Phenotypic and Genotypic Detection of Some Virulence Factors in *Enterococcus faecalis*Isolated From Urine Specimens.

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ABSTRACT

Background: *Enterococci* are responsible for a large proportion of Gram-positive UTI worldwide. Virulence factors and biofilm formation plays a crucial role in the pathogenesis of enterococcal infections, which also prevents antimicrobial agents from penetration**.** This research aimed to determine the incidence of UTIs, and detection of some virulence factors among *Enterococcus faecalis*isolates.

Materials and methods: Twenty*E. faecalis* isolates from patients with urinary tract infection were identified; phenotypic and genotypic characteristics were investigated in Al-Najaf City, Iraq.

Results: The*E. faecalis* isolates showed varying presences of virulence factors. Phenotypically, biofilm formation was exanimated, all of these isolates exhibited their ability to biofilm formation.In addition, it is ability to produce Gelatinase, protease and hemolysin in 30%, 55% and 85% of the isolates, respectively. Virulence factors genes *esp*, *gelE* and *hyl* were found in 60%, 80%, 1% of isolates, respectively.

Conclusion :*E. faecalis* may be have many virulence factors such as production of gelatinase, protease and haemolysin. In addition, to biofilm formation that exhibited by all isolated *E. faecalis.*Data of PCR techniques confirmed the ability of *E. faecalis* isolates to carry virulence factors genes such as (*esp, gelE* and *hyl*).

Keywords: *Enterococcus faecalis*, Virulence factors, Urinary tract infection, Biofilm formation.
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INTRODUCTION

Urinary tract infections (UTIs) are the most prevalent bacterial infections that often affect all components of the urinary system. It is the second most common infection following respiratory tract infections, with a greater susceptibility rate among women than men [1].Infections of the urinary tract produced by gram-positive bacteria are thought to be less common than infections caused by gram-negative bacteria, which are often caused

by *Enterococcus* species, *S. saprophyticus*, and group B *Streptococcus*[2]. *Enterococci* are responsible for a large proportion of Gram-positive UTI worldwide [3].The pathogenic potential of *E. faecalis* and all *enterococci* in general has been largely attributed to their vicious and amazingly adaptable nature, which includes an inherent tolerance to commonly used antibiotics (like cephalosporins), chlorine, alcohol-based detergents, and the ability to survive extremely high and low temperatures, pH levels, oxygen tension, humidity, and nutrient availability [4].Enterococcal surface protein (Esp), hyaluronidase (Hyl), gelatinase (GelE), and cytolysin (CylA) are among the virulence factors that contribute to adhesion, colonization, evasion of the host immune response, extracellular production of enzymes, pathogenicity, and infection severity [5].A major role is played by the enterococcal polysaccharide antigen in the pathogenicity of UTIs, including its adherence to epithelial cells, biofilm formation, and evasion of phagocytosis by neutrophils [6]. In addition to other virulence factors, biofilm development plays a crucial role in the pathogenesis of enterococcal infections, which also prevents antimicrobial agents from penetration [7]. The aim of study include Isolation of *E. faecalis* from patients with UTIS. In addition, phenotypic and genotypic detection of some virulence factors among *E. faecalis* isolates.

MATERIALS AND METHODS

Bacterial isolates

*Enterococcus faecalis*isolates were collected in Al-Kawthar health care center, Al-Najaf Al-Ashraf, Iraq.Fromurine of patients with urinary tract infections, for 3-months period starting from November 2021. Identification of *enterococci* was based on their colony morphology, gram staining, negative catalase activity, ability to hydrolyze esculin and cultural characteristics on Hicrome UTI agar. Further identification done by using PCR technique and VITIC-2 compact system

Phenotypic detection of virulence factors

Gelatinase production

Production of gelatinase was determined by stabbing *E. faecalis*isolates into tubes containing nutrient gelatin medium (12%) and incubation at 37ºC for 24 to 72 hr. Bacteria that produced gelatinase liquidated the culture medium, even after the refrigeration. The negative test showed as intact gelatin medium after refrigeration [8].

Protease Production

Skim milk agar plates inoculated by streaking and incubated at 37ºC for 48 hours. The clear zone exists adjacent the positive result. That indicated the production of protease enzyme [9].

Hemolysin production test:

The presence of haemolysin was detected as phenotypical on blood agar. Inoculated plates were incubated for 24hr at 37ºC. Haemolysin production was determined as a zone of β-haemolysis around the colonies [10].

Biofilm formation

Tissue Culture Plate Method include Transport newly cultivated colonies and inoculate tryptic soy broth with 1% glucose. Incubate inoculated broth at 37°C for 24 hours. Using fresh medium, bacterial suspensions were diluted 1:100. Using sterile polystyrene tissue culture plate (96 wells), appropriate number of wells filled with 200 µl of the prepared bacterial suspension. In addition, only sterile broth used to ensure sterility and to identify non-specific binding. After incubation at 37°C for 24 hours, Plate gently turned to remove wells contents, and then washed with 200µl phosphate buffer saline. The wells washed three times to remove bacteria. Sodium acetate (2%) added to the wells

and kept for 30 minutes in order to fix the biofilms formed by bacteria attached to the wells. Staining of the fixed biofilms using crystal violet (0.1%). After 30 minutes, wells washed with distilled water to remove additional stain. After drying, a microplate reader (at 570 nm wavelength) used to measure the optical densities (OD) of stained bacterial biofilms. Optical densities values indicated bacterial adherence to the wells and biofilm formation. Table (1) show The OD values were calculated and biofilm production was graded into strong, moderate and non/weak [11].

Table (1) Classification of bacterial adherence by TCP method

Optical density	Biofilm formation
< 0.120	**Non/ weak**
0.120- 0.240	**moderate**
>0.240	**strong**

Detection of *E. faecalis* virulence genes

The prevalence of virulence genes among *E. faecalis*isolates were investigated by PCR. PCR was performed to detect virulence genes, including enterococcal surface protein (*esp*), gelatinase (*gelE*) and hyaluronidase (*hyl*). were detected among *E. faecalis* isolates. Primer pairs used are listed in Table (2).

Primers	Sequence (5'-3')	Product size(bp)	References
16s rRNA for *E.faecalis*	F: TGGCATAAGAGTGAAAGGCGC R: GGGGACGTTCAGTTACTAACGT	290	[10], [12]
hyl	F: ACAGAAGAGCTGCAGGAAATG R: GACTGACGTCCAAGTTTCCAA	276	[13]
esp	F: TTGCTAATGCTAGTCCACGACC R: GCGTCAACACTTGCATTGCCGAA	933	[14]
Gel E	F: ACCCCGTATCATTGGTTT R: ACGCATTGCTTTTCCATC	419	[14]

Table (2): primers used in this study

RESULTS

Out of 412 urine specimens, 110 specimens (78 females and 32 males) had positive urine culture and the etiological agents that caused the urinary tract infection were as follows: out of 110 specimens 60 (54.54%) gram-negative isolates and 50 (45.45%) gram-positive isolates.

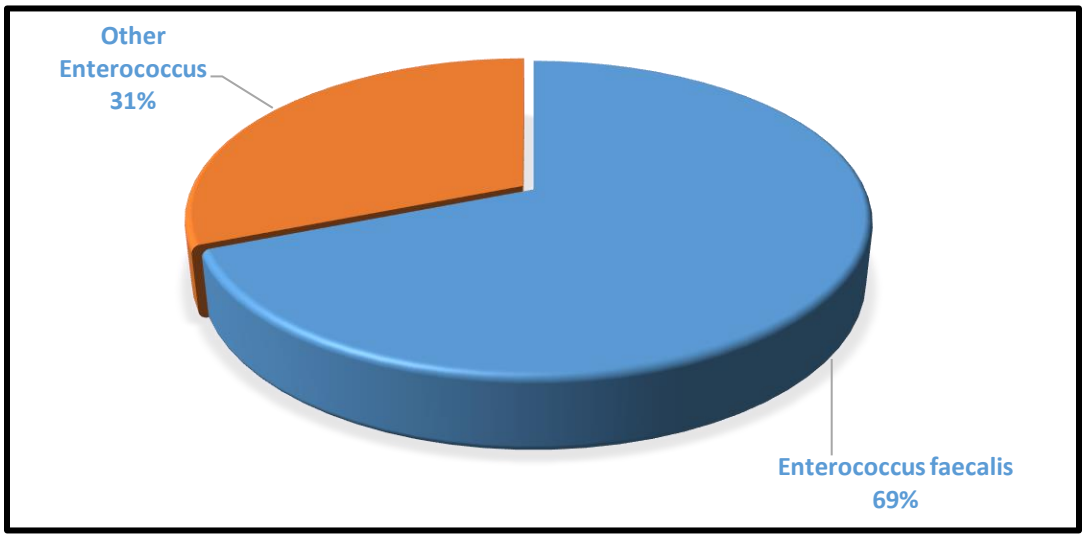
Table (3): The number of patients

		Female	Male	Total
Status	**Significant pyuria**	**78**	**32**	**110**
	Insignificant pyuria	**137**	**165**	**302**

Total		**215**	**197**	**412**
p-value: 0.000 Chi-Square: 21.086 significant differences between gender groups at p-value <0.05				

Enterococcus species were identified as causative agents of urinary tract infection in present study: 20 *Enterococcus faecalis* and 9 other *Enterococcus* accounted for 29 *Enterococcus ssp.*figure (1).

Figure (1): The percentage of *E. faecalis* that cause UTI.



Phenotypic detection of some virulence factors

All isolates of *E.faecalis* were displayed ability to form a biofilm were two (1%) isolates strong biofilm formation, 9 (45%) isolates intermediate and nine (45%) isolates weak.

It was found that 30% of *E. faecalis* isolates were have ability to gelatin hydrolysisand 55% of *E. faecalis* isolates were positive for protease enzyme production. Haemolysin production detected phenotypically on human blood agar as a clear zone of β-haemolysis around the colonies. It was found that 85% of *E. faecalis* isolates were positive for this haemolysin as shown in figure (2)

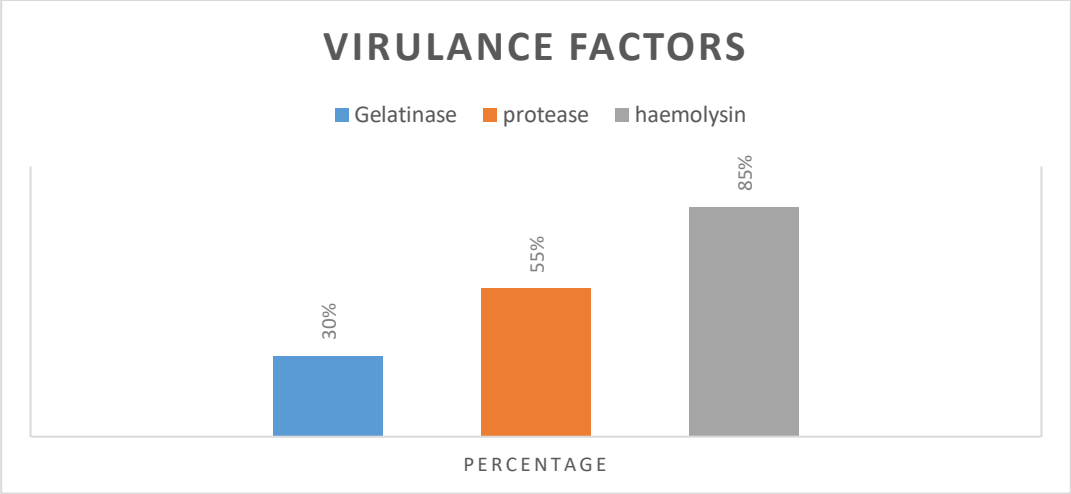


Figure (2): Percentage of virulence factors among *E. faecalis* isolates

Molecular Detection of virulence factors Genes

This study included the detection of genes encoding for virulence factors genes among 20 isolates of *E. faecalis* using specific primers for recognizing those genes.

The results of PCR amplification of *esp* gene (Enterococcal surface protein) revealed that most isolates of *E. faecalis* were positive. The figure (3) demonstrated that the results of PCR amplification for *esp* gene were obvious in an amount reaching 12 (60%) of isolates have positive results.

Detection of *esp* gene in *E. faecalis*

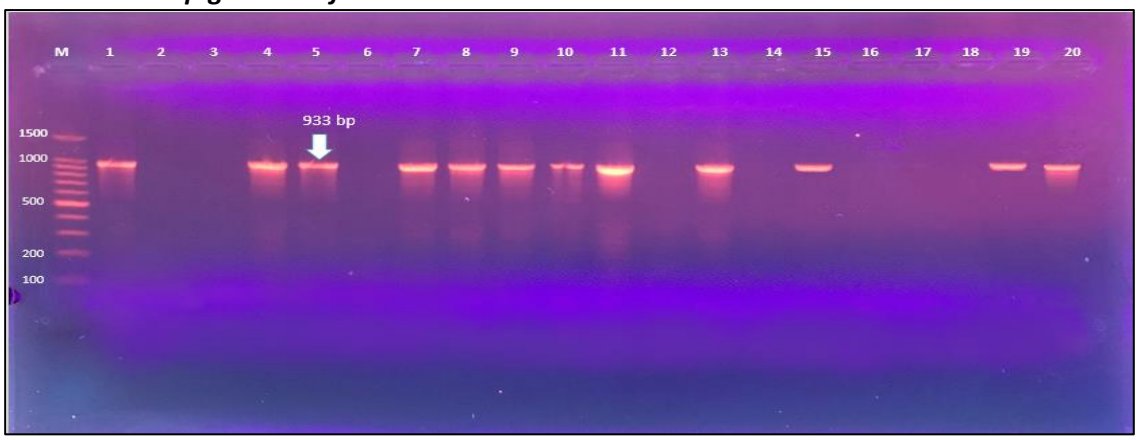


Figure (3): Electrophoresis diagram of PCR amplified products for extracted DNA of 20 isolates of *E. faecalis*using specific *esp* gene primer show positive products at 933 bp. Line M: molecular size DNA marker and products migrated at 75 volt for 80 minutes and stained with ethidium bromide.

Detection of *gel E* gene in *E. faecalis*

The results of PCR amplification of *gelE* gene (Gelatinase E) revealed that most isolates of *E. faecalis* were positive. The figure (4) demonstrated that the results of PCR amplification for *gel E* gene were obvious in an amount reaching 16 (80%) of isolates have *gelE* gene.

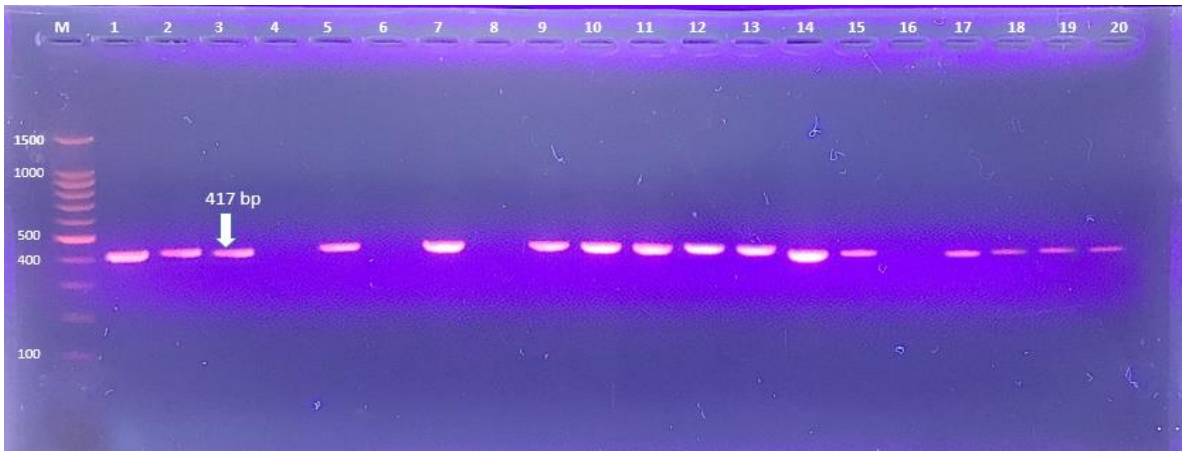


Figure (4): Electrophoresis diagram of PCR amplified products for extracted DNA of 20 isolates of *E. faecalis* using specific *gel E* gene primer show positive products at 417 bp. Line M: molecular size DNA marker and products migrated at 75 volt for 80 minutes and stained with ethidium bromide.

Detection of *hyl* gene in *E. faecalis*

The results of PCR amplification of *hyl* gene (hyaluronidase) revealed that only one isolate of *E. faecalis* were positive. The figure (5) demonstrated that the results of PCR amplification for *hyl* gene were only one (5%) of isolates harboring this gene.

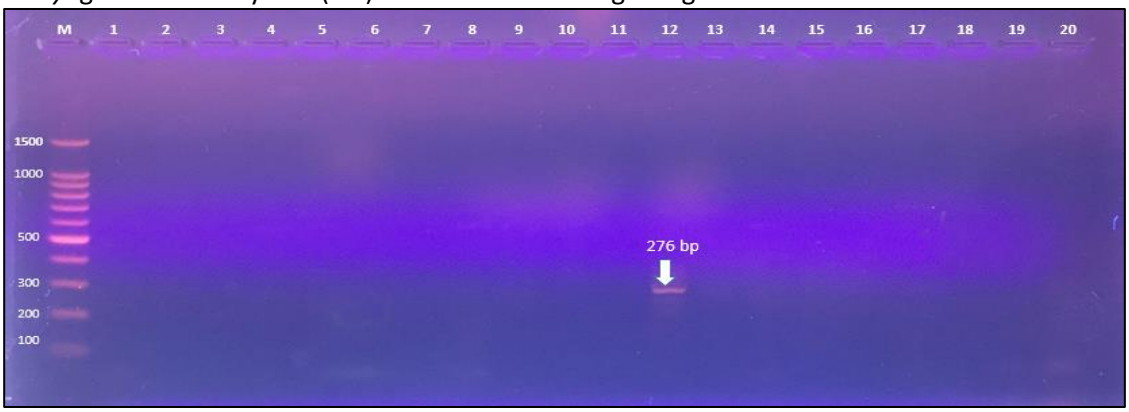


Figure (5): Electrophoresis diagram of PCR amplified products for extracted DNA of 20 isolates of *E. faecalis* using specific *hyl* gene primer show positive products at 276 bp. Line M: molecular size DNA marker and products migrated at 75 volt for 80 minutes and stained with ethidium bromide.

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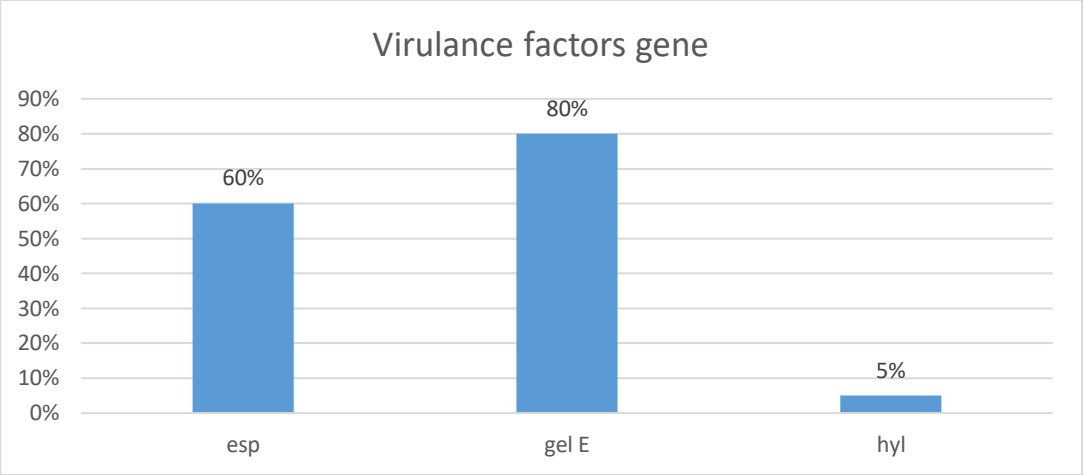

Figure (6): Distribution of virulence factors genes among*E. faecalis* isolates.

DISCUSION

Results show that gram-negative bacteria are more likely to cause urinary tract infections than gram-positive bacteria. which is compatible with a local study conducted by [15], that show Bacterial growth among urine specimens were Gram negative higher than Gram positive, and the spreading of Gram-negative pathogens among clinical specimens due to its ability to cause disease by lipopolysaccharides (LPS) which act as endotoxin.*Enterococcus faecalis* is the commonest species isolated from human clinical samples, followed by *E. faecium*[16]**.** Several previous international and local researches and studied have demonstrated that *E. faecalis* isolates are one of main causing infection especially urinary tract of human [15], [17].

These results seemed to agree with several studies that reported *Enterococci* are nosocomial pathogens that can form biofilms, which contribute to their virulence and antibiotic resistance, and show that all isolates of *E. faecalis* formed biofilms [18], [19]. All these investigations observed that *E. faecalis* is an important microorganism which creates biofilms which somewhat narrows the therapy options of antimicrobials to treat infections.

These results in agreement with other studies that reported the ability of *E. faecalis* to produce this enzyme [7], [20], [21].these results show agreement with [22] that reported *E. feacalis* is able to produce active protease.Toxins (hemolysins) produced by microbial strains with β- hemolytic activity lyse blood cells, and damaging the host's immune system [23].These observations in agreement with the other studies that revealed there are difference in the type of hemolysis among *E. faecalis*[24], [25].

Many studies conducted by[8], [26], [27] that reported the presence of *esp* gene in *E. faecalis* at percentage (53.5%),(81.9%) and (68.70%) , receptively.The percentage of *E.faecalis* isolated in this study that harboring *gelE* gene was (80%), while only (30%) of *E. faecalis* isolates had gelatinase activity, that agree with the study[19] that reveal the detection of *gelE* gene was detected in 87% of the isolates, only 22% of the isolates had gelatinase activity.The hyaluronidase is an enzyme encoded by *hyl* gene and plays a role in colonization and in spreading of bacteria [28]. The result in current study show only one isolate (5%) of *E. faecalis* harboring *hyl* gene, wich is less

than results reported by [29] that include 17.5% of isolated. *E faecalis* had *hyl* gene. While other study that conducted by [30] reported that *hyl* gene was no detected in all isolated *E. faecalis*.

CONCLOSION

Urinary tract infection is more frequent in female than male, and UTI caused by gram-positive bacteria more than gram-negative. *E. faecalis* may be have many virulence factors such as production of gelatinase, protease and haemolysin. In addition, to biofilm formation that exhibited by all isolated *E. faecalis.*Data of PCR techniques confirmed the ability of *E. faecalis* isolates to carry virulence factors genes such as (*esp, gelE* and *hyl*).

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