International Journal of Biotechnology and Microbiology
www.biotechnologyjournals.com
Online ISSN: 2664-7680, Print ISSN: 2664-7672
Received: 12-08-2023, Accepted: 28-08-2023, Published: 13-09-2023
Volume 5, Issue 3, 2023, Page No. 54-60

Molecular detection of some virulence factors of gram positive bacterial species causing urinary tract infection in diabetic patients

Musaab Obaid Hamad[1*], Mohammed Nadhir Maaroof[2]
[1] Department of Biology, College of Education for Pure Science, University of Tikrit, Iraq
[2] College of Medicine, Microbiology, University of Tikrit, Iraq

Abstract
Staphylococcus aureus and *Enterococcus faecalis* were isolated as Gram positive causative agent of UTIs from urine samples of type 1 and type 2 diabetic patients. Phenotyping detection of virulence factors β- hemolysin, biofilm and β-Lactamase was done followed by molecular detection of these factors by PCR using the primers *hlB*, *cyl A, esp* and *blaZ.*

β-hemolysin was highest virulence factors it was produced by all *Staphylococcus aureus* and *Enterococcus faecalis* isolates which isolated from both type 1 and type 2 diabetic patients separately. All *Enterococcus faecalis* isolates which isolated from both type 1 and type 2 diabetic patients separately were produced of biofilm, while *Staphylococcus aureus* isolates which isolated from both type 1 and type 2 diabetic patients were produced of biofilm with a rate of 40% and 57% respectively. All *Staphylococcus aureus* isolates which isolated from both type 1 and type 2 diabetic patients separately were produced β-Lactamase, while *Enterococcus faecalis* isolates which isolated from both type 1 and type 2 diabetic patients were not produced of β-Lactamase.

The *hlb* gene was appeared in all *Staphylococcus aureus* which isolated from both type 1 and type 2 diabetic patients separately. The *cyl A* and *esp* genes were appeared in all *Enterococcus faecalis* which isolated from both type 1 and type 2 diabetic patients separately. The lowest appearance of genes was for *blaZ* gene which was in 60% of *Staphylococcus aureus* isolated from type 1 diabetic patients, and in 71.4% of isolates which isolated from patients with type 2 diabetes.

Keywords: UTIs, diabetic patients, molecular detection, PCR, *blaz* gene, *cyl a* gene

Introduction

Diabetes mellitus (DM) is a worldwide health problem. There are two main types of diabetes they are type 1, and type 2, various impairments in the immune system, glycosuria and bladder dysfunction, may contribute to the increase of infectious disease in diabetic patients (Selman *et al.*,2023) [36]. Urinary tract infection (UTI) is the most common infection among patients with DM and is responsible for considerable morbidity, particularly if it is unrecognized or untreated (Berbudi *et al.*,2020) [9]. The UTI is caused by microbial colonization and proliferation in the urinary system (Al-Zubaidi and Al-Salman, 2022) [8]. The commonest cause of UTIs represented by Gram negative. However Gram positive bacteria such as *Staphylococcus aureus, Enterococcus faecalis* and species of coagulase negative Staphylococci can be a cause of these infection (Kline and Lewis, 2017) [19]. These bacteria encode widespread virulence factors closely related to colonization, persistence and pathogenesis of bacteria in the urinary tract (Govindarajan and Kandaswamy 2022) [15]. These factors are encoded by genes carried on mobile genetic elements such as plasmids, transposons, and phages, or carried in locations on the bacterial chromosome called pathogenicity islands (PAIS) (Arunachalam *et al.*, 2023) [4]. The most important of these factors toxins such as β -hemolysin, biofilm formations and enzymes such as β-Lactamase (Bien *et al.*, 2012) [10].

β -hemolysin: is one of the cytolytic toxins and one of the most important factors of virulence for bacteria that cause infections it works to destroy the urothelial cells, and facilitates the invasion of bacteria into the urinary tract (Alshaibah *et al.*,2021) [3]. This factor encoded by a group of genes an examples of which are *hlb* gene in *Staph. aureus* and *cyl* genes in *Entero. faecalis* (Hashem *et al.*,2021; Kafil and Mobarez, 2015) [16, 18].

Biofilm: Biofilms are complex microbial communities typically attached to a surface and embedded in an extracellular matrix which protects embedded bacterial cells by preventing the penetration of antibiotics and host immune cells (Costerton *et al.*,1995) [11]. A lots of genes coding for different substances in a single bacterial species or different species working together to form a biofilm (Abbood and Alwan, 2023) [1], from within these genes is the *esp* gene responsible for coding Extracellular surface protein in *Entero. faecalis*. (Samani *et al.*,2021) [35]

β-Lactamase: is an enzyme produced by bacteria that provide multi-resistance to beta-lactam antibiotics such as penicillins, cephalosporins, cephamycins, monobactams and carbapenems by break the beta-lactam ring necessary for the action of the antibiotic (Poirel *et al.*,2022) [30]. This enzyme encoded by a group of genes an examples of which is *blaZ* in *Staph. aureus* (Pugazhendhi *et al.*,2020) [33].

Detetion of virulence factors of bacterial pathogens at the molecular level is important to success development of new strategies to enhance the elimination of bacterial infection, and this is of importance with the global emergence of microorganisms resistant to antibiotics (Medeiros *et al.*,2014) [24].

As a result of the lack of studies on the molecular detection of uropathogenic gram positive virulence factors in both type 1 and type 2 diabetic patients in the city of Mosul, this knowledge aimed at revealing these factors.

Materials and Methods.

1. Study population

Patients with Type 1 and type 2 diabetes mellitus patients who came to Al-Wafa·a Specialization Center For Diabetes and Endocrinology, Mosul, Iraq, for the purpose of periodic review. pregnant women and patients who had undergone catheterization were excluded of the study.

2. Culture media

2.1 The commercially prepared culture

MacConkey agar, Nutreint Agar, Mannitol Salt Agar, and Brain heart infusion broth were used, as the media were prepared and pH values were adjusted sterilized as per manufacturer's instructions.

2.2 Culture media prepared in the laboratory Blood agar medium

It was prepared by adding sterilized sheep blood with a concentration of (5% v/v) to the prepared blood agar base medium according manufacturer's instructions (Atlas,2004) [5].

3. Specimen Collection

One hundred fifty urine samples (58 of them were from type 1 diabetic patients and 92 were from type 2 diabetic patients) were collected from the midstream. (McPherson and Pincus, 2017) [25].

4. Culturing and Identification Procedure

Specimens were cultivated on both Blood agar and MacConkey agar, and incubated at 37°c for 24 hours in an aerobic environment (Lough *et al.*,2019) [21]. Significant bacteriuria was defined as colony counts that produced bacterial growth of 10^5 CFU/mL of urine (Worku *et al.*,2019) [40].Using the VITEC 2 compact system identification was completed.

5. Virulence factors

5.1 Phenotypic detection of virulence factors of Gram positive isolated species.

The phenotypic detection procedures of virulence factors of only Gram positive isolated species were done.

Biofilm Formation

The tube method (TM) which described by (Ponnusamy *et al.*, 2013) [31] was used to detectin of biofilm formation.

Hemolysin production

A blood agar plate was injected with bacteria, and it was left to grow overnight at 37°C. The appearance of transparent zone around the colonies considered a sign for the presence of β- hemolysin (Tula *et al.*, 2023) [39].

β- lactamase production

β -lactamase production test was performed using the filter paper method described by (Otu *et al.*, 2021) [29] Penicillin solution was prepared by addition of 1g of penicillin to dissolving 0.2% of starch solution. Strips of filter paper (1 x 5 cm) were cut and soaked in Penicillin solution. They left to two-hour for drying. The colonies were rubbed into the paper strip in a circular motion to cover an area measuring about 5 mm in diameter, and then the strips were infected with a loop-full of the freshly isolated test organism, and it was then incubated at 37° C for 30 minutes. The filter paper was covered with an iodine solution flood, and any extra solution was drained. When the injection site became colorless after 10 minutes while the other non-beta-lactamase producers kept their purple color, the test for beta-lactamase was deemed positive.

5.2 Molecular detection of virulence factors of Gram positive isolated species

After conducting the phenotypic detection of the virulence factors, the species that showed a high percentage of these factors were selected in order to conduct the molecular detection of these factors. Table (1).

Table 1: The virulence factors and the bacterial species they possess and their number according to the source of the isolation and the target genes.

bacterial type	Number of isolates causing UTI		virulence factors	Gene name
	from T1DP n=	from T2DP n=		
Staph. aureus	5	7	Hemolysin	*hlB*
			β- lactamase	*blaZ*
E. faecalis	3	4	Hemolysin	*cyl A*
			Biofilm	*esp*

Abberviation: T1DP = type 1 diabetic patients T2DP = type 2 diabetic patients

DNA extraction from bacteria

Presto Mini gDNA Bacterial Extraction Kit from Geneaids company was used. The extraction process was carried out according to the steps provided by the manufacturer.

Agarose gel electrophoresis of DNA

Mix 8 microliters of each of the DNA samples with 2 microliters of loading buffer, The mixture of each sample was carried on agarose gel 1%, then turn on the power supply and adjust the device. The gel was examined in a dark room by exposing it to a (UV transilluminator) at a wavelength of 260 nm. The gel was photographed using the Gel documentation. The molecular size of the DNA segments was estimated by comparing the position and of the bands with the standard size guide (100 bp. (DNA). The molecular weights of the DNA were estimated depending on the distances made by these particles in the gel (Lee *et al.*, 2012) [20].

Polymerase Chain Reaction

Table (1) shows the virulence factors to be investigated and the bacterial species they possess and their number according to the source of the isolation and the target genes**.** The primers prepared from Macrogen company shown in table (2) were used, and they were re dissolved according to manufacturer instruction to obtain a stock solution with a concentration (100 pmol/ µl). The Premix solution prepared by Bioneer company consisting of PCR buffer, deoxynucleotides dNTPS, and Taq DNA polymerase also used. The main reaction mixture consisting of (19 µl nuclease free water, 2 µl premix solution, 1 µl of ***10*** pmol/ µl for each forward primer and 1 µl of ***10*** pmol/ µl for each reverse primer) was prepared and distributed on 0.2 ml Eppendorf tubes with a volume of 23 microliters for each sample, then 2 microliters of DNA for each sample was added to each tube to make the final volume 25 microliters. Then it was input in the thermocycler using the appropriate program and as shown in table (3).

Five microliters of each sample of the PCR products were loaded into agarose gel 2% and added to it the safe red dye (2) microliters installed in the electrophoresis, after the end of the electrophoresis, the gel was removed and examined in a dark room by exposing it to (UV transilluminator) at a wavelength of 260 nanometers and the gel was photographed using the Gel documentation.

Estimate the molecular size of the PCR product by comparing bundle position and thickness with a standard 100bp DNA Ladder size guide.

Table 2: Primers were used for PCR

Primers	sequence	Amplicon size bp	Reference
hlB	F:GTG CAC TTA CTG ACA ATA GTG C R:GTT GAT GAG TAG CTA CCT TCA GT	309	Alshaibah *et al*., 2021 [3]
blaZ	F:AAG AGA TTT GCC TAT GCT TC R:GCT TGA CCA CTT TTA TCA GC	*559*	Dehbashi *et al*., 2021 [12]
cyl A	F:TGG CGG TAT TTT TAC TGG AG R: TGA ATC GCT CCA TTT CTT C	*186*	Hashem *et al*. 2021 [16]
esp	F: TTG CTA ATG CTA GTC CAC GAC C R: GCG TCA ACA CTT GCA TTG CCG AA	*955*	Samani *et al*., 2021 [35]

Table 3: PCR thermocycler programs

Primers	Denatur.1	Denatur.2	Annealing	Exten.1	Exten.2
hlB	72 c^0 /6 m.	72c^0/60 sec	52 c^0/45sec	95c^0/30sec.	72 c^0 /6 m.
	1 cycle	35 cycle			1 cycle
blaZ	72 c^0 /5 m.	72c^0/60 sec	55 c^0/30 sec	94c^0/30sec	72 c^0 /5 m.
	1 cycle	35 cycle			1 cycle
cyl A	72 c^0 /7 m.	72c^0/45 sec	55 c^0/60 sec	94c^0/60sec	72 c^0 /7 m.
	1 cycle	35 cycle			1 cycle
esp	72 c^0 /7 m.	73c^0/45 sec	54 c^0/90 sec	95c^0/60sec	72 c^0 /7 m.
	1 cycle	35 cycle			1 cycle

Results

1. Culture results

Thirty of the fifty eight (51.7%) urine samples cultured from type 1 diabetic patients showed significant bacteriuria. A total of 9 (30%) Gram positive isolates were found *Staph. aureus* being the most common isolate in 5 (55.6%) and *Entero. faecalis* was 4(44.4%).Significant bacteriuria was found in 46 of 92 (50%) urine samples from patients with type 2 diabetes. A total of 11 (23.9%) Gram-positive isolates were found, *Staph. aureus* accounting for the majority in 7(63.63%) and *Entero. faecalis* was 4(36.37%).

2. Results of phenotyping screening of virulence factors

Table (4) shows percentage of virulence factors produced by Gram positive causative agent of UTIs which isolated from both type 1 and type 2 diabetic patients.

Table 4: Rate of virulence factors product from gram positive isolates which isolated from type 1 and type 2 diabetic patients

Pathogens / Virulence factors		*Staph. aureus*		*E. faecalies*		Total
	T2D n=11 n (%)	T1D n =9 n (%)	T2D n=4 n (%)	T1D n=4 n (%)	T2D n =7 n (%)	T1D n=5 n (%)
β-hemolysin	11(100)	9(100)	4(100)	4(100)	7(100)	5(100)
Biofilm	8(72.7)	6(66.7)	4(100)	4(100)	4(57)	2(40)
B-lactamase	7(63.6)	5(55.6)	0 (0)	0 (0)	7(100)	5(100)

Results of the molecular study

The isolates that showed high levels of virulence factors production were selected in order to conduct a molecular detection of the genes responsible for encoding these factors. Genomic DNA of the isolates shown in Table (1) has been isolated.

4. Results of DNA isolation

The results of electrophoresis showed that all samples contained DNA, Figure (1). The purity and concentration of the DNA were measured using the Nanodrop device. The nucleic acid concentrations were adjusted to a concentration of (50 ng/ul) using Free nuclease water. The nucleic acid was used after adjusting the concentration in the Polymerase chain reaction.

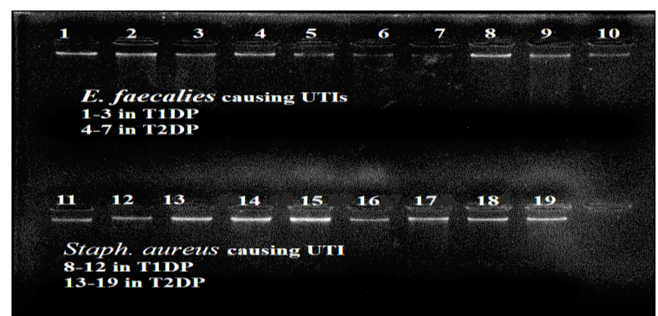

Fig 1: Results of electrophoresis of genomic DNA on an agarose gel 1% for *E. faecalies*

1-3 causing UTI in type 1 diabetic patients 4-7 causing UTI in type2 diabetic patients. and for *Staph. aureus*

8-12 causing UTI in type 1 diabetic patients 13-19 causing UTI in type2 diabetic patients.

Results of Polymerase chain reaction

PCR Detection *hlb* and *blaZ* genes in *Staph. aureus*.

in our study the presence of *hlb* gene was detected through the appearance of a band of 309 base pairs, as shown in Figure (2 A), all isolates (100%) causing UTI in patients with type1and type 2 diabetes mellituse contains this gene.

The presence of *blaZ* gene in our study was detected through the appearance of a band of 559 bp, as shown in the Figure (2 B). It was found that three out of five isolates with a rate of 60% isolated from patients with type 1 diabetes contain this gene, and five out of seven isolates with a rate of 71.4% isolated from patients with type 2 diabetes contain this gene.

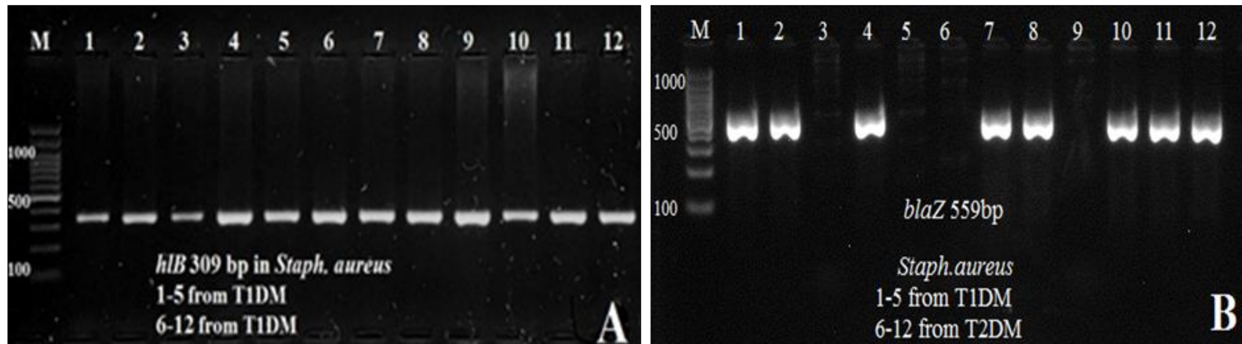

Fig 2: the product of electrophoresis on agarose gel with a concentration of 2% of the *hlb* gene (A) and *blaZ* gene (B) in *Staph. aureus* after amplification of samples by PCR.

1-5: causing UTI in type1 diabetic patients.6-12: cause UTI in type2 diabetic patients.M: marker (100bp).

PCR Detection of *cylA* and *esp* genes in *Entero. faecalies*

The presence of *cylA* gene was detected through the appearance of a band of 186 bp, as shown in Figure (3A). It was found that all isolates (100%) causing UTI in patients with type1 and type 2 diabetes all alone contains this gene.

In our study the presence of *esp* gene was through the appearance of a band of 955 base pairs, as shown in the Figure (3 B). It was found that all isolates (100%) causing UTI in patients with type1 and type 2 diabetes mellituse contains this gene.

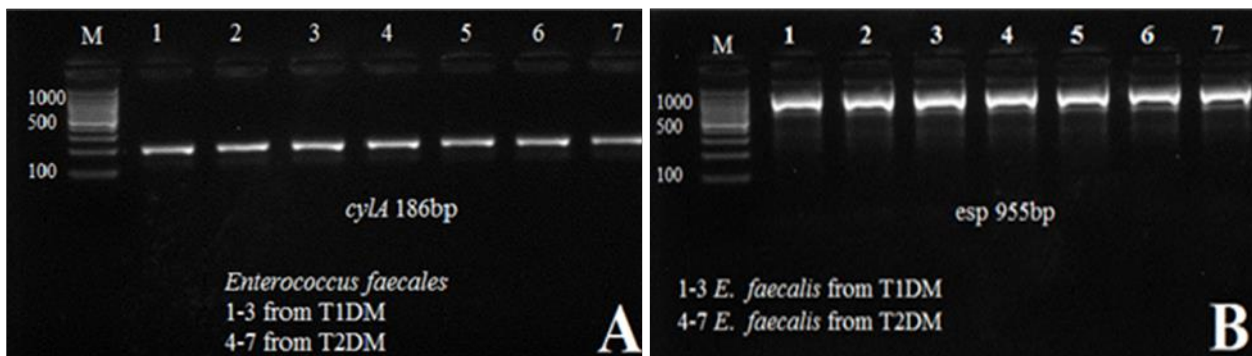

Fig 3: the product of electrophoresis on agarose gel with a concentration of 2% of the primer *cylA* gene (A) and *esp* gene (B) in *Entero. faecalies* after amplification of samples by PCR.

1-3: causing UTI in type 1 diabetic patients. 4-7: cause UTI in type2 diabetic patients.M: marker (100bp).

Discussion

We isolated *Staph. aureus* and *Entero. faecalis* as Gram positive causative agent of UTIs in both type 1 and type 2 diabetic patients. The ability of *Staph. aureus* and *Entero. faecalis* to adherence with epithelial cells of urinary system and colonize inside it explain the ability of these bacteria to cause UTIs and evade immune system (Ognenovska *et al*., 2022) [27], these results were similar to a prior study (Luty *et al*., 2020) [22] at Al-Basrah Governorate and (Al-Azawi and Abbas, 2020) [6] at Wasit Governorate.

β-hemolysin is considered an important factor for bacteria to overcome the host's defense mechanisms, as the lysis of red blood cells increases the availability of iron in the bacterial environment and tissue damage, and this facilitates their survival, proliferation and the possibility of invasion in tissues host (Tula *et al*., 2023) [39]. In this study *Staph. aureus* and *Entero. faecalies* showed their ability to production of β-hemolysin for all isolates causing UTIs isolated from both type1 and type 2 diabetic patients, These results were in concurrenced with a local studies (Hussien and Makhrmash, 2023 and Obaid, 2023) [17,26], but it disagreed with the study (Abead *et al.,* 2013) [2]. Production of β-hemolysin is encoded by *hlb* gene in *Staph. aureus* (Alshaibah *et al*., 2021) [3].This gene located on a 4kb ClaI fragment, contains 330 amino acids having a predicted molecular weight of 39kd (Projan *et al*., 1989) [32], in our study *hlb* gene was detected in all isolates (100%) causing UTI in patients with type1and type 2 diabetes, these results are in concurrence with a study of (Rasheed and Hussein, 2020) [34] in Duhok Governorate but it differed from a study of (Obaid, 2023) [26] that they conducted in Wasit Governorate.

The cylA is a bacterial toxin expressed strains of *Entero. faecalis*, it has β-hemolytic properties in humans and is bactericidal against other Gram-positive (Abead *et al.,* 2013) [2]. Production of cylA toxin is encoded by *cylA* gene

(Hashem *et al*. 2021) [16], It was found that all isolates (100%) causing UTI in patients with type1and type 2 diabetes contains this gene. (Abead *et al.,* 2013) [2] and (Hashem *et al*.2021) [16] indicated that all hemolytic *Entero. faecalis* that they isolated from patients with urinary tract infection possessed the *cylA* gene, while (AL-HAMDANI and TUWAIJ, 2020) [7] indicated that the presence of this gene was in 90.476% of hemolytic *Entero. faecalis*.

Bacterial growth within the biofilm community provides certain degree of shelter and homestasis to the bacteria residing in biofilm and increases bacterial resistance to antimicrobial agents (Makia *et al*., 2013) [23]. In this study *Entero. faecalis* isolates were able to form a biofilm for all isolates causing UTI isolated from both type 1and type 2 diabetic patients separately. These results were agreed with a prior study from Iran (Samani *et al*., 2021) [35]. *Staph. aureus* isolates were able to form a biofilm in (40%) and (57%) for isolates isolated from both type 1and type 2 diabetic patients respectively, these results were closed to a prior study (Fox *et al*., 2005) [13]. Enterococcal surface protein (ESP) is the larger surface protein with repetitive domains which is provides bacterial persistence to antibiotics and facilitate the *E. faecalis* adherence to epithelial cells in bladder leads to form a biofilm, this protein encoded by *esp* gene (Kafil and Mobarez, 2015) [18]. We found that all isolates (100%) causing UTI in patients with type1 and type 2 diabetes contains this gene, these results agreed with a local prior study (Al-Azawi and Abbas, 2020) [6]. in the city of Essaouira, Wasit Governorate.

Beta-lactam antibiotics are the largest family of antimicrobial agents and the most widely used in current clinical practice. These drugs have a slow, time-dependent bactericidal action, generally good distribution in the body, and low toxicity, whose mechanism of action is inhibition of the last stage of bacterial cell wall synthesis, (Suarez and Gudio, 2009) [38]. The β-lactamases are the major defense of bacteria against β-lactam antibiotics by covalently binding to their carbonyl section and degrading the β-lactam ring, thus enabling bacterial resistance to these antibiotics (Gharavi *et al*.,2021) [14]. In this study *Staph. aureus* showed its ability to produce this factor for all isolates isolated from both type 1and type 2 Diabetic patients separately, these results agreed with the results of a prior studies such as (Otu *et al*., 2021) [29] in Nigeria and (Pugazhendhi *et al*., 2020) [33] in Saudi Arabia.

β-lactamases encoded by *blaZ* gene in *Staph. aureus* (Dehbashi *et al*., 2021) [12]. Four types of blaZ product (A, B, C, D) have been distinguished by serotyping and differences in hydrolysis of selected b-lactam substrates types A, C and D are usually located on plasmids, whereas type B typically resides in the chromosome. (Olsen *et al*., 2006) [27], in this study the *blaZ* gene was found with a rate of 60% of isolates isolated from type 1 diabetic patients, and of 71.4% isolated from patients with type 2 diabetes, these results agreed with what was mentioned by (Snoussi *et al*., 2023) [37] in saudi Arabia**.** The presence of other β-lactamases producing genes such as *MecA* and *MecC* explains production of this enzyme inspite of lack of *blaZ* gene (Snoussi *et al*., 2023) [37]. Some islates also lose their ability to produce thes enzyme in the case of long preservation, because this gene is carried on the plasmid (Gharavi *et al*.,2021) [14], and this is what we noticed when the phenotypic redetection of this enzyme in the isolates that showed possession of this enzyme phenotypically, but they do not possess this studied gene, as it was found two isolates lost the ability to produce β-lactamase although they were producing it in the initial detection, and this indicates that they lost this gene during the long preservation**.**

Conclusions

Staph. aureus being the most common of Gram positive causative agent of UTI in both type 1 and type 2 diabetic patients.

The *hlb* gene is found in all uropathogenic *Staph. aureus* in both type 1 and type 2 diabetic patients.

The *blaZ* gene is not only gene responsible for β-lactamase production in *Staph. aureus* and may be lost in the event of prolonged preservation.

The *cyl A* and *esp* genes were appeared in all *Enterococcus faecalis* which isolated from both type 1 and type 2 diabetic patients separately

Acknowledgment

We would like to express our thank and forever grateful to Directorate of Ninava Healh and to the Administration of Al-Wafa·a Specialization Center For Diabetes and Endocrinology in Mosul, and its employees to assist us in completing the research.

References

1. Abbood DH, Alwan ZH. Molecular detection of genes encoding for adhesion factors in biofilm formation among uropathogenic Escherichia coli isolates. Medical Journal of Babylon,2023:20(2):258.
2. Abead SA, Abdul-Razzaq MS, Tarrad RJK. Molecular Detection of cylA and Investigation of Heamolysin Type in Enterococcus faecalis.Medical Journal of Babylon,2013:10(4):992-996.
3. Alshaibah LH, Al-Khafaji ZK, Aljelehawy QH. Evaluation of virulence factors among Staphylococcus aureus strains isolated from patients with urinary tract infection in Al-Najaf Al-Ashraf teaching hospital. Cellular, Molecular and Biomedical Reports,2021:1(2):78-87.
4. Arunachalam K, Pandurangan P, Shi C, Lagoa R. Regulation of Staphylococcus aureus virulence and application of nanotherapeutics to eradicate s. aureus infection. Pharmaceutics,2023:15(2):310.
5. Atlas RM. Handbook of microbiological media. CRC press, 2004.
6. Al-Azawi IH, Abbas MH. Determination Enterococcus faecalis in Asymptomatic Urinary Tract Infection Associated with Diabetes Type 2 Patients in Suwayrah General Hospital-Iraq. Medico-Legal Update,2020:20(1):661-666.
7. AL-HAMDANI ZA, TUWAIJ NS. Molecular Screening of Virulence Genes among Uropathogenic Enterococcus faecalis Isolated from patients in Al-Najaf City, Iraq. International Journal of Pharmaceutical Research (09752366),2020:12(4):331-341.
8. Al-Zubaidi OH, Al-Salman AR. Assessment of parents' awareness about urinary tract infections in children of Babylon Province. Med J Babylon,2022:19:50-7.
9. Berbudi A, Rahmadika N, Tjahjadi AI, Ruslami R. Type 2 diabetes and its impact on the immune system. Current diabetes reviews,2020:16(5):442-449.
10. Bien J, Sokolova O, Bozko P. Role of uropathogenic Escherichia coli virulence factors in development of

urinary tract infection and kidney damage. International journal of nephrology, 2012.

11. Costerton JW, Lewandowski Z, Caldwell DE, Korber DR, Lappin-Scott HM. Microbial biofilms. Annual review of microbiology,1995:49(1):711-745.
12. Dehbashi S, Tahmasebi H, Zeyni B, Arabestani MR. Regulation of virulence and β-lactamase gene expression in Staphylococcus aureus isolates: cooperation of two-component systems in bloodstream superbugs. BMC microbiology,2021:21(1):192.
13. Fox LK, Zadoks RN, Gaskins CT. Biofilm production by Staphylococcus aureus associated with intramammary infection. Veterinary Microbiology,2005:107(3-4):295-929.
14. Gharavi MJ, Zarei J, Roshani-Asl P, Yazdanyar Z, Sharif M, Rashidi N. Comprehensive study of antimicrobial susceptibility pattern and extended spectrum beta-lactamase (ESBL) prevalence in bacteria isolated from urine samples. Scientific reports,2021:11(1):578.
15. Govindarajan DK, Kandaswamy K. Virulence factors of uropathogens and their role in host pathogen interactions. The Cell Surface,2022:8:100075.
16. Hashem YA, Abdelrahman KA, Aziz RK. Phenotype–genotype correlations and distribution of key virulence factors in Enterococcus faecalis isolated from patients with urinary tract infections. Infection and Drug Resistance,2021:10:1713-1723.
17. Hussien HM, Makhrmash JH. The role of haemolysin in pathogenesis of staphylococcus aureus and s. haemolyticus causing urinary tract infections in pregnant and non-pregnant women. Ann. For. Res., 2023:66(1):4431-4443.
18. Kafil HS, Mobarez AM. Spread of enterococcal surface protein in antibiotic resistant Entero-coccus faecium and Enterococcus faecalis isolates from urinary tract infections. The open microbiology journal,2015:9:14.
19. Kline KA, Lewis AL. Gram-positive uropathogens, polymicrobial urinary tract infection, and the emerging microbiota of the urinary tract. Urinary tract infections: Molecular pathogenesis and clinical management,2017:15:459-502.
20. Lee PY, Costumbrado J, Hsu CY, Kim YH. Agarose gel electrophoresis for the separation of DNA fragments. JoVE (Journal of Visualized Experiments),2012:20(62):e3923.
21. Lough ME, Shradar E, Hsieh C, Hedlin H. Contamination in adult midstream clean-catch urine cultures in the emergency department: a randomized controlled trial. Journal of Emergency Nursing,2019:45(5):488-501.
22. Luty RS, Fadil AG, Najm JM, Abduljabbar HH, Kashmar SA. Uropathogens antibiotic susceptibility as an indicator for the empirical therapy used for urinary tract infections: a retrospective observational study. Iranian Journal of Microbiology,2020:12(5):395.
23. Makia RS, Fadhil AM, Ismail MC. Biofilm production as a virulence factor in Uropathogenic bacteria and yeasts. Journal of Biotechnology Research Center,2013:7(1):29-34.
24. Medeiros AW, Pereira RI, Oliveira DV, Martins PD, d'Azevedo PA, Van der Sand S, Frazzon J, Frazzon AP. Molecular detection of virulence factors among food and clinical Enterococcus faecalis strains in South Brazil. Brazilian Journal of Microbiology,2014:45:327-32.
25. McPherson RA, Pincus M R. Clinical Diagnosis and Mangent Laboratory Method. ELSEVIER Inc; 23rd ed., 2017.
26. Obaid HA. Investigation of some physiological, biochemica characteristics and multiple antibiotic resistance of Staphylococcus aureus isolated from wound samples. Journal of Advanced Education and Sciences,2023:3(3):09-16.
27. Ognenovska S, Mukerjee C, Sanderson-Smith M, Moore KH, Mansfield KJ. Virulence Mechanisms of Common Uropathogens and Their Intracellular Localisation within Urothelial Cells. Pathogens,2022:11(8):926.
28. Olsen JE, Christensen H, Aarestrup FM. Diversity and evolution of blaZ from Staphylococcus aureus and coagulase-negative staphylococci. Journal of Antimicrobial Chemotherapy,2006:57(3):450-460.
29. Otu JU, Izevbizua EV, Ekpiken ES. Carriage of Plasmid-mediated β-lactamase genes of Staphylococcus aureus isolates from Outpatients and Inpatients of UCTH, Calabar, Cross River State, Nigeria. Int. J. Curr. Res. Med. Sci.,2021:7(2):5-18.
30. Poirel L, Ortiz de la Rosa JM, Sadek M, Nordmann P. Impact of acquired broad-spectrum β-lactamases on susceptibility to cefiderocol and newly developed β-lactam/β-lactamase inhibitor combinations in Escherichia coli and Pseudomonas aeruginosa. Antimicrobial Agents and Chemotherapy,2022:66(4):e00039-22.
31. Ponnusamy P, Nagappan R. Extended spectrum beta-lactamase, biofilm-producing uropathogenic pathogens and their antibiotic susceptibility patterns from urinary tract infection-an overview. Int J Microbiol Res.,2013:4(2):1836-842.
32. Projan SJ, Kornblum J, Kreiswirth B, Moghazeh SL, Eisner W, Novick RP. Nucleotide sequence: the beta-hemolysin gene of Staphylococcus aureus. Nucleic Acids Research,1989:17(8):3305.
33. Pugazhendhi A, Michael D, Prakash D, Krishnamaurthy PP, Shanmuganathan R, Al-Dhabi NA, Duraipandiyan V, Arasu MV, Kaliannan T. Antibiogram and plasmid profiling of beta-lactamase producing multi drug resistant Staphylococcus aureus isolated from poultry litter. Journal of King Saud University-Science,2020:32(6):2723-2727.
34. Rasheed NA, Hussein NR. Characterization of different virulent factors in methicillin-resistant Staphylococcus aureus isolates recovered from Iraqis and Syrian refugees in Duhok city, Iraq. PloS one,2020:15(8):e0237714.
35. Samani RJ, Tajbakhsh E, Momtaz H, Samani MK. Prevalence of virulence genes and antibiotic resistance pattern in Enterococcus faecalis isolated from urinary tract infection in Shahrekord, Iran. Reports of Biochemistry & Molecular Biology,2021:10(1):50.
36. Selman NA, Albayati AH, Alsaffar Y, Farhan AH. The risk impact and benefits of Ramadan fasting on patients with type 2 diabetes in a sample of Iraqi patients. Medical Journal of Babylon,2023:20(2):352.
37. Snoussi M, Noumi E, Bouali N, Bazaid AS, Alreshidi MM, Altayb HN, *et al*. Antibiotic Susceptibility Profiling of Human Pathogenic Staphylococcus aureus

Strains Using Whole Genome Sequencing and Genome-Scale Annotation Approaches. Microorganisms,2023:11(5):1124.

38. Suarez C, Gudiol F. Beta-lactam antibiotics. Enfermedades in fecciosas y microbiologia clinica,2009:27(2):116-29.
39. Tula MY, Filgona J, Kyauta SE, Elisha R. Screening for some virulent factors among bacterial isolates from surfaces of hospital fomites and hands of healthcare workers. Cellular, Molecular and Biomedical Reports,2023:3(1):9-16.
40. Worku YG, Alamneh BY, Abegaz EW. Prevalence of bacterial urinary tract infection and antimicrobial susceptibility patterns among diabetes mellitus patients attending Zewditu memorial hospital, Addis Ababa, Ethiopia. Infection and drug resistance,2021:15:1441-1454.