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Molecular detection of some virulence genes of Enterococcus *faecalis* isolated from different clinical samples in Babylon province/ Iraq

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***Abstract*---**A total of 120 clinical samples were collected during this study which obtained from patients suffering from different infection such as UTI, vaginitis, wound infection, gastro enteritis who admitted to two main hospitals of AL-Hilla City: Al-Hilla Surgical Teaching Hospital and A1-Hashimiyah General Hospital during a period extending from (Ougust 2021 to November 2021). All sample were subjected to aerobic culturing on different media and it was found that out of the total 120samples, 87 (72.5%) samples showed positive bacterial culture. No growth was seen in other 33(27.5%) samples which indicate the presence of microorganisms that may be cultured with difficulty such as virus, fungi and other agent or may be due to difference in the size and nature of the samples. Among (87) positive culture were culturing on chromogenic ager medium (selective media), 20(22.98%)positive samples was identified as *E. faecalis.* Identification of *E. faecalis* depends mainly on the cultural, and biochemical characteristics and also microscopic patterns. These isolates then subjected to molecular detection method using specific primer based on *D-alanine D-alanine ligase* gene as a genetic marker for confirmed identification of *E. faecalis* by PCR, the results revealed that 20(100%) were positive for *ddl* .

***Keywords*---**UTI, vaginitis, wound infection.

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Introduction

Enterococcus is a genus of Gram-positive bacteria, with 67 species, belonging to the lactic acid bacteria from the phylum Firmicutes (Arias and Murray, 2012; Parte, 2014). *Enterococcus* species are non-spore-forming facultative anaerobes tolerant to a wide range of environmental conditions (Bondi *et al.*, 2020).

Enterococcus faecalis is an aerotolerant, Gram-positive bacteria that is distributed widely in the natural environment, and in the gastrointestinal tracts of humans, animals, and insects. Among different enterococcal species, *E. faecalis* causes urinary tract infections, bacteremia, prosthetic joint infection, abdominal-pelvic infections, and endocarditis (Arias *et al.*,2010; Tornero *et al.*,2014).The most important features of *E. faecalis* are their high adaptability under harsh environmental conditions and their potential development of antibiotic resistance (Arias *et al.*,2012; Miller *et al.*,2014).

Enterococcus can express alpha, gamma, or beta hemolysis on blood agar. Haemolysin producing strains of enterococci have been shown to be virulent in a human infections, and to be associated with increased severity of infection. Trypticase soy agar or Columbia agar with 5% (v/v) defibrinated sheep blood may be used to assess the haemolysis produced by enterococci. If human or horse blood is used, haemolysis is based on cytolysin activity and may cause a β-haemolytic reaction, Bacterial colonies appear gray on blood agar and Turquoise color on Chromogenic agar medium (Atlas and Snyder, 2015).

Conventional culture-based methods for the identification of *Enterococcal* spp. require 2-3 days to yield results, while PCR has provided a method for culture independent detection of *Enterococcal* bacteria in a variety of clinical specimens. This assay is capable of yielding accurate results in few hours . Hence, PCR technology provides high specificity and sensitivity and is faster than the conventional methods currently used in hospitals and laboratories (Temimay *et al* .,2018; Bayram *et al.*,2017).

Phenotypic methods are not highly sufficient, it is recommended to use polymerase chain reaction technique with primers for *ddI E. faecalis* hat provides a rapid, accurate , more sensitive, and less time-consuming detection of these bacteria. *E. faecalis* molecular detection of virulence genes also showed significant correlations between the presence of *gelE* and *sprE* genes and the strength of biofilm formed, and between *fsrB* and gelatinase activity, but confirmed prior findings that the presence of *gelE* is not sufficient to predict gelatinase activity, whereas the quorum sensing *Fsr* locus was an important predictor(Hashem *et al* .,2021). The *fsr* locus of *E. faecalis* encodes a two-component regulatory system that senses the cell density and regulates virulence . The fsr locus is 2.8 kb in size and comprises four genes: *fsrA, fsrB, fsrD*, and *fsrC* . The fsrA gene encodes the *FsrA* protein, which belongs to the LytTR family of DNA-binding domains (Papa *et al.*,2011).

Sortase family proteins (*SrtA,B,* and *C*) play an important role in initial attachment of planktonic bacterial cells, and subsequent biofilm formation . In *E.*

faecalis, the cell wall anchoring of virulence factors such as aggregation substance and pili were facilitated by Sortase enzymes. Therefore, Sortase family protein was considered as the docking receptor and the antibiofilm active peptides were used as ligands. (Kurcinski *et al.*,2019). studies have also proved that the both *SrtA* and *SrtC* were focally localized in *E. faecalis* and essential for efficient bacterial colonization and biofilm formation on the host tissue surfaces and was identified as an attractive drug target(Natarajan *et al.*,2017).

Atl gene are endogenous lytic enzymes that break down the peptidoglycan components of biological cells which enables the separation of daughter cells following cell division. They are involved in cell growth, cell wall metabolism, cell division and separation, as well as peptidoglycan turnover and have similar functions to lysozymes. Autolysin is formed from the precursor gene, Atl. Amidases ,gametolysin, and glucosaminidase are considered as types of autolysins(*Clarke*.,2018). Autolysins exist in all bacteria and are potentially considered as lethal enzymes when uncontrolled. They target the glycosidic bonds as well as the cross-linked peptides of the peptidoglycan matrix(Atilano *et al.,*2014*)* .The peptidoglycan matrix functions for cell wall stability to protect from turgor changes and carries out function for immunological defense(Zhang *et al.,*2019; Pazos *et al.,* 2019*).*

Methods:

from (Ougust 2021 to November 2021), 120 clinical samples were collected from patients suffering from different infection such as UTI, vaginitis, wound infection, gastro enteritis who admitted to two main hospitals of AL-Hilla City: Al-Hilla Surgical Teaching Hospital and A1-Hashimiyah General Hospital(2021). *E. faecalis* genomic DNA was isolated using the Geneaid Genomic DNA Purification Kit (UK) according to the manufactures instructions; the bacterial culture was seeded in 10 ml brain hart infusion medium and cultured at 37c overnight in a shacking incubator .

Primer Sequences

Molecular assay in this study includes 4 genes; one for diagnosis, three for virulence. The oligonucleotide primers for all genes used in this study were obtained from previous studies and pubmlst.org, each one has specific nucleotide sequences and product size. The primer sequences and PCR conditions that used are listed in Tables (2-1), (2-2),(2-3).

Table (2-1) Primer used in PCR assays for the detection of *E. faecalis*

Genes	Primer sequence (5′-3′)	Size bp	Reference
ddl **F**	ATCAAGTACAGTTAGTCT	941	(Saffari *et al*., 2017)
ddl R	ACGATTCAAAGCTAACTG		

Table (2-2) Virulence genes primers sequences with their amplicon size base pair (bp)

Genes	Primer sequence (5'-3')	Size bp	Reference
fsr **F**	CAAGGCACTATTTCTTACTTAGG	1016	(Song *et a*l.,2019)
fsr **R**	AGCGCATAAATCAACCAAG		
srt **F**	GTATCCTTTTGTTAGCGATGC	612	(Hashem *et al*., 2017)
srt **R**	TGTCCTCGAACTAATAACCGA		
Atl **F**	CTGCTCCAGCTGTTACACCA	206	(Design this study)
Atl **R**	ACCCCAACCAGATTCAACAA		

Table (2-3): The PCR amplification conditions performed with a thermal cycler were specific to each single primer set depending on their reference procedure, as follows:

Gene	Initial denaturation	Denaturation	Annealing	extension	Final extension	Cycle
ddlE	94°C / 60 s	94°C / 60 s	54° C / 60s	72°C / 60 s	72°C/7 m	30
fsr	94°C / 5 m	94°C / 1 m	55° C / 1 m	72°C / 1 m	72°C/10m	35
srt	94°C / 5 m	94°C / 1 m	56° C / 1 m	72°C / 1 m	72°C/10 m	35
Atl	94°C / 5 m	94°C / 1 m	60° C / 1 m	72°C / 1 m	72°C/10 m	35

Resalts:

There are 87 positive resalts out 120 clinical specimens, with 87 (72.5%) isolates belonging to Enterococcus faecalis , 33(27.5%) isolates belonging to other bacterial genera, and 33(27.5%) samples no growth as shown in Table (3-1),(3-2).

Table (3-1): prevalence of *E. faecalis* among other etiological agents associated with isolated sample.

No. of samples	Negative bacteria culture	Positive culture of other bacteria	Positive culture of *E. faecalis*	on molecular (*D-alanine ligase* gene) positive
120	33 (27.5%)	87 (72.5%)	20 (22.98%)	20 (100%)

Table (3-2): *E. faecalis* isolated from different sites of infection

Site of infection	**No. of samples**	***E. faecalis***	**%**
Urine	41	6	30 .0%
Wound	21	4	20.0%
Blood	12	0	0.0%
Vagina	28	6	30.0%

Stool	18	4	20.0%
Total	**120**	**20**	**100%**

Confirmation and Diagnosis of *E. faecalis* by PCR using specific primer:

All the isolates of *E.faecalis* were used to produce a specific size of the 941-bp fragment of the *ddlE* gene 20(100%) as shown in Figure (3-1), and Table (3-3).

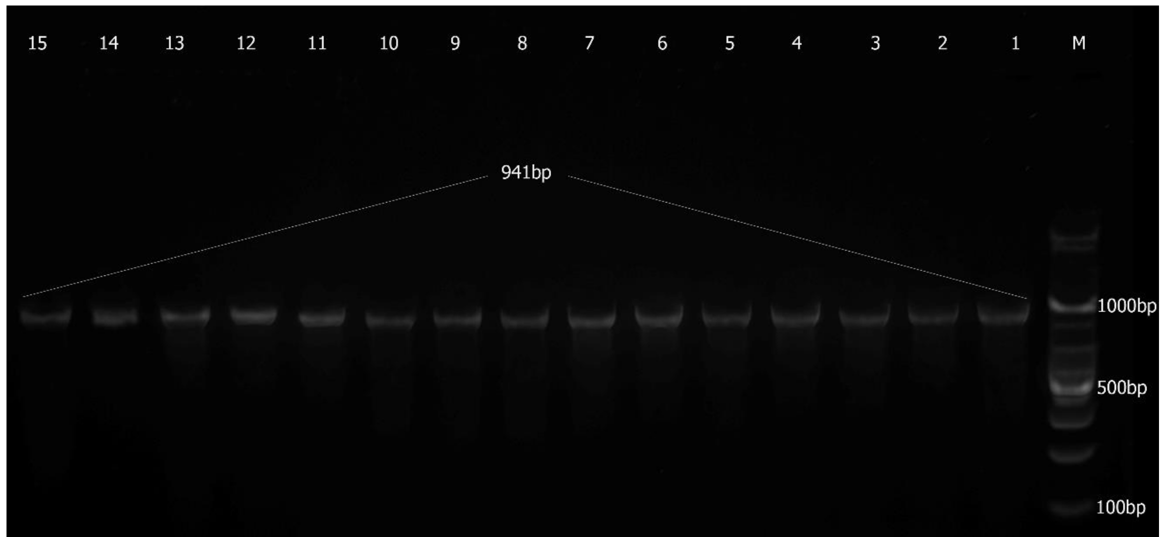

Fig. (3-1):1.5% Agarose gel electrophoresis image at 75V for 1 houre that showed PCR products analysis of D-alanine D- alanine ligase gene in *E. faecalis* isolated from clinical samples.WhereM: (100-1500bp)and Lane (1-15) showed (1,2,3,4,5,6 vagina , 7,8,9,10 wound , 11,12,13,14 stool , 15 urine) at (941bp) .

Table (3-3): Identification of *ddlE* gene gene of *E. faecalis* in patients with different sample sources.

Results	*ddlE* N (%)Positive	*ddlE* N(%)Negative
Urine	6 (100)	0(0%)
Vagina	6 (100)	0(0%)
Wound	4 (100)	0(0%)
Stool	4 (100)	0(0%)
Total	20/20	0/20

Molecular detection of virulence genes in *E. faecalis*

The genotypic characters were tested for all *E. faecalis* isolates in this study in order for detection of some virulence factors. The specific primers were used for screening the presence of (*fsr, srt,* and *Atl*) genes as shown in Table (3- 4).

Table (3-4): Distribution of Virulence factors *(fsr, srt* and *Atl*) among *E. faecalis* isolates.

genes	No.	(%)
fsr	9	45
srt	20	100
Atl	9	45

Molecular Detection of (*fsr* gene) in *E. faecalis*

In this study, *fsr w*as been, and the results showed that 9 isolates in a rate of 45%) had *fsr* gene as shown in Figure (3-2), and Table (3-5).

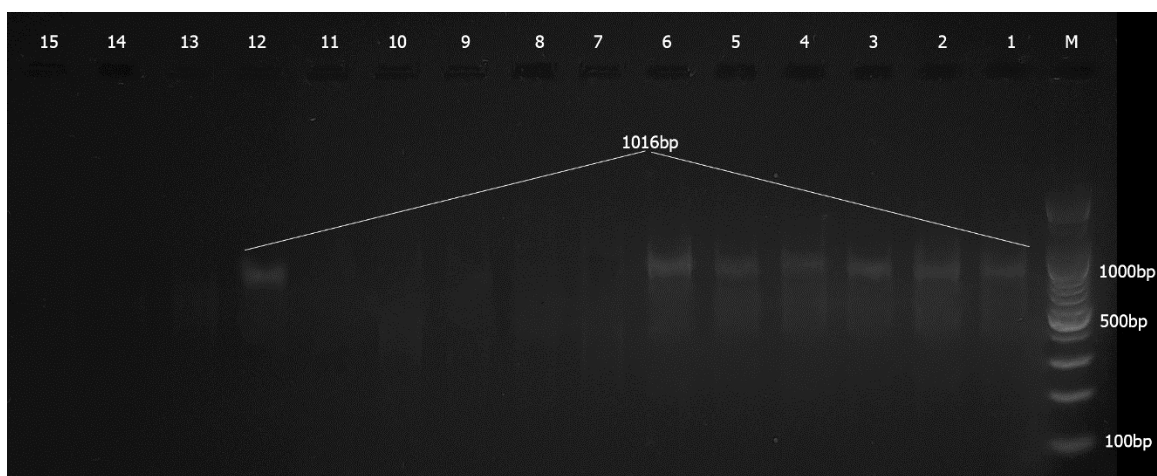

Fig. (3-2): Agarose gel electrophoresis of PCR products obtained by using Fsr-specific primer. lanes 1-6 and 7 represent the identified Fsr gene products with 1016bp, Lane M represent 100bp DNA ladder.

Table (3-5): Identification of *Fsr* gene gene of *E. faecalis* in patients with different sample sources.

Results	*Fsr* N (%)	P value
Vagina	6 (100)	<0.0001*
wound	0 (0)	
Stool	1 (25)	
Urine	2 (33.3)	
Total	9/20	

Molecular Detection of (*srtA* gene) in *E. faecalis*

The results show that 20(80%) of *Proteus mirabilis* isolates gave positive result at 100bp in PCR amplification of *srt* gene, as shown in Figure (3-3), and Table (3-6).

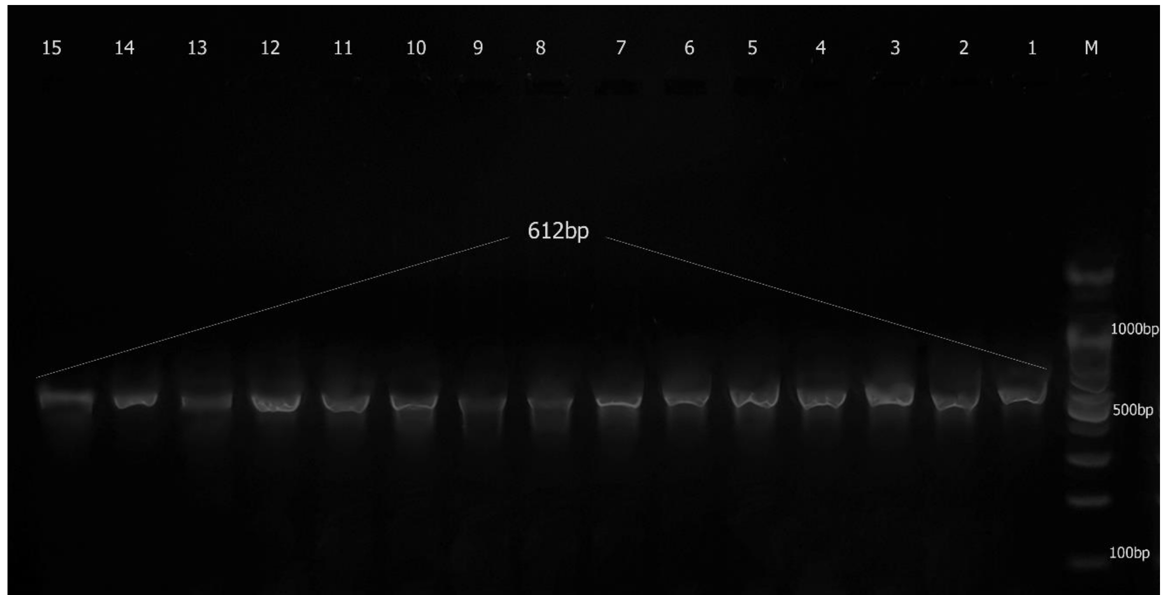

Fig. (3-3): Agarose gel electrophoresis of PCR products obtained by using Srt -specific primer. lanes 1-15 represent the identified *Srt* gene products with 612bp, Lane M represent 100bp DNA ladder.

Table (3-4): Identification of *Srt* gene gene of *E. faecalis* in patients with different sample sources.

Results	*Srt* N (%)	P value
Vagina	6 (100)	1.000
wound	4 (100)	
Stool	4 (100)	
Urine	6 (100)	
Total	20/20	

* represent a significant difference at p<0.05.

Molecular Detection of *(Atlgene)* in *E. faecalis*

The result of the current study was shown that *Atl* gene was present in 9 isolates out 20 isolates of *E. faecalis* at rate (45%) from different samples sources with long length in (206bp) as shown in Figure (3-4) and Table (3-7).

Fig. (3-4): Agarose gel electrophoresis of PCR products obtained by using ATN-specific primer. lanes 1-15 represent the identified *Atn* gene products with 206bp, Lane M represent 100bp DNA ladder.

Table (3-4): Identification of *ATN* gene of *E. faecalis* in patients with different sample sources.

Sample Source	ATN N (%)	P value
Vagina	6 (100)	<0.0001*
wound	0 (0)	
Stool	1 (25)	
Urine	2 (33.3)	
Total	9/20	

* represent a significant difference at p<0.05.

Discussion

87 of 120 clinical specimens were found to be positive, with 20(22.98%) belonging to *Enterococcus faecalis* and the remaining 87(72.5%) to various bacterial species, with 33(27.5%) samples showing no growth.

Seenaa and Lamees,(2020) in Babil/Iraq observed that all 47 isolates were recorded related to *E. faecalis*, collected from the following site, 11 isolates (23.40%) obtained from urine samples, 10 isolates (21.28%) from stool, 10 isolates (21.28%) from wound, 6 isolates (12.76 %) from vagina, 10 isolates (21.28%) from pus. While no bacteria 0(0.0%) were isolated from blood samples .

AL-Khafaji,(2021) observed that 27 isolates were recorded related to *E. faecalis*, it was collected from the following site, out of 50 specimens of urine, 14(51.85%)

positive culture for *E. faecalis* were isolated, 40 specimens of stool, 10(37.03%) positive culture for this bacteria .

Variations in *Enterococcus* isolation between studies can be attributed to a variety of factors, including sanitary practices in hospitals and staff, environmental conditions, isolation and identification techniques, social and cultural level of patients, and use of multidrug (antibiotics) that may lead to bacterial resistance development, or differences in sample size; all of these factors may combine and play an important role in inhibiting or stimulating bacterial resistance development.

Distribution of *E. faecalis* Isolated from Different Clinical Samples

6(30%) isolates from patients with urinary tract infection, 6(30%) isolates from patients with vaginal infection, 4(20%) isolates from patients with wound infection, and 4(20%) isolates from patients with stool infection were obtained from the total of 20 isolates of bacteria *E. faecalis.*

AL-saadi, (2013) found that 39 *E. faecalis* isolates that isolated included 18 isolates (46%) obtained from stool samples, 14 isolates (36%) from urine samples, and 7 isolates (18%) from vaginal swabs.

Kandela, (2012) in Baghdad/ Iraq was found that 50 isolates belonged to *Enterococcus faecalis* of which, 23 isolates (46%) from stool, 27(54%) isolates from clinical cases distributed between 16 (32%) from urine, 6 (12%) from wounds and 5(10%) from vaginal. Reinseth *et al.*, (2021) have witnessed increased interest in *enterococci* not only because of their ability to cause serious infections but also because of their increasing resistance to many antimicrobial agents . Urinary tract infections are the most common cause of infectious disease produced by enterococci both inside and outside hospital settings. Changes in vaginal microflora that show a critical role in promising vaginal colonization (Aiyegoro *et al.,* 2007), and my hypothesis is that the reason intestinal bacteria are associated with urinary tract and vaginal infections is due to the close proximity of the anal opening to the vagina and urethra, so contamination from the anus can lead to bacteria being found in the vagina area, though this is a much less common occurrence((Inabo and Obanibi, 2006).

D-alanine D-alanine ligase gene (ddl) is present in *E. faecalis* and this gene is specific for *E. faecalis*, So it can facilitated downstream analyses such as molecular detection. *E. faecalis* is an opportunistic bacterium considered as pathogen for significant infection to human. Increasing research on *E. faecalis* in the past attested. To create a precise size of the 941-bp segment of the ddl gene 20, all *E. faecalis* isolates were used (100%).

Identification to the species level using PCR with species-specific primers is a valuable method and can replace complex molecular clustering techniques and conventional microbiological tests that are otherwise necessary to identify species that are difficult to distinguish using phenotypic approaches (Iacumin *et al.*, 2015).

Seenaa and Lamees (2020) found the results that all isolates 47 were produced the specific 941bp DNA fragment when compared with allelic ladder.

The result obtained by (Khalid, 2016) in Duhok City, Kurdistan Region/Iraq who found that 25 isolates of *E. faecalis* from urine samples were confirmed by successfully amplification of 914bp amplicon of *ddl* gene which used as species specific primer for detection of *E. faecalis*.

[PCR] widely used to identify bacteria .Since conventional and cultivation identification methods have been proved to have several limitations with respect to microbiological diagnosis, more sensitive techniques may be necessary for accurate characterization of the microbial assembly. Species-specific PCR primers such as *ddlE. E. faecalis* were documented as competent for enterococcal species identification by different studies. The use of PCR with these primers may be the simplest molecular approach for highly effective identification of distinct *Enterococcus spp*.

Gelatinase activity is known to be co-controlled by *gelE* and *fsr* genes, and lack of *fsr* affects the production of gelatinase . The fsr gene product, which hydrolyzes gelatin, casein, hemoglobin, and other bioactive peptides, was detected in 64% of *E. faecalis* isolates. Among *E. faecalis* isolates harboring the *esp* gene, which contributes to enterococcal biofilm formation, resistance to environmental stresses, and adhesion to eukaryotic cells (Song *et al.*, 2019).

Hashem *et al.*,(2021) found when take *E. faecalis* from UTI samples the gene *fsrA* in 42%, *fsrB* in 28% and *fsrC* in 48% of the isolates. *E. faecalis*. Molecular detection of virulence genes also showed significant correlations between the presence of gelE and sprE genes and the strength of biofilm formed, and between *fsrB* and gelatinase activity, but confirmed prior findings that the presence of *gelE* is not sufficient to predict gelatinase activity, whereas the quorum sensing *Fsr* locus was an important predictor(Hashem *et al* .,2021).

The *Fsr* and cytolysin regulatory systems in *E. faecalis* regulate much of their pathogenicity and have been documented in several studies , while the role of the LuxS regulatory mechanism in *E. faecalis* is less certain Fsr Mediated Quorum-Sensing The *fsr* locus of *E. faecalis* encodes a two-component regulatory system that senses the cell density and regulates virulence (Tornero *et al.*,2014).

The quorum-sensing system is the population density-dependent regulatory mechanism by which bacteria communicate via signaling molecules, called autoinducers . Generally, in quorum-sensing, bacteria produce autoinducers, and these molecules accumulate in the environment with the increase in the cell density. The role of these autoinducers depends on the location of their receptors, which are present on cell surface or in the cytoplasm. An autoinducer activates its cognate receptor specifically, which then activates the transcription of quorum-sensing genes(Piras *et al.*,2012). This phenomenon provides a mechanism for bacteria to synchronize their social behavior, to communicate among themselves, and to regulate gene expression in response to their population density. Gram-positive bacteria contain another type of quorum-

sensing, in which autoinducers are transported back into the bacterial cytoplasm, where they interact with a specific transcription factor, thereby regulating gene expression (Piras *et al.*,2012).

Sortase peptide is a virulence factor in *E. faecalis*, Our study showed that all 20 isolates, gave positive resalts to this gene, which gave molecular length 612bp

Sortases are membrane-bound transpeptidases that cleave the sorting signal of the secreted protein to form an isopeptide bond between the secreted pro- tein and peptidoglycan. They are either responsible for covalently anchoring specific surface proteins or polymerizing pilin sub-units to form a proteinaceous structure

Sortase-displayed surface structures play a pivotal role in displaying virulence and pathogenesis properties without affecting the growth and viability of cells. They are responsible for cell attachment, heme transport, nutrient uptake, sporulation and aerial hyphae formation . The surface proteins recognized by the sortase enzyme contain a C-terminal pentaglycine recognition motif followed by a stretch of hydrophobic amino acids and a positively charged tail (Susmitha *et al.*, 2021). Stępień *et al.*,(2019) found when isolated *E .faecalis* from birds specimens *srt* was present in *E. faecalis* (96.3%, 26 isolates). Hashem *et al* .,(2017) found *srt* gene in *E. faecalis* isolated from clinical samples in 94% of the isolates.

Sortase family proteins (*SrtA,B,* and *C*) play an important role in initial attachment of planktonic bacterial cells, and subsequent biofilm formation . In *E. faecalis*, the cell wall anchoring of virulence factors such as aggregation substance and pili were facilitated by Sortase enzymes. Therefore, Sortase family protein was considered as the docking receptor and the antibiofilm active peptides were used as ligands. (Kurcinski *et al.*,2019).

One of the most well characterized *SrtA* substrates in *E. faecalis* are the endocarditis and biofilm associate pili. Pili in *E. faecalis* are made up of three subunits *EbpA*, *EbpB*, and *EbpC* and are co-transcribed along with *SrtC* in a polycistronic mRNA . *SrtC* polymerizes the three subunits to form large molecular weight structures that are greater than 200kDa in mass and over 10μm in length (Flores-Mireles *et al.*, 2014).

In *E. faecalis*, *Atl* is the major peptidoglycan hydrolase. The ability to cleave peptidoglycan makes *Atl* pivotal in separating dividing cells . As such, *Atl* requires strict modulation to regulate its function in cell division. Recent evidence suggests that multiple mechanisms control *Atl* activity, including glycosylation .The results presented here provide strong evidence that post-translational modification of *AtlA* by GelE directly affects the function of *AtlA* during cell division(Salamaga *et al.*,2017).

When *GelE* cleavage of *AtlA* has not yet occurred and long cell chains are observed. As GelE reaches maximum activity levels at later growth stages, after colonization is established, *AtlA* would be cleaved and short cell chains would result. These short chains could potentially aid in bacterial dissemination or help *E. faecalis* evade host immune defense. *GelE* has previously been shown to be associated with an increase in bacterial burden at dissemination sites . Thus,

GelE could aid in bacterial cell dissemination by cleaving *AtlA* at a later growth stage, resulting in short chains. The short chained *E. faecalis* cellshave been shown to have decreased phagocytosis in a zebrafish infection model , further suggesting that the GelE processing of *AtlA* may impact *E. faecalis* virulence(Salamaga *et al.*,2017).

Conclusions

This study reached the following conclusion:

1. Using of (*ddlE)* gene for earlier identification of *E. faecalis* infections.
2. It was found that the presences of some virulence factor genes in *E. faecalis* as (*fsr, Atl* and *srt*) at high percentage increase pathogenicity of this pathogen.
3. The *srt* showed the higher percentage among *E. faecalis* isolates.

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