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Bacteriological and molecular study of *Staphylococcus aureus*

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ABSTRACT

Background: In the United States, approximately 400,000 patients are infected with *S. aureus* in hospitals each year, and approximately 100,000 of these die from complications caused by the infections (Haque et al., 2018). *S. aureus* remains a deadly pathogen for humans, more than a century after it was identified. S.aureus infections continue to be associated with high morbidity and mortality, both in hospitals and in the community, A total of 200 fresh urine samples were collected from both male and female patients with UTI of different ages included in this study during February-June 2021. Of the sampled patients, 133 (66.5%) showed positive growth. No significant growth was observed in the remaining 67 patients (33.5%). Demographic information of patients who applied to AL-Hussein Teaching Hospital with symptoms of UTI, The current findings of this study showed that 133 (66.5%) isolates had positive growth of the pathogen collected from UTI patients, while 67 samples (33.5%) showed no significant growth. Statistically, there were no significant differences in bacterial growth distribution ($P \leq 0.01$). The distribution of patients according to gender and bacterial growth Thereupon, 85 suspected isolates, seen under the microscope as G(+) with the coke shape mainly grouped in clusters, compared to ten mannitol salt agar, were able to ferment mannitol and produced a yellow color due to acid production. They were catalase positive due to the production of the enzyme catalase, which distinguishes them from Streptococcus spp., and they were coagulase positive due to the production of the enzyme staphylothrombin, which reacts with prothrombin and forms staphylothrombin.

Introduction

In the United States, approximately 400,000 patients are infected with *S. aureus* in hospitals each year, and approximately 100,000 of these die from complications caused by the infections **(1)**. *S. aureus* remains a deadly pathogen for humans, more than a century after it was identified. S.aureus infections continue to be associated with high morbidity and mortality, both in hospitals and in the community, despite continuous improvements in patient care. The clinical and genetic epidemiology of *S. aureus* infections has changed significantly as community-associated methicillin-resistant *S. aureus* has become more common in the last two decades **(2)**

UTI is a common bacterial disease that affects various parts of the urinary tract in both men and women. UTIs can be asymptomatic or symptomatic, and symptoms can range from minor

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space irritation to sepsis, bacteremia, and death **(3).** Although both men and women are susceptible to infection, women are more susceptible than men due to their anatomy and reproductive systems. Urinary tract bacterial invasion, which includes the lower and upper urinary tract, is the most common cause of infection.

Cystitis is a term that means bladder infection. This inflammatory process is usually triggered by a bacterial urinary tract infection. If the cystitis infection progresses to the kidneys, a urinary tract infection called pyelonephritis occurs. **(4).** Educating people about raising awareness about the risk factors and pathogens of UTI has an important place in the prevention of the disease. Progression of colonization to UTI can be prevented by simple methods such as adequate fluid intake to dilute the urine, providing appropriate personal hygiene, and emptying the bladder completely **(5).**

Among natural applications, citrus juice can be used as a urinary acidifier, berries such as cranberries and blueberries and their extracts can help reduce symptoms of women with recurrent UTI infections and bacteriuria. It has been observed that the components found in these fruits prevent the attachment of bacteria to uroepithelial cells and inhibit the progression of the infection (**6**).

MATERIALS AND METHODS

Preparation of Cultural Environments

Blood Agar

In our study, blood agar medium was prepared for the initial isolation and culture of hemolysin producing bacterial isolates. For the preparation of the medium, firstly, 40 g of blood agar powder was dissolved in 1 liter of distilled water. In the dissolution process, the medium was heated to boiling point by shaking and then sterilized at 121°C for 15 minutes. When the ambient temperature drops to 50 °C5% v\v sterile human blood was added and mixed thoroughly until homogeneous. The blood agar medium solidified at room temperature in sterile petri dishes was incubated at 37°C for 24 hours to ensure sterilization. At the end of the incubation, the media were stored at 4 °C until use .

Brain Heart Infusion Agar

Brain heart infusion agar was prepared according to the manufacturer's instructions for use in our study. The medium, heated to boiling point with frequent shaking, was autoclaved at 121°C for 15 minutes, dispensed into sterile tubes, and the tubes were cooled to 50°C. After sterilization, the tube was placed in an inclined position and left at room temperature until solidified. Tubes were incubated at 37°C for 24 hours and stored at 4°C at the end of the incubation, so they can be used to culture a variety of finicky organisms. In particular, it has been used to culture streptococci, pneumococci and meningococci, which can be difficult to grow.

Mannitol Salt Agar (MSA)

For the preparation of Mannitol Saline Agar medium, 111 g of MSA was dissolved in 1 liter of distilled water and sterilized in an autoclave at 121 ˚C for 15 minutes. It was cooled to 50 °C before pouring into sterile petri dishes. Subsequent processing is the same as the preparation of the media mentioned above, used as a selective medium for the isolation of pathogenic Staphylococci.

MacConkey Agar

In order to grow gram-negative bacteria, 51 grams of medium was dissolved in 1 liter of distilled water and sterilized in an autoclave. The medium was cooled to 50°C before pouring into petri plates and allowed to solidify at room temperature. The next steps are the same as for the preparation of the aforementioned media. The crystal violet in this medium inhibits the growth of gram-positive bacteria while allowing the growth of Gram-negative bacteri

Muller-Hinton Agar (MHA)

MHA was prepared to be used in the antibiotic susceptibility test study. While preparing the medium, 38 grams of MHA powder was dissolved in 1 liter of distilled water and heated to boiling point with frequent shaking. The culture medium was then sterilized at 121°C for 15 minutes and cooled to 50°C before pouring into petri dishes. The next steps are the same as for the preparation of the aforementioned media.

Cysteine Lactose Electrolyte Deficient Agar (CLED Agar)

CLED agar is a type of differential media recommended for the diagnosis of urinary bacteriology. The medium supports the growth of all potential urinary pathogens and provides the distinct morphology of the colony. For the detection of pathogenic microorganisms in urine samples, 36 grams of medium powder was dissolved in 1 liter of distilled water. Sterilized in autoclave at 121°C, 15 minutes, then cooled to 50°C. The next steps are the

same as for the preparation of the aforementioned media.

Staphylococcus Medium No. 110 I

Staphylococcus Medium No. 110 I is a selective medium used for the isolation of pathogenic Staphylococci. It was prepared by dissolving 150 g of powdered medium in 1000 ml of distilled water for the isolation of staphylococci. It was cooled to 50°C after being sterilized in an autoclave at 121°C, 15 minutes after it was brought to the boiling point for complete dissolution. The next steps are the same as for the preparation of the aforementioned media.

Preparation of Reagents, Solutions and Buffers

Catalase Reagent

It was prepared by mixing 1 ml of 30% H2O2 with 9 ml of distilled water to obtain a final concentration of 3%, and then stored in a dark bottle at 4˚C. Catalase reagent was used to determine the ability of bacteria to produce catalase enzyme

Oxidase Reagent

The oxidase reagent was prepared by adding one gram of oxidase powder (NNNN tetramethyl-pphenylenediamine dihydrochloride) to 100 ml of distilled water. Used to detect the susceptibility of bacteria to oxidase production.

Turbidity Standard (McFarland)

Standard McFarland solution (No.0.5) was prepared as standard guidelines by mixing 0.5 ml 1.175% (w/v) barium chloride dehydrate solution (BaCl2.2H2O) with 99.5 ml 1% (v/v) sulfuric acid. covered with parafilm and stored at room temperature in the dark."(22-25˚C) Stored up to 6 months. no. Visually comparable to 0.5 McFarland standard, sterile saline or bacterial nutrient broth suspension.

Tris-Borate -EDTA (TBE)

"Working buffer (1X) was prepared from TBE buffer (10X) by adding 100 ml to 900 ml distilled water.

Gel Electrophoresis Solution

Agarose powder (1 g in case of DNA extraction and 2 g for PCR product detection) was mixed in 100 ml of 1X TBE buffer and boiled for a short time to dissolve the agar. After cooling to 50˚C, 2-3 µL of ethidium bromide (nucleic acid staining solution) was added.

Hydrochloric Acid (HCL)

Hydrochloric acid was used as a reagent in the DNase test. HCL was prepared by mixing 8.98 ml with 91.02 ml distilled water.

Collection of Samples

The strains used in our study were isolated from urine samples from 200 patients who applied to the AL-Hussein Teaching Hospital in AL-Nasiriyah, Southern Iraq, with the complaint of UTI between February and June 2021. After the patients showing UTI symptoms were examined by the physician, consent was signed by the patients and the patients were asked to urinate midstream in a sterile container. Samples were appropriately labeled at the collection point and quickly forwarded to the microbiology laboratory for microscopic examination, culture and susceptibility analysis. During the collection of samples, a questionnaire including age and background information was applied to all patients.

Identification of Staphylococci

All collected urine samples were inoculated on blood agar, MSA, MacConkey agar and Staph.110 agar media. After 24 hours of incubation at 37°C, naive isolated colonies were passaged into brain heart infusion agar to confirm identification. At the end of the incubation, Staphylococci were identified according to the size, transparency, color, shape, marginal structure, hemolysis status and height of the colonies in the culture medium .

Microscopic Examination

Gram staining was performed for microscopic examination of staphylococcal strains. A colony of pure staphylococci taken with a sterile loop was spread on a slide with a drop of water. Then, gram staining of slides dried at room temperature was performed. Dried slides were examined under a light microscope (100x) by dripping imversion oil.

Biochemical Tests

All biochemical tests used in this study were prepared according to Atlas, (2010) and Markey et al., (2014).

Catalase Test

Hydrogen peroxide solution was used in this test, which was carried out by spreading a pure colony using a wooden stick on a microscope slide. The observation of O2 bubbles when 3% hydrogen peroxide drops are poured on the slide was considered catalase positive (Markey et al., 2014).

Oxidase Test

In this test, in which the presence of cytochrome C oxidase enzyme was investigated, a piece of filter paper was immersed in a 1% aqueous solution of tetra methyl-p-phenylenediamine dihydrochloride (oxidase reagent) in a petri dish. The test bacteria spread on the soaked filter paper and the color change (light purple to dark purple) observed within 10 seconds was considered oxidase positive **(7**).

Coagulase Test

The coagulase test was performed with the aim of revealing the coagulase enzyme (staphylocoagulase), which is especially found in staphylococci and coagulates the blood plasma, and to distinguish between pathogenic and nonpathogenic ones. After 0.5 ml of plasma is placed in a clean tube, the same amount of *S. aureus* cultures is dropped on it and homogenized. Tubes are incubated at 37 °C in a water bath for 4 hours and visually inspected every hour. Coagulation status is determined by bending the tubes very slightly. The appearance of the coagulation shows a positive result compared to the control (**8**).

Deoxyribonuclease (DNase) Test

This test was used to measure the ability of microorganisms to synthesize the heat-stable DNase enzyme. The enzyme decomposes deoxyribonucleic acid (DNA) in cell nuclei by depolymerizing it. It is also used to measure the thermonuclease stability of *S. aureus* DNases against heat. DNase activity is also used to determine the pathogenicity of S. aureus, especially coagulase-negative reaction.

Microorganism cultures to be examined are cultivated on DNA agar. The Petri dish is incubated at 37 °C for 18-24 days. After this time, a few drops of HCl (1N) were added to the culture and monitored for 15-30 minutes for hydrolysis of DNA by the bacterial DNase enzyme. While an open area is formed around the growth in positive cultures, an opaque color occurs around the growth in negative cultures (**7**)

Novobiocin Test

The novobiocin test was used to differentiate coagulase-negative staphylococci and to identify resistant (R) S. saprophyticus. Disk diffusion method was used in the realization of the test. After the Diagnostic Sensitivity Test agar plate was dried, the sterile swab was dipped in a 24-hour nutrient broth culture and used to inoculate evenly across the surface of the medium. The plate was then allowed to dry. The Novobiocin "5µg" disc was placed on the mid-surface with sterile forceps and gently pressed, and kept at room temperature for 15 minutes. It was reported that the isolates were susceptible or resistant by incubating at 37°C for 24 hours and measuring the zone diameter in millimeters in the inhibition zone (**8**).

Urease Test

In the urease test, a pure bacterial colony seeded on urea agar was incubated at 37°C for 24-48 hours. At the end of the incubation, the color of the medium changed from yellow to pink positively (**7**).

Hemolysis Test

For the hemolysis test of bacterial isolates, they were seeded on blood agar plates and incubated at 37°C for 18-24 hours. The development of transparent areas around the bacterial colonies indicates the susceptibility of the bacteria to the secretion of hemolysis.

RESULTS

Patient Results

A total of 200 fresh urine samples were collected from both male and female patients with UTI of different ages included in this study during February-June 2021. Of the sampled patients, 133 (66.5%) showed positive growth. No significant growth was observed in the remaining 67 patients (33.5%). Demographic information of patients who applied to AL-Hussein Teaching Hospital with symptoms of UTI are summarized in Table1.

Table 1. Characteristics of UTI patients included in this study

Parameter		**Number of patients**	**Percentage (%)**
sample size		200	one hundred
Bacterial Growth	Growth	133	66.5
	no growth	67	33.5
Gender	Female	136	68
	Boy	64	32
resident	Urban	124	62
	Rural	76	38
hospital status	Outpatient treatment	118	59
	Inpatient	82	41

Significant differences (p ≤ 0.01)

Distribution of Patients by Bacterial Growth

The current findings of this study showed that 133 (66.5%) isolates had positive growth of the pathogen collected from UTI patients, while 67 samples (33.5%) showed no significant growth. Statistically, there were no significant differences in bacterial growth distribution (P≤ 0.01). The distribution of patients according to gender and bacterial growth is given in Table 2.

Table 2. Distribution of patients by gender and bacterial growth

Gender	Culture		Total
	Growth%	No growth%	
Boy	33	31	64
	16.5%	15.5%	32%
Female	one hundred	36	136
	50%	18%	68%
Total	133	67	200
	66.5%	33.5%	100.0%

Cal.X2: 9.41 Tab.X2: 6.63 df: 1 P_value: 0.01

Distribution of Patients by Age

The highest UTI rates were seen between 55 patients (27.5%) and the age group (51-70). On the other hand, the lowest infection rate for this disease was found in the age group (11-20) with 25 cases (12.5%) as shown in Table 3. There is no statistically significant difference in age group distribution (P≤0.01).

Table 3. Urinary tract infection status varies according to age groups.

Age group	Hsubordinate case		No. Tested %
	Number of Positives %	Number of Negatives %	
11-20	13	12	25
	6.5%	6%	12.5%
21-30	29	11th	40
	14.5%	5.5%	20%
31-40	27	14	41
	13.5%	7%	20.5%
41-50	26	13	39
	13%	6.5%	19.5%
51-70	38	17	55
	19%	8.5%	27.5%
Total	133	67	200
	66.5%	33.5%	100.0%

Cal.X2: 3. Tab.X2: 13.28 df: 4 p_value: 0.01

Isolation and identification of Staphylococcus aureus

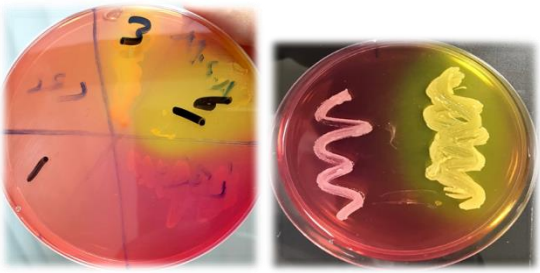
A total of 200 clinical specimens from UTI patients showed beta hemolytic activity on blood agar, with 133 specimens showing positive cultures due to traditional cultural procedures. It was observed that the isolates produced a clear zone surrounding the isolates as a result of hemolysis. Positive cultures were first identified by gram staining and biochemical tests.

*Staphylococcus aureus*Bacteriological examination on clinical specimens for isolation was carried out using the following procedures:

Staphylococcus culture

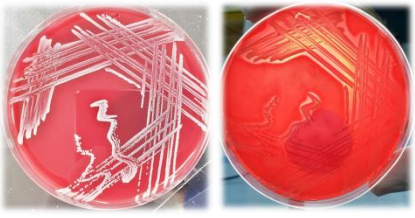
Staphylococcus colonies on Mannitol Salt Agar Medium had different morphological shapes and colors compared to bacteriological culture. As seen in Figure 1., *S. aureus* isolates fermented mannitol and turned red agar into yellow. Colonies appear round and smooth in yellow with a yellow zone.

Figure 1. Mannitol fermentation of *Staphylococcus aureus* on Mannitol Salt Agar



On blood agar, *S. aureus* isolates appear as large, round, smooth, white to yellow, shiny, opaque, and clear hemolysis zones as shown in Figure 2.

Figure 2. Appearance of *S. aureus* isolates on blood agar



Macroscopic Examination

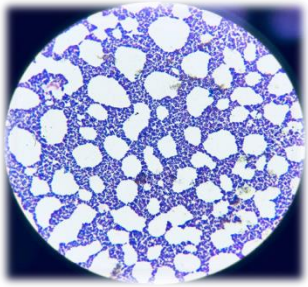
When the morphological images of *S. aureus* growing on different media are examined, it grows fastest at 37°C, and the temperature range where the best pigment formation is seen is between (20–25°C). Colonies seen on solid media appear spherical, smooth, raised and shiny. Colonies of *S.*

aureus were usually gray to dark golden yellow in color and showed varying levels of hemolysis.

Microscopic Examination

After cultural cultivation of the isolates, gram staining of pure colonies was made and examined under a microscope. Under the microscope, the bacterial cells were seen as gram-positive cocci and as irregular clusters similar to bunches of grapes as seen in Figure 3.

Figure 3. Gram stain image of *Staphylococcus aureus* under the light microscope



Biochemical identification

*Staphylococcus aureus*Biochemical tests were carried out on cultures. As seen in Figure 4.6, catalase, gelatinase, DNase, coagulase tests and hemolysis were found positive in the isolates, respectively. Images of oxidase test results are given in Figures 4., 5.,

Table 4. Biochemical tests of *Staphylococcus aureus* isolates

biochemical test	Conclusion
Gram stain	+
catalase	+
Novobiocin	S
oxidase	-
DNAse	+
coagulase	+
Gelatin liquefaction	+
hemolysis	+
Mannitol fermentation	+

Figure 4. Tube coagulase test

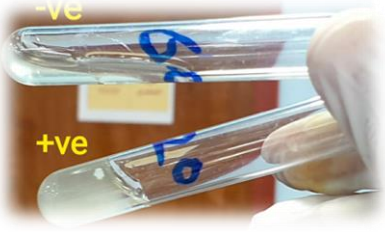


Figure 5. Positive result for *Staphylococcus aureus* catalase test

DISCUSSION

Urinary tract infections (UTIs) are a common disease and a common cause of morbidity, usually in outpatients and hospitalized patients. It affects every person from all age groups and different geographical regions (**9**). In UTI, patients may have symptomatic or asymptomatic manifestations. UTI is more common in women than men, due to genetic variation. It is one of the most common bacterial infectious diseases occurring in humans today (**10**).

The present study was conducted for the morphological and molecular detection of *Staphylococcus aureus* isolated from UTI patients in AL-Nasiriyah city. The findings of this study showed a very high level of positivity of 66.5%. In a similar study conducted in Iraq, the positivity rate in cultures isolated from urine culture was 57.9%. These rates are similar to our study (**11**) 13). On the other hand, in another study, the positive culture rate was found to be 41.6% (**12**). This rate is quite low compared to our positive rate. UTI positivity rates were determined as 39.7%, 26%, and 30.69%, respectively, in different studies performed outside of Iraq **(13-15).** These differences in pathogen distribution, As it can be explained by different fields of studies, it can also be caused by drugs used in treatment and having an important role in the distribution and spread of the pathogen. The risk of loss of a microbial agent may depend on the antibiotics used by patients during the sampling period. In humans, UTIs are very common but vague

or neglected, which can lead to unwanted complications due to neglect or misdiagnosis. Early diagnosis and treatment of UTI will help to significantly reduce the incidence of high blood pressure and renal bleeding in the future (Al-Rawi, 2010). In humans, UTIs are very common but vague or neglected, which can lead to unwanted complications due to neglect or misdiagnosis. Early diagnosis and treatment of UTI will help to significantly reduce the incidence of high blood pressure and renal bleeding in the future (**16**). In humans, UTIs are very common but vague or neglected, which can lead to unwanted complications due to neglect or misdiagnosis. Early diagnosis and treatment of UTI will help to significantly reduce the incidence of high blood pressure and renal bleeding in the future (**16**).

*S. aureus*In addition to different biochemical tests, all isolates were identified by numerous features such as microscopic examination, morphology of the culture medium. Especially in hospitals, Colony morphology, gram staining and biochemical reaction of *S. aureus* isolated in this study are similar to the results reported in other studies (**17,7**).

Thereupon, 85 suspected isolates, seen under the microscope as G(+) with the coke shape mainly grouped in clusters, compared to ten mannitol salt agar, were able to ferment mannitol and produced a yellow color due to acid production. They were catalase positive due to the production of the enzyme catalase, which distinguishes them from Streptococcus spp., and they were coagulase positive due to the production of the enzyme staphylothrombin, which reacts with prothrombin and forms staphylothrombin, which converts fibrinogen to fibrin and causes blood clotting. Coagulase positivity of the isolates caused *S. aureus* to be detected by other Staphylococcus spp. Therefore, tubes with coagulase production are considered the "gold standard" for the identification of S. aureus, describing the ability of the tested isolates to produce the nuclease enzyme, suggesting that these isolates have the ability to cleave DNA.

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