Phenotypic And Molecular Characterization of Methicillin-Resistant Staphylococcus Aureus Isolated from Clinical Samples in Samarra City

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Abstract
The study was conducted in the laboratories of the Department of Life Sciences - College of Education / Samarra University and the laboratories of the Department of Pathological Analyzes - College of Applied Sciences / Samarra University for the period from November 5/11/2021 to February 5/2/2022 by collecting 100 samples from (swabs of burns, wounds, skin infections and inflammation tonsils) from patients arriving at Samarra General Hospital, and according to the phenotypic, cultural and biochemical characteristics, 55 isolates (55%) were identified as Staphyloccocus. aureus.Methicillin-resistant Staphylococcus aureus MRSA was diagnosed by methicillin disc spread and the results showed that among 55 S. aureus isolates, 20 isolates (36%) were diagnosed as methicillin-resistant while 35 isolates (64%) were sensitive to methicillin.The results showed that the highest percentage of MRSA was in burn samples (45%). DNA was extracted from MRSA isolates and PCR technique was used to determine the presence of the MecA resistance gene, and the MecA gene appeared in all MRSA isolates. The study showed that the highest prevalence of MRSA bacteria was in the first age group, young at age, at a rate of (45%), then followed by the second and third age groups, at a rate of (30%) (25%). The study also showed that the percentage of MRSA isolates among females carrying S. aureus bacteria was greater than its percentage among males, as it was in females by (60%), while the percentage of its presence in males was (40%).
Keywords: clinical samples; MARSA, P.C.R, MecA

1. Introduction

Staphylococcus ureus is one of the most well-known and widespread bacterial pathogens (1). Although this bacterium is often part of the normal flora, it is found in the nose and throat of (20-70%) of adults and on the skin without No symptoms (2) but it has the potential to cause a variety of infections ranging from mild local infections of the skin and soft tissues to severe systemic infections such as endocarditis, toxic shok syndrome (TSS), and osteomyelitis, which may be fatal (3) Certain strains of this bacteria have developed a resistance known as Methicillin-resistant S.aureus, and MRSA is a source of great concern in the community, as the number of cases of it has increased worldwide and now constitutes 90% of human Staphylococcus aureus (4). MRSA is generally resistant to many antibiotics and has appeared among people in hospitals, nursing homes and other health care institutions since the 1960s. It is difficult to treat due to the high number of drug-resistant strains. These methicillin-resistant bacteria are determined by genes, including the mec A gene (5).

2. Materials and methods

Collection and identification of bacterial isolates

Clinical samples were collected by 100 samples of different ages and for both sexes from patients arriving at Samarra General Hospital and attending private clinics for the period from November 5/11/2021 until February 5/2/2022 The samples were collected using sterile cotton swabs container on a transport medium, the samples were planted after They were collected on Mannitol salt agar medium as a differential and selective medium for the isolation of S.aureus bacteria and Blood Agar medium. Then the dishes were incubated under aerobic conditions at a temperature of 37 °C for 24 hours. After the incubation process, morphological and biochemical tests were performed. Mannitol salt aga medium and Blood Agar medium in terms of shape, size, color, edge and its dissolution to the blood. A microscopic examination was also carried out. A number of biochemical tests were conducted, such as the catalase test, the oxidase test, the hemolysin production test, the mantol sugar fermentation test, and the investigation of the coagulant enzyme based on its coagulase. (6.).

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Diagnosis of methicillin-resistant Staphylococcus aureus (MRSA)

Diagnosis by diffusion of methicillin tablet

The purified bacteria were cultured on Muller-Hinton's medium and incubated at 37°C for 18–24 hours to determine the methicillin-resistant species using the antibiotic (methicillin) (7).

Molecular diagnosis by polymerase chain reaction (PCR) method

A- DNA extraction according to the manufacturer's protocol **(Taiwan-Geneaid)** According to the method described by (8)

B- Measuring the concentration of DNA

The measurement of DNA concentration and purity for all samples was determined by using a (Nanodrop) device. 1 µl of the extracted DNA was placed and mixed with 199 µl of diluted Quantifluor dye after 5 minutes of incubation at room temperature, then the DNA concentration values were detected(9).

C- Primer preparation

The mecA gene primers prepared from lyophilized mecrogen were dissolved in Nuclease free water to give a final concentration of 100 picomoles as the stock solution. Store at 20° C. In order to avoid repeated freezing and thawing, the working solution was prepared by adding 10 µl of the storage solution to 90 µl of Nuclease free water to obtain a solution with a concentration of 10 pmol/µl and stored at 20 ° C.

D- PCR Reaction Mixture

The main reaction mixture for DNA samples was prepared for the PCR polymerase chain reaction by mixing the reaction components in tubes to obtain a final volume (20) µl as shown in Table (1). The reaction solution drops on the wall of the tube and then the tubes are placed in the thermocyer to start the reaction according to the steps of the specified program.

Table (1) of the main reaction components of the PCR technique

Volume (µl)	Component	
6	Nuclease-free water	1
10	(2x) Master mix	2
2	(25-50 ng) DNA sample	3
1	(10Pmol/µl) Forward primer	4
1	(10 Pmol/µl) Reverse primer	5
20	Total volume	6

E- Molecular detection of the mecA. gene

Molecular detection of the mecA gene, which is responsible for the resistance of S.aureus bacteria to the antibiotic methicillin and other types of beta-lactams, was carried out.

Table: (2) Optimum conditions for the detection of the mecA. gene (10)

Cycles	Time	Temperature (°C)	Steps	
1	5 min	95	Initia denaturation	1.
30	30 sec	95	DNA Denaturationl	2.
	30 sec	60	Annealing	3.
	30 sec	72	Extension	4.
1	7 min	72	Final extension	5.
	10 min	10	Hold	6.

F- Preparation of agarose gel

Acarose gel was prepared at a concentration of 1.5% and used to ascertain the size of the DNA bundles, by dissolving 1.8 g of agarose in 100 mL of 1X TBE buffer. Heat the agarose using a microwave oven to boiling and cooled to 55-50 °C, then 1 µl of dye was added to it. Ethidium bromide at a final concentration of 0.5 µl/ml, the solution was then added to the Tray vessel of the comb-containing electrophoresis to form a pit wall and left to cool for 30 minutes to solidify. Approximately 1-2 mm covering the entire surface of the agarose gel (11)

G- Electrophoresis for the detection of bacterial DNA

Agarose Gel Electrophoresis
Transfer 6 microliters of the PCR product (Ampilcon) to the holes designated for it and also transfer 3 microliters of the volume guide solution (1500-100) base pair Ladder, which is used to determine the sizes of the phased DNA pieces, then pass an electric current at a voltage of 100 volts for a period of time 80 minutes, then examine the agarose gel using a UV-Transilluminator at a wavelength of 260 nm.(12)

3. Result and discussion

1- Isolation

100 samples were collected from patients with infections of burns, wounds, skin infections and tonsillitis in Samarra General Hospital for the period from 5/11/2021 to 5/2/2022, 55 (55%) were isolated from S.aureus, the result agrees with (13), the number of isolates reached 68, with a percentage of (96.53 percent) of the total isolatesal samples, also in agreement with a similar study (14), which obtained (50%) of the total samples, and the fact that bacteria are one of the main causes of hospital infections, this is due to their increase in the samples taken (15), in addition to the fact that S. aureus has the ability to develop strains resistant to antibiotics and produce enzymes Beta-lactamases, and the reason is also due to their ability to multiply rapidly and to possess many virulence factors such as the production of toxins, Toxins, enzymes that include Deoxy ribonuclease, Catalase, Coagulase, Lipase, Enterotoxin, PVL, Leukocidin, Hemolysin, Surface proteins, Hyaluronidase, the capsule that plays a surface role. important in invading host tissues The spread of bacteria, and the β-lactamase enzymes

that these bacteria possess contribute to their resistance to many different antibiotics (16). These bacteria constitute 30% of the bacteria naturally present on the skin and mucous membranes lining the nose. Opportunistic Bacteria causes many diseases ranging from simple skin infections such as boils and impetigo to life-threatening diseases such as bacteremia and endocarditis (17). Table (3) shows the type of disease, number of samples, numbers and percentages of S isolates.aureus and MRSA.

Twenty (36%) isolates of S. aureus gave resistance to methacillin after growing on Muler Hinton agar medium. Figure (1) shows the percentage of emergence of S.aureus and MRSA. in the initial isolation. The obtained results were close to what was reached (18), where the percentage of MRSA isolates from S.aureus bacteria was (47.19%) and less than reached (19) where the percentage was (66%) while (7) the rate of MRSA was (84%), and the reason for the emergence of these numbers and percentage may be due to the fact that the majority of those infected with these infections resort to taking broad-spectrum antibiotics without consulting the specialist. The ease of taking antibiotics from available pharmacies and the low cost of these antibiotics due to their poor origins make it easier for the patient to take treatment and use it sometimes, according to his mood. This is attributed to the high percentage of MRSA isolates (45%), as burn swabs were taken from patients lying in the hospital. This confirms that resistant bacteria are widely present in the hospital environment. The most common complications that occur in the hospital are called hospital-acquired infections. A reason for the spread of these bacteria (20)

Table (3): Type of infection, number of samples, numbers and percentages of S.aureus and MRSA					
percentage	The number of MRSA isolates	percentage	Number of S.aureus isolates	number of samples	The type of idsease
45.00%	9	25.45%	14	21	burns
25.00%	5	18.18%	10	19	wounds
10.00%	2	20.00%	11	20	skin infections
20.00%	4	36.36%	20	40	tonsillitis
100.00%	20	100.00%	55	100	Total

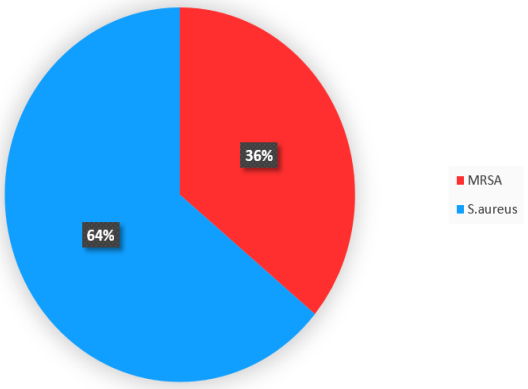

Figure (1) Percentage of appearance of S.aureus and MRSA in primary isolate.

for Table (4) it shows the numbers and percentages of S.aureus and MRSA bacteria by sex. The results showed that the prevalence of MRSA that the percentage of MRSA isolates isolated from males carrying S.aureus bacteria was (40%), while it was from females. (60%) that the obtained results were close to what was reached (13) and by (55.11%) females were isolated, and (44.89%) were isolated from males.the percentage of infection difference between the sexes may be due to the quantity and quality of the natural flora in the bodies of the different sexes, as well as the difference in the method of collecting samples for both sexes during the study (21)..

Table (4) Distribution of S.aureus and MRSA bacteria by sex				
Sex	Isolated S.aureus number	percentage	MRSA number	percentage
Male	30	54.5	8	%40
Female	25	45.5	12	%60
Total	55	100	20	%100

Table (5) shows the numbers and percentages of the distribution and prevalence of S.aureus and MRSA by age groups. The patients were divided into three age groups (10-29) (30-49) (≤50) and the results showed that the highest rate of MRSA infection is in the first age group (45%), and this percentage decreases as we progress in age groups and (%) 25) (30%) for the second and third age groups, respectively.

These obtained results were similar to what was reached (19), where the percentages were (50%) (41%) (33.3%) for the first, second and third age groups, respectively. Likewise, it is similar to what was reached (22). All of the MRSA isolates that were isolated were in the first age group.

Table (5) Numbers and percentages of the distribution and prevalence of S.aureus and MRSA by age groups.					
	Age group	Number of S.aureus	percentage	Number of MRSA	percentage
1	29-10	30	%54.5	9	%45
2	49-30	15	%27.3	6	%30
3	≤50	10	%18.2	5	%25
Total	-	55	%100	20	%100

2- *S.aureus* Laboratory diagnosis

S.aureus were identified morphologically, microscopically and biologically to confirm the isolated species, as shown in Table (6)

Table (6) Results of culture, microscopic and biochemical tests to diagnose S.aureus

Test type	The result
Agricultural properties on: mannitol medium, blood agar medium	golden yellow colonies Complete hemolytic cream
microscopic properties	Spherical in shape, in clusters or pairs
Coagulase	+
Oxidase	-
Catalase	+
(+) the result is positive for the test, (-) the result is negative for the test	

Colonies appeared on solid local manitol salt agar in a circular shape, smooth edges, slightly raised on the surface, golden in colour. As this medium is considered one of the selective and differential media for isolating the bacteria S. aureus, where Staphylococcus aureus, S. aureus, is fermented for mannitol sugar and produces acid, and then leads to an increase in the acidity index, leading to the conversion of the color of the medium from red to yellow, and this is consistent with (23) As for the colonies isolated and grown on blood agar medium, they are large, with a creamy color close to golden, surrounded by a clear transparent halo, indicating hemolysis. This result is consistent with (24) that it was found that S. aureus isolates produce B. haemolysin enzyme.

The results of the microscopic examination of all the glass slides prepared for 55 isolates after staining them using Gram stain showed that the bacteria cells are single or arranged in pairs or in the form of tetramers or are arranged in the form of clusters resembling grapes, which are positive for the Gram stain as they appear in violet color and this corresponds to (7). All isolates of S. aureus bacteria were positive for the catalase test, as this test is important in distinguishing Streptococci (negative for the catalase test) and Staphylococcus aureus (positive for the catalase test), as all the isolates produced the catalase enzyme that catalyzes the decomposition of hydrogen peroxide into water and oxygen (25). These results are consistent with (18), where all isolates were positive for the catalase test. All isolates of S. aureus bacteria were negative for the oxidase test, and the negative result in this test is caused by the inability of S. aureus to change the blue or violet color of the oxidase detector, and this means the inability to have the cytochrome oxidase that stimulates the transfer of electrons from these donor compounds to the electron acceptors (26). All S. aureus isolates were positive for this test. A Coagulase blood clotting test was conducted to distinguish between S. aureus that is positive for this test, that is, it is able to produce the Coagulase enzyme and convert plasma into a thrombus, and Staphylococcus aureus that is negative for the Coagulase test (27). And that the production of this enzyme is a criterion for the diagnosis S. aureu (28)

3- Identification of Methicillin Resistant S.aureus

Method of spreading the antibiotic Methicillin

The results of Figure (2) show that all 55 isolates of S.aureus were tested for resistance to the antibiotic thistle and it was found that out of 55 isolates of S.aureus, 20 isolates (36%) were MRSA, and this result does not agree with what was found by (7). It was found that out of 46 S.aureus isolates, 39 (84.8%) isolates were MRSA.

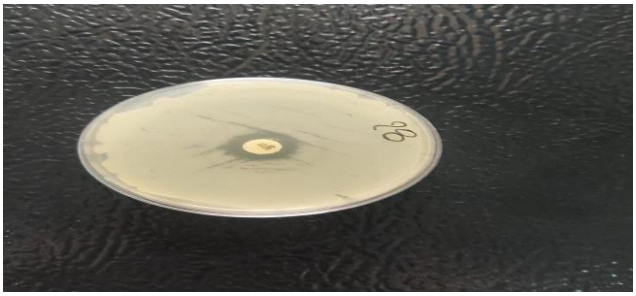

Figure (2): shows the mode of spread of the antibiotic Methicillin.

Diagnosis Using Polymeras Chain Reaction molecular.

A- DAN extraction Figure (3) shows that the DNA extraction method is effective for all 20 MRSA isolates. These isolates were grown on the brain heart infusion broth for 24 hours, in order to increase the numbers of bacterial cells and then increase the transcription of the results of the DNA target template for the binding of complementary primers in the PCR reaction (29). After that, the DNA was extracted using the solutions supplied by Promga Companywhich proved its accuracy and effectiveness in extracting DNA. (30) indicated that the best DNA purity is confined between (1.7-2.0) and it has been proven that this purity was obtained using a spectrophotometer. The average concentrations of DNA for the extracts were (25.35) nm. As well as the appearance of carryover DNA bands on 1.5% agarose gel before amplification using P.C.R technique.

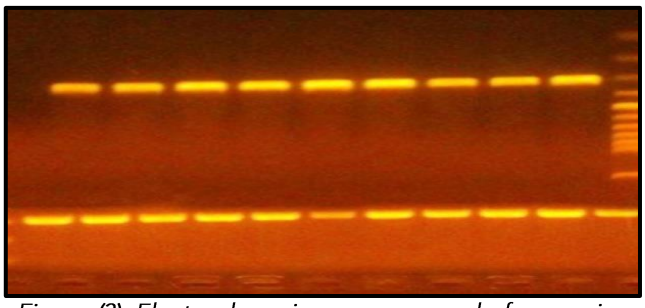

Figure (3): Electrophoresis on agarose gel of genomic DNA samples of MRSA isolates

B-Genetic investigation of the Mec A gene using the Polymerase Chain Reaction P.C.R

Figure (4)(5) show the results of electrophoresis of DNA extracted from the 20 isolates on agarose gel at a concentration of 1.5% after amplification using the polymerase chain reaction (P.C.R.) technique and based on the forward and reverse primers mec A to identify the corresponding sequence In the gene responsible for the character of antibiotic resistance, the results obtained showed the emergence of gene bundles by (100%), that is, 20 out of 20 MRSA isolates that have this gene appeared within the expected region for this gene (310 base pairs) and according to the results of P.C.R, from Among 55 S.aureus isolates, 20 isolates were identified as mecA-positive and 35 mecA-negative. The confirmed isolates of MRSA through P.C. R can be compared with the initial identification of MRSA by the method of metecillin disc diffusion, where the study showed that the 55 isolates of S.aureus In it, 20 isolates were resistant to methicillin MRSA, a percentage (36%) of the two methods, and these results differ from the results of (19). Where the percentage of MRSA by the traditional method was 66.6% and by using P.C.R (37.1%), that is, the results of the P.C.R are less than the results of the traditional methods, and these results are consistent with the results of (7), where the percentage of isolated MRSA by the disc diffusion method was found (84.4 %) and all of them gave a positive result for mec A by the P.C.R method and also almost corresponds to (18) where 42 isolates of MRSA were isolated by the high chro agar medium method and 41 isolates were positive for mec A. The mec A gene is the special genetic relationship for the discovery of MRSA (31) identification of the mec A gene is the most reliable method for detecting MRSA isolates (32)

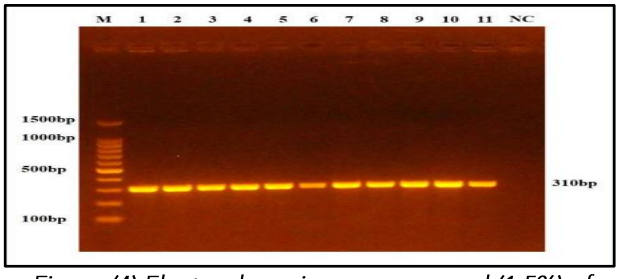

Figure (4) Electrophoresis on agarose gel (1.5%) of mecA gene amplifiers of MRSA isolates for 1 hour at a voltage of 100 bp

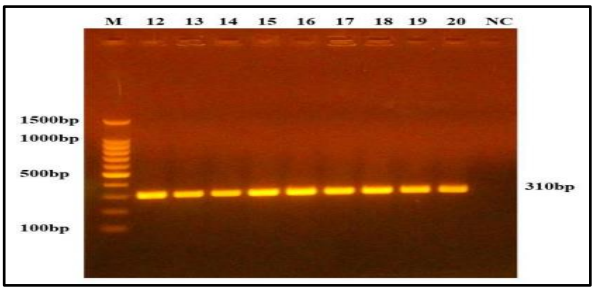

Figure (5) Electrophoresis on agarose gel (1.5%) of mecA gene amplifiers of MRSA isolates for 1 hour at a voltage of 100 bp

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