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THE ROLE OF HAEMOLYSIN IN PATHOGENESIS OF *STAPHYLOCOCCUS AUREUS* AND *S. HAEMOLYTICUS* CAUSING URINARY TRACT INFECTIONS IN PREGNANT AND NON-PREGNANT WOMEN

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Abstract

Background: Urinary tract infections (UTIs) are one of the most common infections afflicting women. Hemolysins or haemolysins was a potential virulence factor produced by microorganism (M.O).

Aims: To identify gram positive bacterial species isolated from urinary tract infections, and also to evaluate *in-vitro* the antimicrobial activity of antibiotic and biofilm production and hemolysin gene detection.

Methods: A total 200 urine specimens were collected from patients with UTIs, that include pregnant (n=100, 50%), non- pregnant (n=100, 50%), bacterial colonies were Gram stained and microscopically examined. Biochemical tests were done to identify pathogen species. The microtitre plate method (MTP) was used to detect biofilm formation by bacteria and Detection of some virulence genes of bacterial isolates by PCR.

Results: The result of identification revealed that the number of patients with significant bacteriuria among 200 urine specimens with positive culture was 60% and the Gram positive bacteria (50.83%), *S. aureus* (12.5%), *S. haemolyticus* (8.3%), and *S. epidermidis* (12.5%). In *S. aureus*, the percentage of isolates produces strong biofilm (86.7%). In *S. haemolyticus*, the strong biofilm was 80%. Diagnosis using PCR of 16S rRNA of isolates was 100% positive for all isolates. For *S. haemolyticus*, *Hly-A* was 80% and *Hly-B* was 0%. The virulence factor in *S. aureus* was *Hly-A* (100.0%) for all bacteria, while the *Hly-B* was 53.3%.

Conclusion: The most isolates were resistant to colistin Nalidixic acid and Azithromycin, This investigation showed *Hly-A* and *Hly-B* are a gene found in bacterial cells that allows them to be resistant to antibiotics.

Keywords: *Hly-A*, *Hly-B*, *in-vitro*, Biofilm, Iraq

Introduction

Urinary tract infections (UTIs) are one of the most common infections afflicting women. UTIs in women are one of the most prevalent infections occurring at different stages of life. Female are much more prone to UTIs than male, mainly due to the female lower urinary tract anatomy and its proximity to the reproductive organ (Czajkowski *et al.,* 2021), and to the short urethra and colonization of the peri-urethral area by pathogens from the gastro-intestinal tract. From the peri-

urethral area, pathogens ascend to colonize the urinary bladder or kidneys (Wing *et al*. 2013; Czajkowski *et al*., 2021). In addition, UTIs incidence is estimated about 150 million persons per year (Al-Tulaibawi, 2019). Hemolysins or haemolysins was a potential virulence factor produced by M.O., it was lipids and proteins that cause lysis of red blood cells by forming pores and disrupting the cell membrane (Stipcevic *et al.*, 2005). Alpha-hemolysin of *Staphylococcus aureus*, α-hemolysin (*αHL*) was encoded by the *hla* and acts as a pore-forming cytotoxin the major M.O that produce hemolysins that can cause cystitis, pyelonephritis, and sepsis and β-hemolysin (*hlb*) was a phospholipase C toxin (Kebaier *et al*., 2012), expression of hemolysin correlates with severity of infection, as up to 78% of UPEC isolates from pyelonephritis patient cases express hemolysin, harbor infectious isolates with up to 78% encoding operon hemolysins gene (Ristow and Welch, 2016).

On the other hand, M.O aggregate as architectural structure was known as biofilm, biofilms are heterogeneous with 15% of cells, usually in microcolonies, and 85% of polymeric extracellular substances. The composition of the biofilm matrix varies among different species, but in general contains proteins, polysaccharides, and nucleic acids (Vetrivel *et al*., 2021). *Staphylococcus aureus* is a G+ve spherically shaped bacterium, member of the microbiota of the body, frequently in the upper respiratory tract and on the skin. It is often positive for catalase and nitrate reduction (Tong *et al.,* 2015). Pathogenic strains often promote infections by producing virulence factors such as potent protein toxins, and the expression of a cell-surface protein that binds and inactivates antibodies. Furthermore, *S. aureus* is one of the leading pathogens for deaths associated with antimicrobial resistance and the emergence of antibiotic-resistant strains such as methicillin-resistant *S. aureus* (MRSA) is a worldwide problem in clinical medicine. (Lepelletier *et al.,* 2020) Moreover, *S. aureus* can cause a range of illnesses, from minor skin infections, such as pimplesto life-threatening diseases such as pneumonia, meningitis, osteomyelitis, endocarditis, toxic shock syndrome, bacteremia and sepsis (Tong *et al.,* 2015). The ability of *S. aureus* to cause infections is probably due to the expression of a wide range of virulence factors, including adhesions and toxins (hemolysin). Hemolysin genes produced by *S. aureus* are a crucial virulent factor. They have cytotoxic action responsible for lysing red blood cells and culminating in worsening of clinical conditions, *S. aureus* has been shown to produce alpha, beta, gamma, and delta toxins (Ahmed *et al*., 2022). *Staphylococcus haemolyticus* is one of the coagulase-negative staphylococci (CoNS) that inhabit the skin as a commensal. It is increasingly implicated in opportunistic infections. In contrast to the abundance of information available for *S. aureus* and *S. epidermidis*, little is known about the pathogenicity of *S. haemolyticus*, despite the increased prevalence of this pathogen in hospitalized patients. Virulence-related genes were investigated, adhesion and invasion. Most *S. haemolyticus* carried different sets of virulence-related genes (Eltwisy *et al*., 2020). A characteristic feature of *S. haemolyticus* is its ability to form biofilms, which play an essential role in the establishment of infections. The produced exopolysaccharides can inhibit the growth of other bacteria and also decrease their ability to form biofilms (Eltwisy *et al*., 2020).

Material and method

Study design

A total 200 urine specimens were collected from patients with UTIs, that include pregnant (n=100, 50%), non- pregnant (n=100, 50%), with age 13 – 44 years, during the period from 1th August, 2022 to 25th December, 2022 that admitted to Al-Kut Maternity and child hospital in Wasit province.Samples were taken by sterile disposable cotton swabs and transport swab. They were, then, cultured onto Blood agar base, Brain heart infusion Agar, Brain heart infusion broth, Muller-Hinton agar, Nutrient agar plates before incubating aerobically and anaerobically with CO_2 at 37ºC for 24h to 48h. After that, identified based on colony morphology, microscopic Gram stain investigation, capability of blood heamolysis, standard biochemical tests and Vitek 2 system, and 16S rRNA.

Exclusion and inclusion criteria

In order to include all women (pregnant and non-pregnant) patients in the current study who had suspected UTIs with age 13-44, the records were carefully examined. Patients undergoing an antibiotic therapy prior to the study were excluded and bacteria that not cause hemolysis on blood agar excluded too.

Detection of biofilmformation

To detect biofilm formation, preparation of biofilm solution using glacial acetic acid (33%) by adding 33ml of glacial acetic acid to 67 ml of D.W, and used phosphate buffer saline via suspended 9.86g in 1liter of D.W, and autoclaved at 121ºC/15 pounds/inch2 for 15mnts. Then, preserved in refrigerator till used. Finally, this process was used ethanol 96% via mixed 96 ml of ethanol (100%) with 4ml D.W (Christensen *et al*., 1985; Avila *et al*., 2018).

Microtitre plate method

Utilizing a microtitre plate reader, this method is used to estimate biofilm generation. According to Christensen *et al*. (1985), the isolates' capacity to generate biofilm was examined on 96-well flat-bottomed micro-titer polystyrene plates. Add 200µL of bacterial suspensions to BHIB for each isolate in three wells of a microtitre plate. Following that, all microtitre plates were incubated for 24 hrs at 37°C. The controls consisted of wells that were stuffed with BHIB. To get rid of the planktonic bacteria, the contents of each well were then discarded and rinsed three times with phosphate-buffered saline. After fixing the adhesive bacteria with 250µl (96% ethanol) for 5mints, the plates were drained and permitted to dry. We use 100 µl of a 1% crystal violet solution (*w/v*) to stain the plates, and then wait 5 minutes. With sterile distilled water, the excess stain was cleaned. By adding 200µl of glacial acetic acid (33%) per well (*v/v*), microtitre plates were incubated for 15 minutes, and the quantitative analysis of biofilm was completed. The classification of biofilm formation as strongly positive ($OD_{570} \geq 0.24$), weakly positive ($0.12 \leq OD_{570} < 0.24$), or negative ($OD_{570} < 0.12$), as outlined in the Table 1.

Table 1: Analyzing biofilm using the micro-titer plate technique

Mean OD value	Biofilm formation
OD ≤ ODc	None
ODc < OD ≤ 2× ODc	Weak
2× ODc < OD ≤ 4× ODc	Moderate
4× ODc < OD	Strong

***** OD = optical density; ODc = optical density cut off*****

Molecular detection

Polymerase chain reactions (PCR) primers were designed in the current study using NCBI Genbank sequence database design, online software and these primers were synthesized by Scientific Researcher Co. Ltd, Iraq, as summarized in the Table2.

Table 2: Sequences of all primers were used in the present study

Gene	Primer Sequence (5'-3')		Product size	Reference
16S rRNA gene *S. haemolyticus*	F	GCGGTAATACGTAGGTGGCA	532bp	L37600.1
	R	TGCACCACCTGTCACTTTGT		
16S rRNA gene *S. aureus*	F	GGAACTGAGACACGGTCCAG	471bp	LN794238.1
	R	ATCCTGTTTGATCCCCACGC		
Hly-A gene *S. haemolyticus*	F	TGGGCCATAAACTTCAATCGC	72bp	Pinheiro *et al*., 2015
	R	ACGCCACCTACATGCAGATTT		
Hly-B gene *S. haemolyticus*	F	TGGTGGCGTTGGTATTGTGA	541bp	Pinheiro *et al*., 2015
	R	ACCCCAAGATTTCACGGACC		
Hly-A gene *S. aureus*	F	CTGATTATCCAAGAAATTCGATTG	209bp	Puah *et al*., 2016
	R	CTTTCCAGCCTACTTTTTTATCAGT		
Hly-B gene *S. aureus*	F	GTGCACTTACTGACAATAGTGC	309bp	Puah *et al*., 2016
	R	GTTGATGAGTAGCTACCTTCAGT		

PCR thermo cycler program

Polymerase chain reaction thermo cycler conditions for *S.aureus* and *Str. sanguinis* amplification reactions were done using convential PCR thermo cycler system is same for each gene except for annealing temperature as following the Tables 3.

Table 3: PCR thermocycler system of 16SrRNA, *Hly-A gene* and *Hly-B* gene

PCR step	Temperature	Time	No. cycles
Initial denaturation	95	5 min	1
Denaturation	95	30 sec	35
Annealing	58[1]	30 sec	
Extension	72	30 sec	
Final-extension	72	5mnts	1
Hold	4	∞	12

All PCR products were detected by 2% agarose gel (100volts for 45minut) and visualized by staining with 1µl of Ethidium bromide stain then documentation was performed by the gel documentation saving picture (vision, UK).

Statistical analyses

Statistical-Package-for-Social-Science, version 25.0 for windows was used to do statistical analysis on all data. All findings with a significant level ($P \leq 0.05$) were analyzed using Chi Square (Al-Gharban, 2017).

Result

The results were distributed according to the patient age between 13-44 years old. The lowest incidence was among 35-44 age group (17.0%), while the highest incidence was among 25-34 age group (43.5%), as showed in the Table 4.

Table 4: The distribution of patients according to age groups, Pregnant and Non Pregnant

Age Group (Year)	Pregnant		Non Pregnant		Total	
13-24	41	20.5%	38	19.0%	79	39.5%
25-34	50	25.0%	37	18.5%	87	43.5%
35-44	9	4.5%	25	12.5%	34	17.0%
Total	100	50.0%	100	50.0%	200	100.0%

Isolation and identification of pathogenic bacterial from thepregnant and non-pregnant

The cultural characteristics of urine isolates on different media such gram stain and biochemical test and according to the vitek-2 system result and finally by 16s RNA. The result of identification revealed that the number of patients with significant bacteriuria among 200 urine specimens with positive culture was 60% and the negative (no growth) was 40%, may be due to Fungal or viral infections as shown in figure 1.

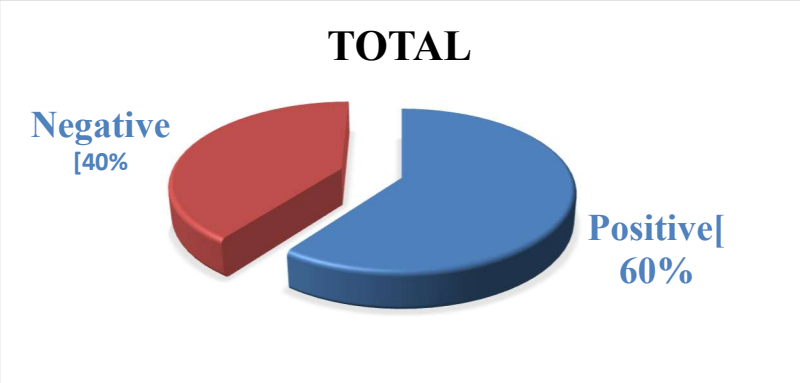

Figure (1): Total percentage of UTI bacterial growth groups N= 200.

The number of patients with significant bacteriuria was higher in pregnant, n= 66/120(55%) compared to non-pregnant n=54/120 (45%), the *P*-value was 0.001.

Cultural result that based on morphological and biochemical tests and Vitek-2 system revealed the mild high incidence of Gram positive bacteria (50.83%) compare with gram negative (49.16%). The cultural characteristics of *S.aureus*, the primarily identification of *S. aureus* was the result of blood agar culturing which appears as circular, round, grey colored with a 0.8-1.0µm in diameter, soft, convex, bright, with a sharp border, and produces a zones of clear β-hemolysis on the agar on blood agar, When streaked on MSA to purified bacterium. In Gram stain appear as bluish/purple, single coccus, in pairs, or in grape-like clusters, biochemically, catalase-positive, oxidase-negative, coagulase-positive, produces a coagulase enzyme that agglutinates/clots blood or plasma (Rasheed and Hussein, 2021).

The cultural characteristics *S.haemolyticus*, Gram-positive cocci in clusters by gram stain, on blood agar cultures beige to white β-haemolytic colonies, about 2-4 mm in diameter, catalase positive with negative coagulase reaction , negative reactions for oxidase on MSA produce small pink or red colonies with no colour change to the medium (Ahmed *et al*., 2019). Sensitive to Novobiocin test (used to differentiate coagulase-negative staphylococci (CONS) *S.haemolyticus* and presumptively identify the isolate as *Staphylococcus saprophyticus* (novobiocin resistant) (Karah *et al*., 2020).

Biofilm production by using microtitre plate (MTP)

In the present study, a total 25 isolates evaluated using MTP method for detection of biofilm production, the results were demonstrated that all 25 of isolates biofilm production, with varied titer and statistical differences ($P = 0.0001$). In *S. aureus*, the percentage of isolates produce weak biofilm was 0%, moderate biofilm 13.3%, and strong biofilm 86.7%. In the S. haemolyticus the percentages of isolates were produce weak biofilm 10%, moderate biofilm110%, and strong biofilm 80% as shown in table 5.

Table 5: MTP Method that used for biofilm production and percent for each type

Bacteria	Biofilm						*P-Value*
	Weak		Moderate		Strong		
S. haemolyticus	1	10%	1	10%	8	80%	0.0001
S. aureus	0	0%	2	13.3%	13	86.7%	

Molecular results

Extraction of DNA in different isolates of pathogenic bacteria, the DNA for *S. haemolyticus* and *S. aureus* were extracted. NanoDrop was used to confirm the nucleic acid purity and concentration (Ibraheim *et al*., 2023).

Molecular detection of 16S rRNA of isolates

Diagnosis using PCR was regarded the golden and confirmatory diagnosis which takes a short period compared to other methods this specific detection of most conserved region of target bacteria recorded *S. haemolyticus* 10(100%), *S. aureus*15(100%).

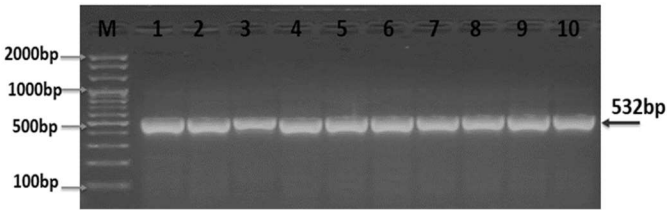

Figure (4): Agarose gel electrophoresis image that showed PCR product analysis of 16S ribosomal RNA gene for detection *S.haemolyticus* isolates. M (Marker ladder 2000-100bp). Lane (1-10) showed some positive Staphylococcus haemolyticus isolates 16S ribosomal RNA gene at 532bp product size.

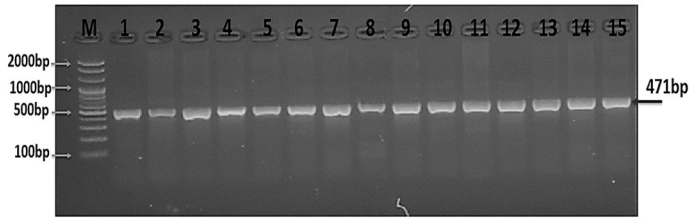

Figure (5): Agarose gel electrophoresis image that showed PCR product analysis of 16S ribosomal RNA gene for detection *S. aureus* isolates. M (Marker ladder 2000-100bp). Lane (1-15) showed some positive Staphylococcus aureus isolates 16S ribosomal RNA gene at 471bp product size.

Detection of some virulence genes of bacterial isolates

PCR was performed to detect the presence of the genes (*Hly -A* and *Hly-B*) for *S. haemolyticus, S. aureus.* For *S. haemolyticus* isolates the result in current study was *Hly-A* was 8(80%) in all isolates, Non Pregnant 3(37.5%) and Pregnant 5(62.5%). Also, the presence of *Hly-B* was 0(0%) as shown in the figure 6. Additionally, the virulence factor *Hly-A* gene in *S. aureus* was *Hly*-A (100.0%) for all bacteria, while the *Hly-B* was 8(53.3%), there were high percentage of *Hly-A* and *Hly-B* gene in pregnant 53% and 62.5% respectively as shown in the figure 7.

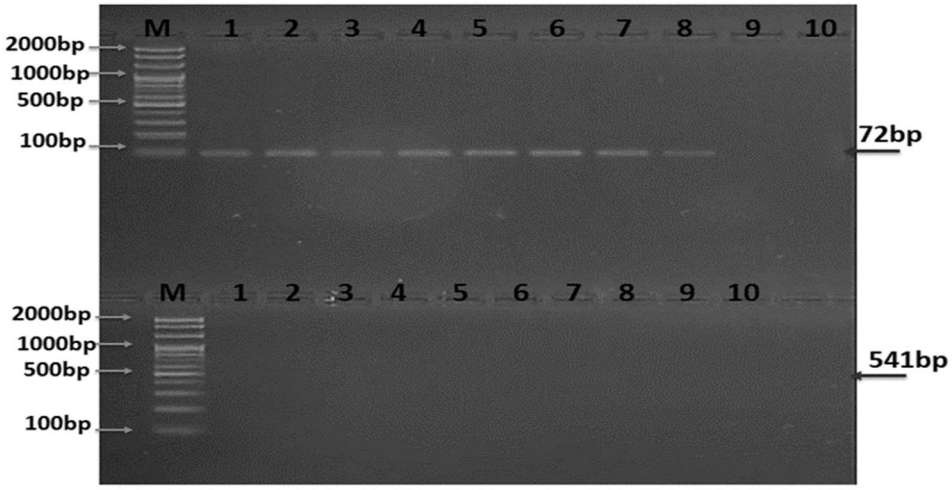

Figure (6): Agarose gel electrophoresis image that showed PCR product analysis of hly-A and Hly-B genes in *S. haemolyticus* isolates. M (Marker ladder 2000-100bp). Lane (1-10) Showed some positive *hly*-A gene *S. haemolyticus* isolates at 72bp product size and Lane (1-10) showed no positive hly-B gene *S. haemolyticus* isolates at 541bp product size.

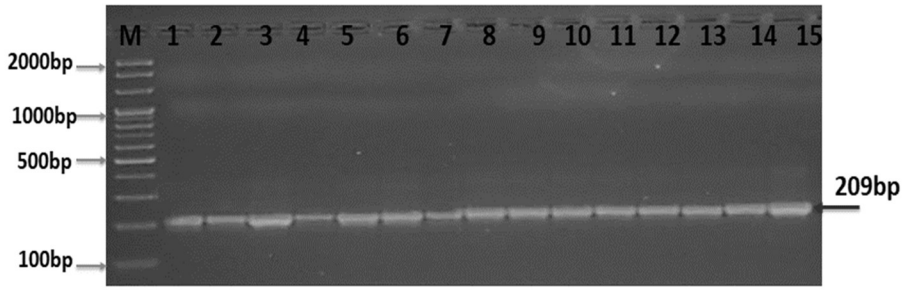

Figure (7): Agarose gel electrophoresis image that showed PCR product analysis of hly-A gene in *S. aureus* isolates. M (Marker ladder 2000-100bp). Lane (1-15) Showed positive hly-A gene Staphylococcus haemolyticus isolates at 209bp product size.

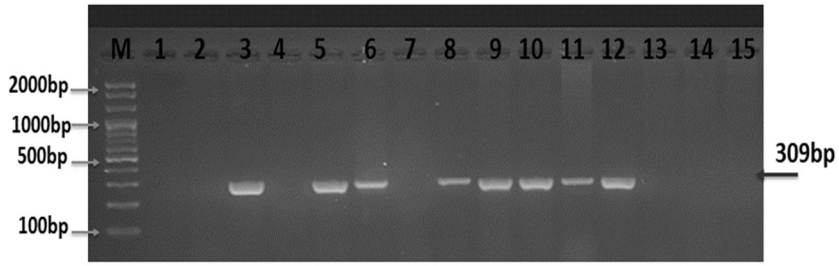

Figure (8): Agarose gel electrophoresis image showed PCR product analysis of hly-B gene in Staphylococcus aureus isolates. M (Marker ladder 2000-100bp). Lane (1-15) showed some positive hly-B gene Staphylococcus haemolyticus isolates at 309bp product size.

Discussion

Higher in pregnant compared to non-pregnant. These result agreed with results that conducted by Abate *et al*. (2020). This study appeared that the pregnant women had a significantly higher infected with UTIs and uropathogenic bacteria than non-pregnant women and can be associated with adverse outcomes for both the mother and fetus (Habak *et al*., 2019). The cultural result in current study revealed high incidence of Gram +ve bacteria that's agreed with result of Ndmason *et al*. (2019). *S. aureus* ferment the mannitol and form yellow zones in the reddish agar due to the production of fermentation acids that contribute in lowering the pH of the medium converting the colour of phenol red to yellow. The microtitre plate was a quantitative technique that reflected the industry standard for identifying biofilms (Al-Dahmoshi, 2013). The results of biofilm of *S. haemolyticus* were close to the result achieved by Shrestha *et al*. (2017), who showed that the percentage of strong biofilm producers for *S. haemolyticus* was 79.4%, and resistant to multiple

antibiotics in comparison to biofilm non- formers. Human colonized by an opportunistic bacterial pathogen *S. haemolyticus* which carries antibiotic resistance genes. *S. haemolyticus*, especially the clinical isolates, are mainly multidrug-resistant, and these isolates produce biofilms, toxins, and enzymes leading to infections that are difficult to treat (Eltwisy *et al*., 2022). The result in the present study for S. aureus biofilm production agreed with another study conducted by Shrestha *et al*. (2019). Also, the disagreed with the result conducted by Yousefi, *et al*., 2016, in IRAN, revealed that 16.6% of the strains were found to be strong, 26.7% were moderate, and 20% were found weakly adherent. *S. aureus* is known to form biofilms on various surfaces. This pathogen can invade renal tissue causing UTI by adherence to uroepithelium and formation of biofilm. Since the ability of biofilm production in S. aureus can increase resistance to commonly used antibiotics, hospitalized patients infected with this organism are at significant risk for treatment failure (Soto, 2014). Furthermore, the results in current study about *Hly-A* and *hlb* gene expressed in *S. haemolyticus* was agreed with the study performed by Eltwisy *et al*. (2020) who showed that *Hly-A* gene expressed in *S. haemolyticus* was 80%. Similar result achieved by Pinheiro *et al*. (2015) in Brazil, who showed that *Hly-A* gene expressed by *S. haemolyticus* were 91.7% and *Hly-B* were 0%. Others result of study Al-Hilu and Al-Shujairi (2020) in Kufa/ Iraq, showed that 47% of CoNS /*S. haemolyticus* isolated form UTIs were isolates had *hla* gene and 41% contain *hlb* gene. Also, the results in the present study agreed with the study performed by Nasaj *et al*. (2020) in Iran, who showed that *S. haemolyticus* (isolated form UTIs) was *hly-A* gene were 94.6% and *hlb*-B was 46%. Similar result of gene amplification by Al-Nashi *et al*. (2017), Iraq, of *hla-A* gene for *S. haemolyticus* isolated form UTIswas 75% and for *hlb*-B was 25%. *S. haemolyticus* produces several toxins and invasive enzymes that help in bacterial pathogenesis by changing the host immune responses and inducing damage in the host cells, cytotoxin or hemolysins are important molecules involved in the pathogenesis (Eltwisy *et al*., 2022). The result in current study for *S. aureus* hemolysin gene agreed with the result conducted by Aubais Aljelehawy *et al*. (2021), the *S. aureus* strains isolated from patients with urinary tract infection in Al-Najaf Al-Ashraf teaching hospital, Iraq, who revealed that among the strains, hla gene with 91% frequency, showed the highest prevalence among pathogenic genes. hlb genes with 13%. Similarity result of AL-Nashi *et al*. (2017) showed that the result of gene amplification of Hly-A and Hly-B gene were 100% and 40% respectivelly. Also, the results in the present study agreed with the study performed by Rahimi *et al*. (2016) Tehran (Iran), who showed that *Hly*-A and *Hly*-B gene expressed in S. aureus were *Hly*-A (79%) and *Hly*-B gene (66%). In addition, regarding the hemolysin genes, similar result *hla* was positive in 93.3%, while hlb was positive in 17 (56.7%) in previous studies reported by Ahmed *et al*. (2022). Alpha hemolysin (*hla*) is involved in the development of osmotic phenomena, cellular depolarization and the loss of vital molecules (ATP) (Aubais Aljelehawy *et al*., 2021).

Conclusion

The results in the current study demonstrated that most isolate produced strong biofilm. All isolates resistant to colistin and most of them wereresistant to Azithromycin, Amikacin, Amoxicillin-

clavulanate and Nalidixic acid, and highly sensitivity rate was to Ciprofloxacin and Imipenem. Also, most isolates express the *hla* gene.

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