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Research Article

Detection of some virulence genes (*esp*, *agg*, *gelE*, *CylA*) in *Enterococcus faecalis* isolated from different clinical cases at Baghdad

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Abstract
The virulent genes are the key players in the ability of the bacterium to cause disease. The products of such genes that facilitate the successful colonization and survival of the bacterium in or cause damage to the host are pathogenicity determinants. This study aimed to investigate the prevalence of virulence factors (*esp, agg, gelE, CylA*) in *E. faecalis* isolated from diverse human clinical collected in Iraqi patient , as well as to assess their ability to form biofilm and to determine their haemolytic and gelatinase activities. Thirty-two isolates of bacteria *Enterococcus faecalis* were obtained, including 15 isolates (46.87%) of the urine, 6 isolates (18.75%) for each of the stool and uterine secretions, and 5 isolates (15.62%) of the wounds from various hospitals in Baghdad, including (Central Children's Hospital, Educational Laboratories, Ibn Al-Baladi Hospital).The isolates were confirmed to belong to the genus *E.faecalis* after performing morphological and biochemical microscopic examinations and for final diagnosis using the VITEC 2 system. The virulence genes viz. *cylA*, *esp*, *gelE* and *agg* were recognized in the *E. faecalis,* and the consequences appeared that the bacteria had *eps* gene in 32 isolates (100%). As for the *agg* gene, 32 isolates (100%) were carriers of this gene, which was responsible for these isolates' aptitude to form the biofilm. While for the *gelE* gene, 27 isolates (84.37%) of the isolates carried this gene, responsible for gelatinase activity whereas, the gene responsible for hemolysis *cyl*, there were 29 isolates (90.62%) of the total isolates. The presemce of genes in the isolates would be helpful to determine the colonization and survival of the bacterium in or causing damage to the host.

Keywords: Biofilm, *Enterococcus faecalis*, Gelatinase, Hemolysin, Virulence genes

INTRODUCTION

Enterococci faecalis is a selective anaerobic bacterium positive for the gram stain that appears under a microscope as single pairs or short chains of cocci (Hashem *et al*., 2021). *E. faecalis* is spherical or oval-shaped and found in pairs or chains of individuals with similar lengths, negative to the catalase test. However, a false reaction can sometimes occur when grown on a blood-containing medium (Kamel and Yaaqoob, 2022).
Enterococci faecalis is a common bacterium that infects the human and animal digestive systems and is generally conveyed in water, soil, food and plant items (Giraffa 2006; Frazzon *et al*., 2009). These bacteria have been related to hospitalized contaminations, bacteremia, endocarditis, surgical wound contagion, and urinary tract diseases (Medeiros *et al*., 2014). It also causes vaginitis, one of the most common venereal diseases and affects women in puberty (Abed and Kandala, 2016). It is found naturally in the digestive system of humans and animals. Yet, it is additionally tracked down in different destinations, including the oral cavity and vagina. It is one of the two most significant sorts among other types that cause opportunistic infections that affect humans, including urinary tract infections, surgical-sit-infection, burn infection, bacteremia, endo-

carditis, cholecystitis, peritonitis, and neonatal meningitis (Strateva *et al*., 2016; Kadhum and Zaidan,2020).

Public interest in *E. faecalis* and the management of enterococcal diseases has expanded because of the development of numerous antibiotic-resistant strains because of their self-resistance from many usually utilized antibiotic agents or their capacity to acquire resistance from all presently accessible antibiotic agents through mutation establishment or by the transmission of genetic substantial through plasmids and transposons (Cetinkaya *et al*., 2000; Dahle´n *et al*., 2012; Kadhem and Flayyih, 2014). The widespread distribution of enterococcal bacterial strains linked to their resistance to numerous antibiotics has become one of the principal issues in managing urinary tract disease (Faisal *et al*., 2013). The pathogenicity ability to produce many virulent factors, including cytolysin, sex pheromone and surface adhesions (Hasson and Kadhem, 2015).

The principal virulence factors described in enterococci are depicted in enterococci collection substances, cytolysin, gelatinase, enterococcal surface protein and hyaluronidase (Georges *et al*., 2022). The enterococci can form biofilms, contributing to their virulence and antibiotic resistance (Hashem *et al*., 2017). It has been proven that biofilms are associated with many diseases that affect humans and can be observed on medical devices (Oleiwi and Abid, 2014). This study sought to identify virulence factors *cylA, esp, gelE*, and *agg* genes in *E. faecalis* isolates from the stool, urine, vaginal swabs and wounds .

MATERIALS AND METHODS

Isolation and biochemical diagnosis of *E. faecalis*

Thirty-two isolates of enterococcus faecal bacteria out of 70 isolated isolates from various clinical sources (stool, urine, vaginal swabs, wounds) were obtained from several hospitals in Baghdad (Central Children's Hospital, Educational Laboratories, Ibn Al-Baladi Hospital). Ethical approval was obtained from the Research Ethical Committee of Ibn Al-Baladi Hospital, University of Baghdad. During the period from 1/7/2022 to 30/9/2022, all isolates were cultured on blood agar and bile esculin agar and incubated at 37°C for 24 hours, and then the bacteria were recognized and established on microscopic diagnosis and biochemical tests, including oxidase and catalase tests, for final diagnosis using a VITEK 2 system to detect *E. faecalis*.

Extraction of DNA

According to the producer's guidelines, bacterial DNA was extracted utilizing a DNA kit (ABIOpure, USA). Quantus Fluorometer Promega, USA was utilized to identify the concentration of extricated DNA. For 1 µl of DNA, 200 µl of diluted Quantifluor dye (Promega, USA) was blended, and after 5min of incubation at room temperature, DNA concentration values were identified.

Preparation of primers and PCR mixture

The stock solution of primers (*agg, gelE, esp, cylA*) was ready as indicated by the guidelines of the producer (Macrogen, Korea) (Table 1). in a lyophilized. Lyophilized primers were disintegrated in nuclease-free water to give a final 100 pmol/µl concentration as a stock solution. A functioning solution of the primers was ready by the addition of 10µl of primer stock solution (keep at cooler -20 °C) to 90µl of nuclease-free water to get a functioning primer solution of 10 pmol/µl.

The preparation of the PCR reaction involved Mastermix (Promega, USA) with all-out volume (20 µl) of the reaction combination, which involved 2 µl of DNA template, 1 µl for each of the primers, 10 µl GoTaq Green mastermix, and 6µl Nuclease Free water (Promega, USA). The PCR polymerase reaction was mixed gently by using the Vortex (Quality Lab System, England). The PCR polymerase reaction mixture for genes was prepared, as shown in Table 2. Then placed in a PCR thermal cycler (Thermo Fisher Scientific, USA).The gene amplification reactions were carried out according to Hashem *et al*. (2021), as shown in Table 3.

Electrophoresis of PCR product

The PCR products (5 µl) were electrophorized on 1.5% agarose gel with 1µl Ethidium bromide (Promega, USA) at 100 vol. for 60 min. The Ethidium bromide-stained bands in gel were envisioned utilizing a Gel imaging system (Significant Science, Taiwan).

RESULTS AND DISCUSSION

Identification

Among seventy isolates obtained from different clinical sources after conducting tests, 32 isolates belonged to *E.faecalis* 6/32 (18.75%) from stool, 5/32 (15.62%) from wounds, 15/32 (46.87%) from urine and 6/32 (18.75%) from uterine secretions. When cultured on blood agar, the appearance of colonies was in white to a gray color and viscous texture, as shown in (Fig. 1A). In contrast, colonies appeared shiny on bile esculin

A B

Fig. 1. *Growth of Enterococcus faecalis (A) on Blood agar, (B) on Bile esculin*

Table 1. Nucleotide sequence of the primers used to detect genes

Genes	Sequencing Primer sequence (5'- 3)	Product size (bp)	Reference
agg-F	TCTTGGACACGACCCATGAT	413	Hashem *et al*., 2021
agg-R	AGAAAGAACATCACCACGAGC		
gelE-F	CGGATTGGTTACACCATTATCC	296	Kim *et al*., 2020
gelE-R	TGCCACTCCTTATCCATTTTT		
esp-F	TCGCTCCAAATGAAAAAGATG	150	Kim *et al.,* 2020
esp-R	CGGTTGAACCTT CTTCTGGT		
cylA-F	ACTCGGGGGATTGATAGGC	700	El-Askary and Zaher, 2022
cylA-R	GCTGCTAAAGCTGCGCTT		

Table 2. PCR mixture of genes

No.	PCR mixture	Volume (µl)
1	Forward primer	1
2	Reverse primer	1
3	DNA	2
4	Nuclease Free Water	6
5	Master Mix	10
	Total volume	20

agar and the color of the medium changed to black, as in Fig. 1B. Microscopic examination of gram stain showed that they were gram-positive cells, and biochemical tests showed a negative result for both oxidize and catalase. In contrast, the results of the VITEK2 system showed a positive result for 32 isolates (100%).

Genotypic detection

cylA gene was found in 29 isolates (90.62%) of *E.faecalis* from diverse clinical sources, as observed in Fig. 2 and Table 4. This consequence was somewhat close to the finding of Al-Saadi (2013), who stated that all *E. faecalis* isolates had *cylA* gene in percentage (100%). This result differed from the finding of Medeiros *et al*. (2014), who found that 54.4% of *E. faecalis* isolates contained the *cylA* gene and Hashem *et al*. (2021), who pointed out that *E. faecalis* isolates possessed this gene in a rate of 54%. The finding of this research also conflicts with a result of Heidari *et al*. (2016), who detected that the *cylA* gene was 30.4% of *E. faecalis*, Cytolysins toxins produced by *E. faecalis* can deteriorate cell membranes to facilitate the contagion manner *CylA* gene present into a chromosome or plasmid of bacteria (Biendo *et al*., 2010). Cytolysin-producing by *E. faecalis* has been shown that virulent in human contagions and related to the expanded seriousness of the disease (Kiruthiga *et al*., 2020).

A virulence gene *gelE* in the present study was found in 27(84.37%), as indicated in Fig. 3 and Table 4. The finding of this study was identical to the finding by Kiruthiga *et al.* (2020), wherever it was found that *E.faecalis* isolates (85.39%) possessed this gene. The finding of the present research was similar to the study of Al-Saadi, (2013) which indicated that all isolates of *E. faecalis* from different clinical sources possessed *gelE* gene at a percentage of 100%.In the present study, results differed from Hussain (2020), who indicated that 63.82% of isolates contained this gene isolated from different clinical sources. The results contradict Kandala *et al*. (2010), who appareled that all *E.faecalis* isolates did not show the capacity to income gelatinase. Gelatinase formed by *E. faecalis* was encoded by *gelE* gene that could hydrolyze gelatin casein collagen and hemoglobulin (Kiruthiga *et al*., 2020). Gelatinase construction by *E.faecalis* participates in virulence of bacteria in humans. This enzyme's ability to degrade collagen and some bioactive peptides suggests that it is involved in the beginning and spread of inflammatory procedures, including *E. faecalis* (Lee and Tan, 2015).

Molecular studies of Enterococcal Surface Protein

Table 3. Optimal conditions of PCR reaction for the genes determination (Hashem *et al*., 2021)

Steps	C°	Minute:Second	Cycle
Initial Denaturation	95	05:00	1
Denaturation	95	00:30	30
Annealing	56*(cylA)* 60*(gelE,esp,agg)*	00:30	
Extension	72	01:00	
Final extension	70	07:00	1
Hold	10	10:00	

Table 4. Proportion of *Enterococcus faecalis* virulence genes isolated from various clinical cases.

Source of isolates	Number of isolations % Percentage	%Percentage per gene			
		cylA	***gelE***	***Esp***	***Agg***
Urine	15(46.87%)	13(86%)	(80%)12	(100%) 15	(100%)15
Wounds	5(15.62%)	4(80%)	(60%) 3	(100%) 5	(100%)5
Vaginal	6(18.75%)	6(100%)	(100%)6	(100%) 6	(100%)6
Stool	6(18.75%)	6(100%)	(100%)6	(100%) 6	(100%)6
Total	32 (100%)	29 (90.62%)	27 (84.37%)	32 (100%)	32 (100%)

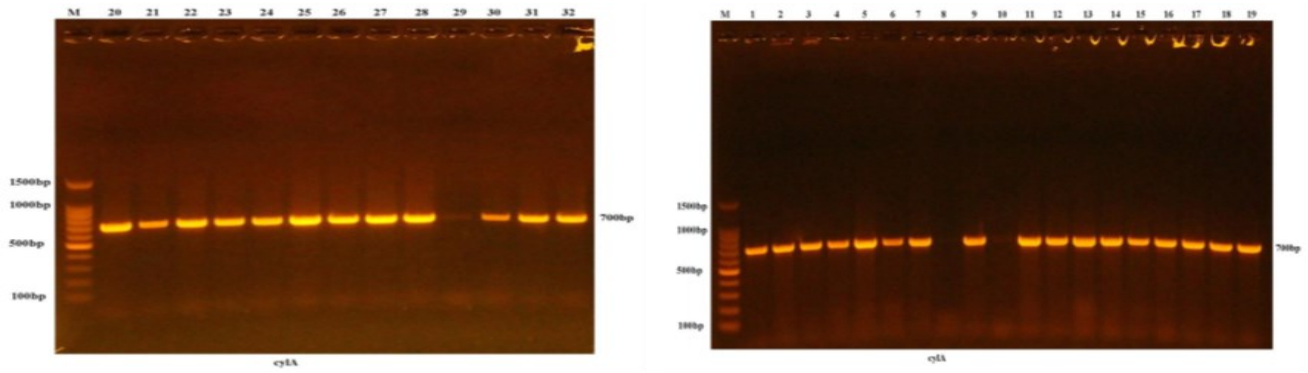

Fig. 2. *Consequences of the amplification of cylA gene 700bp of Enterococcus faecalis were fractionated on 1.5% agarose gel electrophoresis with 1ml Eth.Br (10mg/ml).for 60min M:100bp ladder marker. Lanes 1-32 positive results*

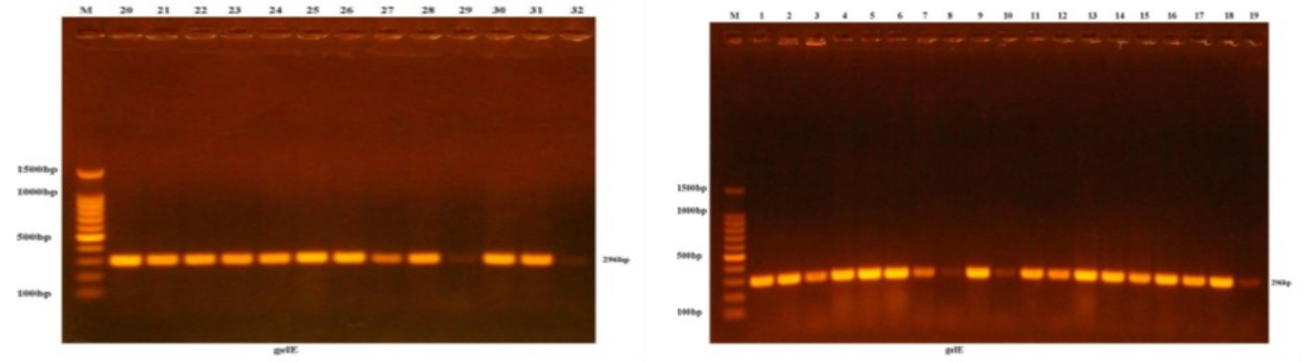

Fig. 3. *Consequences of the amplification of gelE gene 296bp of Enterococcus faecalis were fractionated on 1.5% agarose gel electrophoresis with 1ml Eth.Br (10mg/ml).for 60min M: 100bp ladder marker. Lanes 1-32 had positive results*

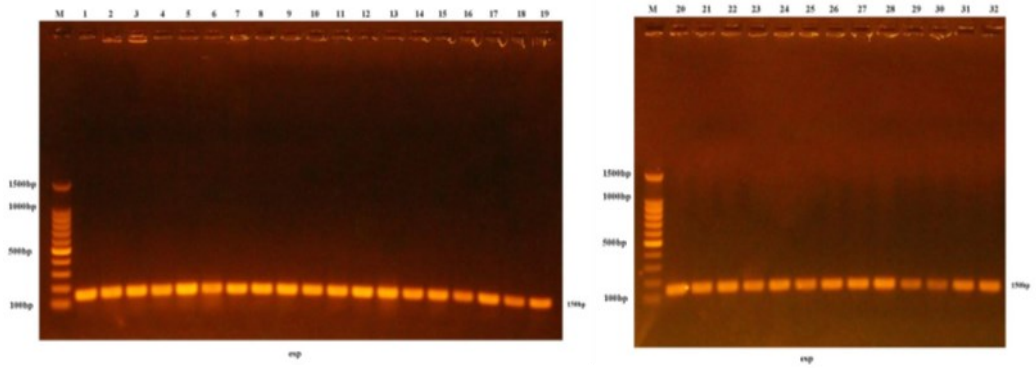

Fig. 4. *Consequence of the amplification of esp gene 150bp of Enterococcus faecalis were fractionated on 1.5% agarose gel electrophoresis with 1 ml Eth.Br (10mg/ml) for 60min M: 100bp ladder marker. Lanes 1-32 positive results*

(*esp*) gene done for all 32(100%) *E.faecalis* isolates indicated that *esp* gene was found in all isolates of these bacteria, as shown in Fig. 4. and Table 4. These findings were similar to consequences of Al-Saadi (2013), who pointed out that all *E. faecalis* isolates from urine, stool and vaginal samples had *esp* gene in percentage (100%). Nevertheless, the findings of the present study differed from the results gained by Khalid

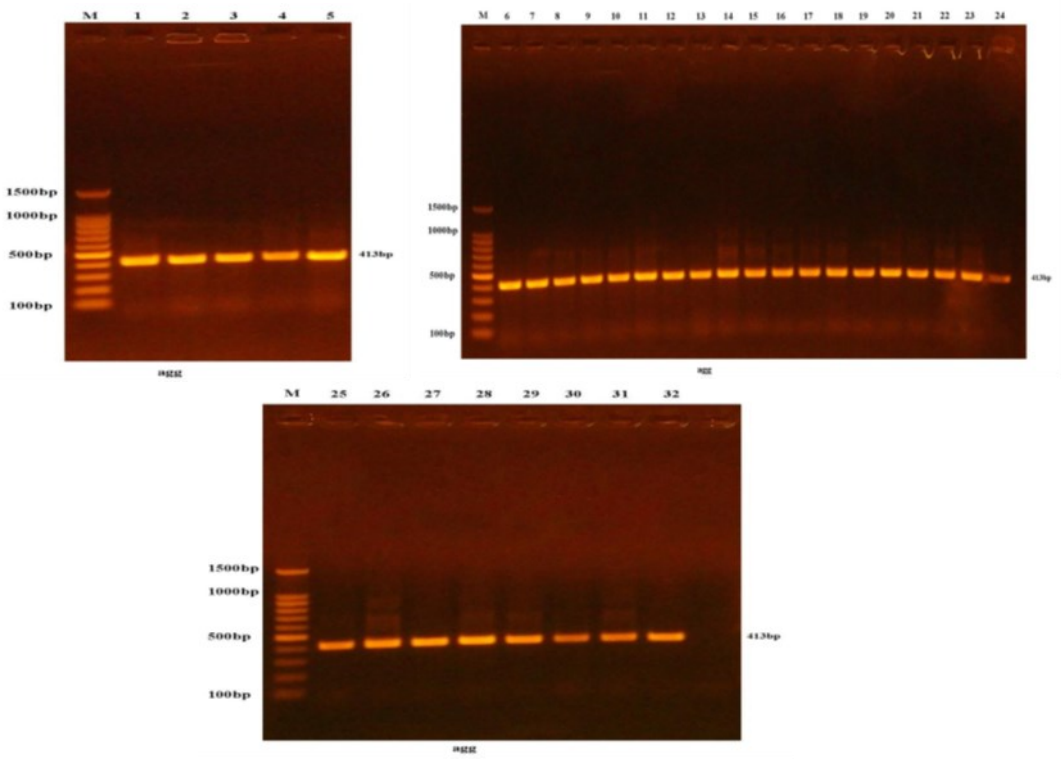

Fig. 5. *Consequences of the amplification of agg gene 413bp of Enterococcus faecalis were fractionated on 1.5% agarose gel electrophoresis with 1 ml*

(2016), who found that isolates (68%) were associated with this gene, and the study of Ferguson *et al*. (2016) stated that *esp* gene encoding the enterococcal surface protein isolated from stool samples found in 11.1%. *Esp* gene is significantly higher amongst clinical isolates than faecal isolates and is linked with expanded virulence colonization and steadiness in the urinary tract and biofilm formation (Kiruthiga *et al*., 2020).

Anderson *et al*. (2016) found that *asal* gene coding the aggregation substance was the utmost common virulence gene. The finding of *asal* gene among some isolates can assist conversation of resistance and virulence gene in emergency clinic situations. In the present study Aggregation Substance gene (*agg*) was also noticed in all isolates of *E.faecalis* 32(100%), as shown in Fig. 5 and Table 4. The consequences were in line with the Al-saadi (2013) study, which displayed that all *E. faecalis* isolates had *asal* gene 100% and results achieved by Khalid (2016), who established that *E.faecalis* 88% was related to this gene. The consequences of this study were in disagreement with the results of Comerlato *et al*. (2013), who stated that *asal* gene was the most prevalent single factor in clinical *E. faecalis* isolates (63%) and another study found that *asal* gene was in 69% *E. faecalis* isolates (Gomez *et al*., 2011). The construction of an accumulation substance (agg) on the surface of the donor cell assists contact with the receiver cell by binding to enterococci material (Clewell, 2007).

The difference between the presence of the virulence gene in *E. faecalis* isolates in the present study and other studies may be due to the influence of geographic conditions; movement of the virulence gene by transposon or by integron or plasmids is a significant mechanism for the spread of virulence gene in bacterial population by conjugation.

Conclusion

The percentage of *E. faecalis* in clinical cases isolated from urine was greater than that of isolates isolated from stool, wounds and vaginal swabs. The virulent genes appeared in most of the isolates. All the isolates possessed *esp* and *agg* genes responsible for biofilm formation. It is an important virulence factor in pathogenicity and antibiotic resistance. *gelE* (Gelatinase) and *cylA* (Cytolysin-producing) genes were also present in most of the isolates. The presence of virulent genes will help to assess their ability to form biofilm and to determine their haemolytic and gelatinase activities.

Conflict of interest

The authors declare that they have no conflict of interest.

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