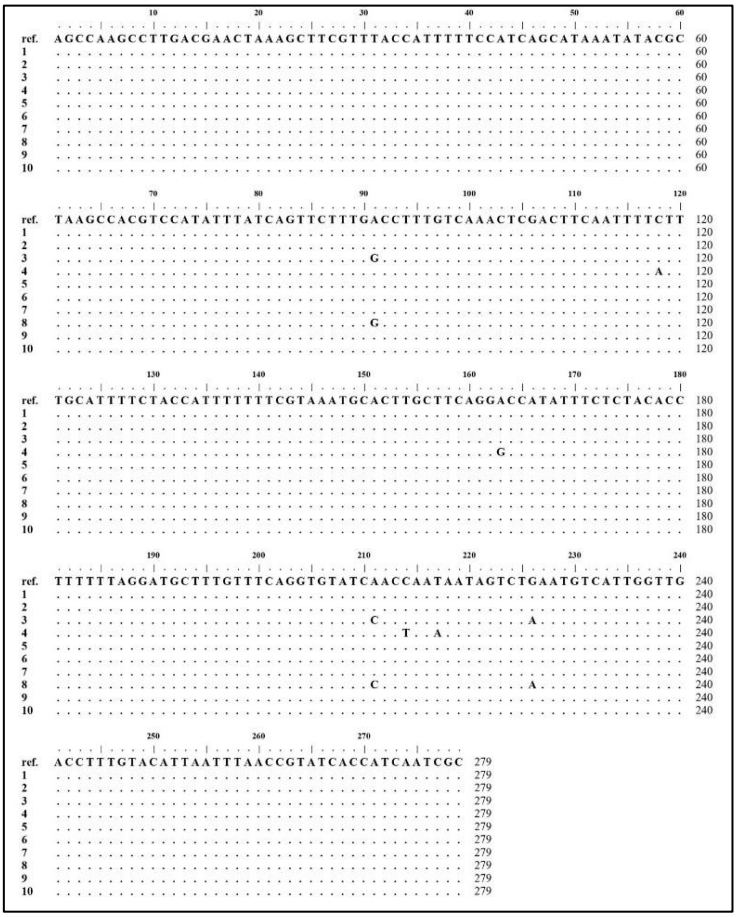

Figure 4: DNA sequences alignment of S. aureus isolates with its corresponding reference sequence of the nuc gene by Bio-Edit software, ref. = reference sequence of nuc gene of S. aureus strain 277.

3.4. Antibiotic Susceptibility of *Staphylococcus aureus*

The Disk Diffusion Test, was used to investigate the susceptibility of forty-five *S. aureus* isolates to different types of antibiotics (11 types). The results of inhibition zone diameter were explained based on CLSI Standards [16], as indicated in figure-5, and table-6.

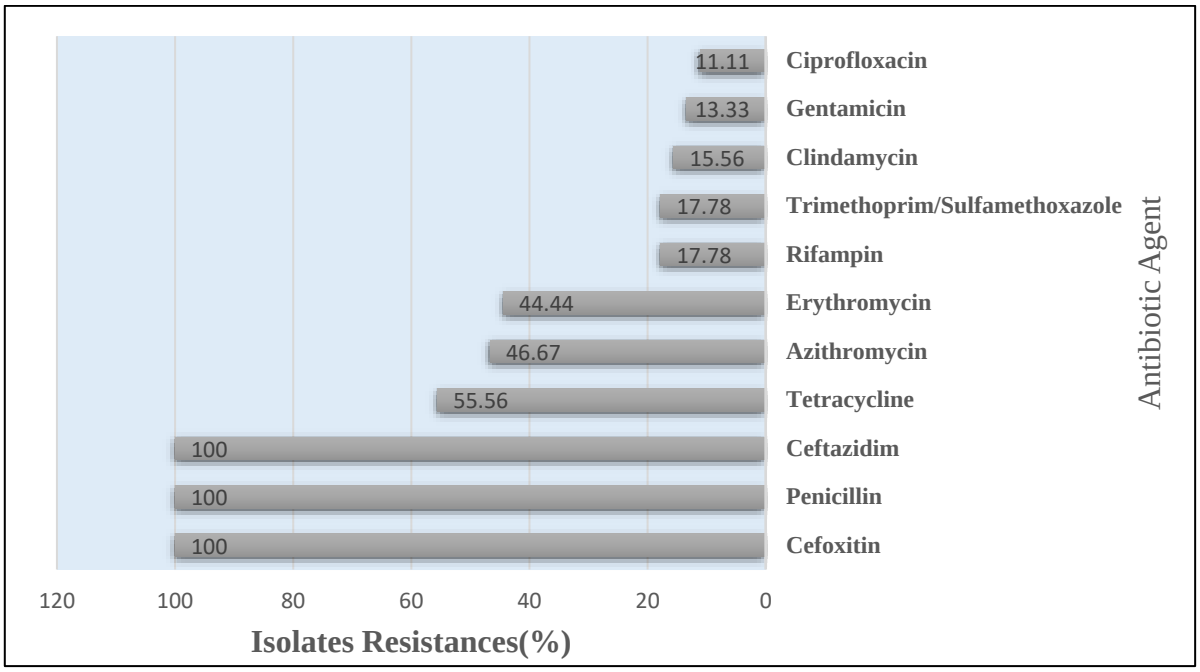

Figure 5: Antibiotic susceptibility of S. aureus isolates to different antibiotics.

Table 6: Antibiotics Susceptibility of S. aureus isolates and percentage of each antibiotic

Antibiotic	No. R	R%
Cefoxitin	45	100
Penicillin	45	100
Ceftazidim	45	100
Tetracycline	25	55.56
Azithromycin	21	46.67
Erythromycin	20	44.44
Rifampin	8	17.78
Trimethoprim/Sulfamethoxazole	8	17.78
Clindamycin	7	15.56
Gentamicin	6	13.33
Ciprofloxacin	5	11.11
R = Resistance.		

Three types of antibiotics (Cefoxitin, Penicillin, and Ceftazidim) were completely ineffective against *S. aureus* isolates. The cefoxitin resistance test is used for the identification of methicillin (oxacillin) resistant *S. aureus* (MRSA) as a phenotypic detection for PBP2a protein or mecA gene [16-18]. Consequently, all isolates that were tested in this study are categorized as MRSA.

4. Conclusion

S. aureus is considered one of the most dangerous pathogenic bacteria that can infect different parts of the human body, causing serious diseases. One of the reasons that increase the importance of the bacteria is its ability to rapidly develop a resistance to many different types of antibiotics. For instance, all isolates that were tested in this study are categorized as methicillin resistant. *S. aureus* (MRSA) is multidrug resistant and has become a real problem, especially in hospitals. The PCR primers that were used in our study for amplification of the *S. aureus* nug gene were very efficient and applicable for fast diagnosis of the bacteria.

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