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Virulence-Associated Genes Characteristics of *Enterococcus faecalis* Isolated from Clinical Sources

A Thesis

Submitted to the Council of the College of Medicine, University of Babylon, in Partial Fulfillment of the Requirements for the Degree of Doctor of Philosophy in Medical Microbiology

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Dedication

I dedicate this effort, which is the conclusion of a long scientific journey, to the Imam Mahdi, peace be upon him.

To the soul of my beloved mother.

To my support and companion, my husband, who has the credit after God for my support and success.

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Summary

Enterococcus faecalis, like other members of the genus *Enterococcus*, is common in healthy individuals but may cause fatal infections, particularly in the nosocomial hospital setting due to its naturally high levels of antibiotic resistance.

This study aimed to screening genes encoding pathogenicity associated factors among *Enterococcus faecalis* isolates.

A total of 200 participants suffering from urinary tract infection and vaginitis with age range (8-56 years old). Urine, vagina and blood samples were collected from Patients who visited to Baghdad's Al-Karama Hospital and Medical City Hospital throughout a three months period (May to July 2022), and 44 blood samples were collected from healthy patient as control groups. The samples were cultured for (18-24) hours. Then, they were incubated at (37ºC) for (18-24) hours on a number of different selective media (blood, MacConkey and CHROM) agar media

When first trying to identify *Enterococcus faecalis* on colony morphology, microscopic examinations, and biochemical tests. All 200 clinical samples cultured positive, only 44(22%) of the isolates were related to *E. faecalis*, the automated Vitek 2 system was employed GP-ID cards containing 64 biochemical tests were used to ensure the isolates of really *E. faecalis*, forty four *E. faecalis* related isolates were identified, 32(72.7%) of the samples were from urine, whereas 12(27.3%) came from the vagina. All previously identified isolates' DNA was taken and used in conventional PCR to amplify the *ddl* gene, the results of gel electrophoresis, which demonstrated that, all 44(100%) isolates at 941bp were related to *E. faecalis*.

The study looked at whether or not certain *E. faecalis* isolates may produce virulence factors. Out of 44 isolates tested, 14(31.8%) were produce hemolysin. 12(27.2%) isolates were able to produce extracellular protease. 14(31.8%) isolates were positive for gelatinase production. In addition, 22(50%) isolates were able to attach to epithelial cells, 18(40.9%) were able to create hemagglutination, 32(72.7%) were able to produce lipase and 16(36.3%) were able to produce hydrophobicity.

In the present study, the capacity to form biofilm was evaluated in 44 isolates, 31(70.4%) of these isolates were strong biofilm producers, 3(6.8%) were moderate biofilm producers, 4(9%) were weak biofilm producers, and 6(13.6%) were non-biofilm former.

Antibiotics resistance were evaluated *in vitro* using a Kirby - Bauer disc diffusion technique for all of the detected 44 *E. faecalis* isolates. Highest rate of resistance was seen to almost antibiotics used in present study, 42(95.4%) isolates were resistant to Synercid, followed by 25(56.8%) isolates were resistant to Levofloxacin, 20(45.4%) isolates were resistant to Erythromycin and Norfloxacin, 19(56.8%) isolates were resistant to Ciprofloxacin, 18(40.9%) isolates were resistant to Vancomycin, 13(29.5%) isolates were resistant to Gentamicin, 9(20.4%) isolates were resistant to Teicoplanin, while no resistance were occur against Nitrofurantoin and Linezolid.

The *E. faecalis* virulence factors genes revealed that, out of 44 isolates, 16(36.3%) had *vanA* gene at (314 bp), 14(31.8%) had *gelE* gene at (213 bp). 36(81.8%) had *cpd* gene at (782bp), 14(31.8%) had *cylA* gene at (688bp), 34(77.2%) had *ebpA* gene at (517 bp), 12(27.2%) had *sprE* gene at (591bp), 20(45.4%) had *fsrA* gene at (474 bp), 15(34%) had *ace* gene at (320bp), 18(40.9%) had *esp* gene at (933bp) and 15(34%) had *efaA* gene at (688bp).

The sequencing of *gelE* gene shows for *E. faecalis* having one transversion A/C, and the effect Missense. From the Gene Bank, found part of *gelE* gene having 99% compatibility with the subject of *gelE* gene in NCBI under sequence ID: CP0881981. Another part of sequencing for *gelE* gene to *E. faecalis*, the results shows compatibility of 100% in Gene Bank of *gelE* under sequence ID: CP088198.1, so no recorded change noticed from the gene in this isolate. The sequencing for *sprE* gene to *E. faecalis*, the results showed compatibility of 100% in Gene Bank of *sprE* gene under sequence ID: CP070621.1, so, no recorded change noticed from the Gene Bank in *sprE* gene. The sequencing for *fsrA* gene for four isolates of *E. faecalis*. The results was coordinated 99% having one transversion A/T, the effect Missense under sequence ID: CP041738.1. Furthermore, neighbour phylogenetic distances in this tree indicated a wide biological diversity of *E. faecalis* sequences.

In this study, determination of IL-23 in serum patients that infected with *E. faecalis* were investigated, and the results showed that, the highest mean of IL-23 level was found among patients with UTI infected with *E. faecalis* (287.54±12.18ng/ml) comparing with the control group (188.08±2.29ng/ml) with a significant difference between the two groups ($P<0.05$).

It was conclude that, identification of *E. faecalis* by using D-alanine ligase gene more specific than other biochemical test, The pathogenicity of *E. faecalis* increased by presence of *vanA*, *gelE*, *cpd*, *cylA*, *ebpA*, *sprE*, *fsrA*, *ace*, *esp* and *efaA* genes and Interleukin-23 is elevated in *E. faecalis* infection.

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List of Abbreviation

Abbreviation	*Meaning*
A	Adenine
Ace	Collagen binding protein
AS	Aggregation substance
AtlA	Autolysin N-acetylglucosaminidase
bp	Base pair
BS	Binding substance
C	Cytosine
CIA	Clumping-inducing agent
CWSS	Cell wall sorting signal
cylA	Cytolysin
DDT	Disk Diffusion Test
DNA	Deoxyribonucleic acid
E. faecalis	*Enterococcus faecalis*
ECM	Extracellular matrix
efbA	Like fibronectin-binding protein
ES	Exclusion substance
esp	*Enterococcus* surface protein
FFAs	Free fatty acids
G	Guanine
gelE	Gelatinase
GI	Gastrointestinal tract
H_2O_2	Hydrogen peroxide solution
HGT	Horizontal gene transfer
ICU	In intensive care unit
IFN-ɣ	Interferon-Gama
IL-1	Interleukin-1
IL-12	Interleukin -12
IL-17	Interleukin-17
IL-18	Interleukin-18
IL-23	Interleukin-23
IL-6	Interleukin -6
LAB	Lactic acid bacteria
LTA	Lipoteichoic acids
MDR	Multidrug-resistant
MIC	Minimum inhibitory concentration
MMP	Matrix metalloproteinase

MurNAc	N-acetyl muramic acids
NF-B	Nuclear factor kappa B
NO	Nitric oxide
OD	Optical density
PBS	Phosphate buffer saline
PCR	Polymerase chain reaction
PMNs	Polymorph nuclear leukocytes
PRRs	Pattern recognition receptors
RBCs	Red blood cells
sprE	Serine protease
T	Thymine
TCA	Trichloroacetic Acid
TCP	Tissue culture plate method
TE	Tris EDTA
UTIs	Urinary tract infections
UV	Ultraviolet
VRE	Vancomycin-resistant Enterococci

Chapter One

Introduction and Literatures Review

1.1 Introduction

Since enterococci are so common in the digestive tracts of humans and animals, they are often blamed as the root cause of life-threatening illnesses including endocarditis, septicemia, and urosepsis (Agyare *et al*., 2018). The bacterium *Enterococcus* is a major nosocomial pathogen and the one of the most common cause of urinary tract infections (UTIs) (Petca *et al*., 2019).

E. faecalis, like other members of the genus *Enterococcus*, is common in healthy individuals but may cause fatal infections, particularly in the nosocomial (hospital) setting due to its naturally high levels of antibiotic resistance (Sangwan *et al.*, 2021). Even though *E. faecalis* is ubiquitous in the human population, it has been linked to serious illnesses such as endocarditis, sepsis, UTIs, and meningitis (Ghalavand *et al*., 2020). However, Enterococci have shown to be very effective in producing opportunistic infections in hospitalized patients, especially in hosts that are already compromised (Ionescu *et al*., 2022).

Antimicrobial resistance and the development of virulence factors are two of the many risk factors for contracting an enterococci infection, and they may help explain why this opportunistic disease has become such a prominent community-acquired and nosocomial pathogen (Hyun & Lee, 2021). Rising levels of antibiotic resistance in enterococci, particularly to β-lactams, aminoglycosides, and, more recently, glycopeptides. The overgrowth and subsequent super infection of enterococci may be attributable to the administration of broad-spectrum antibiotics or several medicines in combination (Peyvasti *et al.*, 2020).

E. faecalis has been identified to produce a variety of virulence factors including collagen binding protein (ace), *Enterococcus* surface

protein (esp), aggregation substance (AS), and gelatinase. Understanding the intricate pathogenic process of these opportunistic microbes may need knowledge of the virulence features of circulating *Enterococcus* strains (Zalipour *et al*., 2019). Observing a clumping response of *E. faecalis* strains during conjugative transfer of plasmids led to the discovery of the sex pheromone system of *E. faecalis*; a subset of *E. faecalis* plasmids, dubbed sex pheromone plasmids, are transmitted by this method (Baquero *et al*., 2021).

Aim of study

This study aimed to screening genes encoding pathogenicity associated factors among *Enterococcus faecalis* isolates, through the following steps:

1. Isolation and Identification of *Enterococcus faecalis* by different methods (culture, vitek system and specific *ddl* primer gene).
2. Phenotypic detection of some virulence factors (Hemolysin, protease, gelatinase, Attached to epithelial cells, hemagglutination, lipase and hydrophobicity.
3. Study of antibacterial susceptibility among different antibiotic groups.
4. Detection of biofilm formation in *Enterococcus faecalis*.
5. Genotypic detection of virulence genes by PCR (*vanA*, *gelE*, *cpd*, *cylA*, *ebpA*, *sprE*, *fsrA*, *ace*, *esp* and *efaA* genes).
6. Determinate of DNA sequencing for *gelE*, *fsrA*, *sprE* virulence genes.
7. Phylogenetic tree of biological diversity of *E. faecalis* sequences.
8. Estimation of some cytokines such as IL-23.

1.2 Literatures review:

1.2.1 *Enterococcus faecalis*

Lancefield used serology to assign Enterococci to group D Streptococci in the 1930s. In his 1937 classification proposal, Sherman suggested that streptococci that can thrive at 10 and 45 degrees Celsius, at pH 9.6 and in 6.5% NaCl, that can withstand 60 degrees Celsius for 30 minutes, and that can divide esculin be given the name "*Enterococcus*." Enterococci were classified as a separate genus, *Enterococcus*, in the 1980s due to their genetic uniqueness compared to streptococci (Russo, 2019) (Figure 1-1).

Enterococcus is a genus of Gram-positive, catalase-negative, non-spore-forming, facultatively anaerobic bacteria that may exist alone or in chains. These organisms not produce endospores, but is sometimes motile by scanty flagella (Giannakopoulos *et al*., 2019).

While the majority of enterococci are anaerobes, there are a few species that require oxygen to survive. Pectin and cellulose are not digested by enterococci, and nitrate reduction is not a typical metabolic process for these bacteria. They are a common, possibly dangerous species that has evolved a resistance to or phenotypic tolerance for various types of disinfectants and physical agents (Deng *et al*., 2022; Al-Azawi and Abbas, 2022).

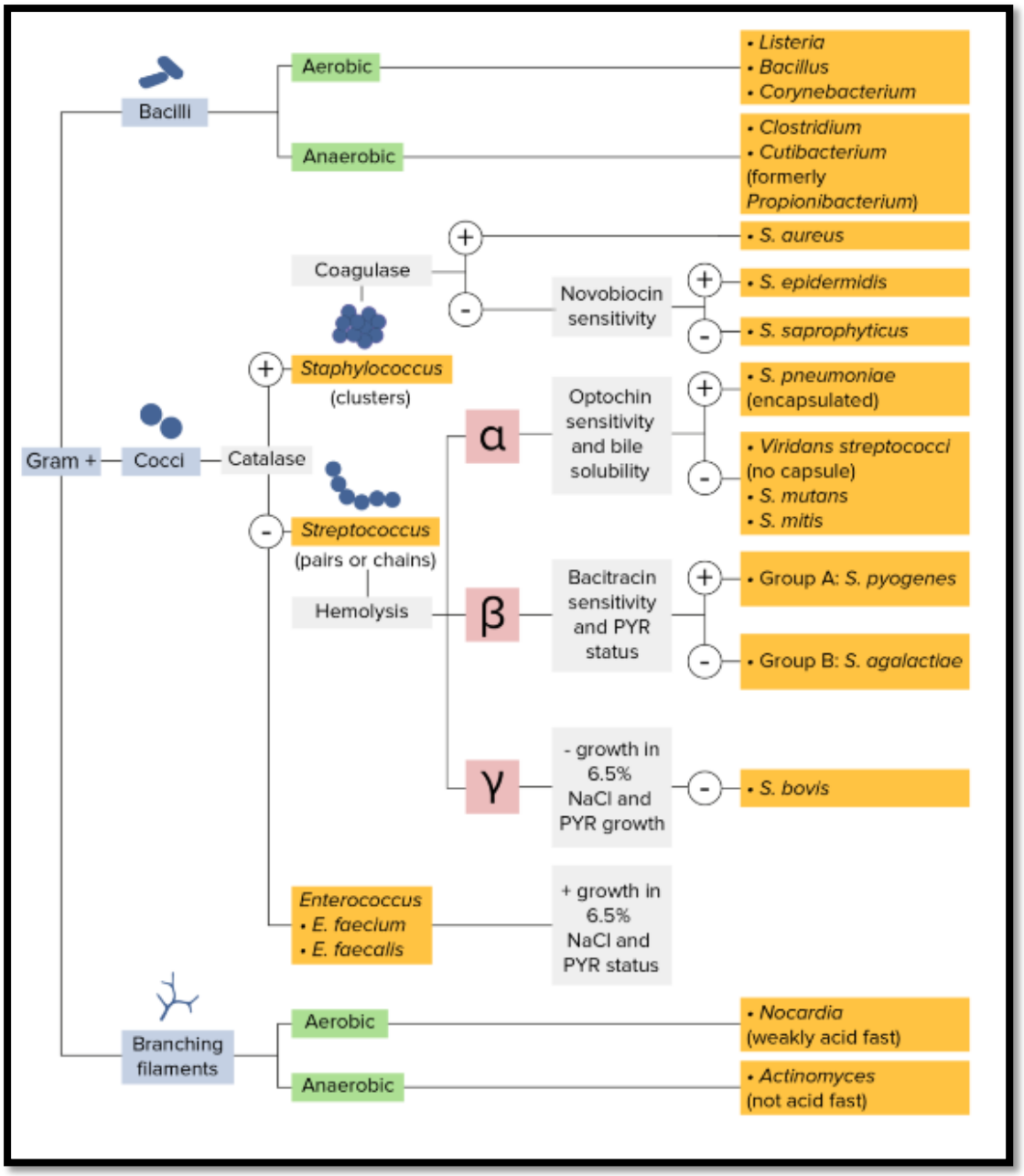

Figure (1-1): Classification of *E. faecalis* (Deng *et al.*, 2022).

Group D carbohydrate cell wall antigen (Lancefield antigen) is a glycerol teichoic acid found in the cytoplasmic membrane of *E. faecalis* bacteria. Peptidoglycan and teichoic acid are found in high concentrations in the cell membrane and the cell wall, respectively. The polysaccharide backbone of alternating N-acetyl glucosamine (GlcNAc) and N-acetyl

muramic acids (MurNAc) that is present in most bacterial cell walls is responsible for helping the bacteria keep its form (Rismondo *et al*., 2021).

Capsular polysaccharides were found to include sialic acid and a carbohydrate backbone structure, similar to glycerol teichoic acid. These polysaccharides aid in the peptidoglycan's dimensionality by being cross-linked through peptide bridges. The trans glycosylation process has been proposed as a possible target for antibacterial medications due to the positioning of the peptidoglycan on the outside of the cytoplasmic membrane and its specificity (Halsr, 2019).

Carbohydrates, glycerol, lactate, malate, citrate, arginine, agmatine, and numerous alpha keto acids are all catabolized by them. The intestinal lumen of humans is home to several *Enterococcus* species [105-108 CFU per gram of feces] that, in the great majority of cases, pose no threat to their hosts (Gunasekaran *et al*., 2019). Enterococci are able to endure severe conditions, such as those with a very alkaline pH or high salt concentrations. As a result, they are impervious to bile salts, detergents, heavy metals, ethanol, azide, and drying out. They thrive between 10 and 45 degrees Celsius, and can tolerate 60 degrees Celsius for 30 minutes (Chayon, 2018).

1.2.2 Epidemiology

Over the last two decades, several research on the ecology and epidemiology of enterococci have been done, most of them in clinical settings. Study that are not related to the healthcare industry demonstrate that enterococci are widespread worldwide (Htun *et al*., 2022).

The evolution of molecular identification and typing technologies has made strain-specific enterococci detection. Despite these advancements, it is still crucial to better characterize ecological reservoirs, comprehend host and bacterial features that favor colonization, and explain transmission mechanisms that facilitate the spread of multi-drug resistance enterococci (Andersson *et al*., 2020).

Over the last two decades, several investigations, mostly in clinical settings, have been undertaken on the ecology and epidemiology of enterococci. Evidence from studies unrelated to the healthcare industry suggests that enterococci are widespread worldwide (Trautmannsberger *et al*., 2022). This resilient bacterium is not only well known to colonize the gastrointestinal tracts (GI) of a wide variety of animals and insects, but it is also commonly collected from beach sands, freshwater and marine water sediments, soil, and aquatic and terrestrial plants (Cattoir, 2022).

Enterococci as indicator bacteria for fecal pollution for sewage waters has been linked to an increase in gastrointestinal and skin diseases in a number of studies. However, it has also been shown that enterococci may occupy ecological niches in the absence of any external contamination (Khan & Gupta, 2020; McKee & Cruz, 2021).

The presence of *E. faecalis* in the gut is considered normal. *E. faecalis* is often an opportunistic pathogen. Nosocomial infections in humans are common, and *E. faecalis* is a major contributor (Gu *et al*., 2018).

1.2.3 Pathogenesis

Due to their generally helpful nature, enterococci make the pathophysiology of enterococcal infections particularly complicated (Krawczyk *et al*., 2021).

This harmonious commensalism may be upset, however, by a number of factors. The failure of host systems that keep commensal organisms in check may be to blame for this disturbance. Patients who have had extensive abdominal surgery or who have received indwelling biomaterial implants, such as drains or catheters, are at risk of having their body's natural barriers, such as the skin or mucosa, compromised (Caldara *et al*., 2022). Genomic features such as bacteriocins, microbial surface proteins, cell wall-anchored proteins, or biofilm-forming proteins contribute to *E. faecalis's* versatility as a colonizer and pathogen of a broad range of host organisms (Zrelovs *et al*., 2021).

Virulence is the measurement of the ability to cause diseases in the host. It describes the quantitative negative effect on the host. To cause disease, two factors are important: the nature of the pathogen and the nature of the host. Moreover, the genetic makeup of both pathogen and host is important for a disease to occur. The defense systems in hosts (e.g. immunity systems in an animal or phenolic compound in a plant) will alter the ability to contract a disease. However, high virulence may result in host mortality, and it affects negatively to host transmission, which leads to pathogen fitness (Andersson *et al*., 2020).

Pathogenicity is the ability to cause diseases in the host organism. The pathogenicity is a qualitative measurement. Moreover, it is measured by virulence. A disease is an outcome of the relationship between the virulence of a pathogen and the resistance of the host. Furthermore, many

factors in a pathogen give a reasonable contribution to cause the disease. Those are called virulence factors. Virulent factors include toxins that kill the host cell, enzymes that act on the host cell walls, and substances that alter the normal cell growth (Cattoir, 2022). (Figure 1-2).

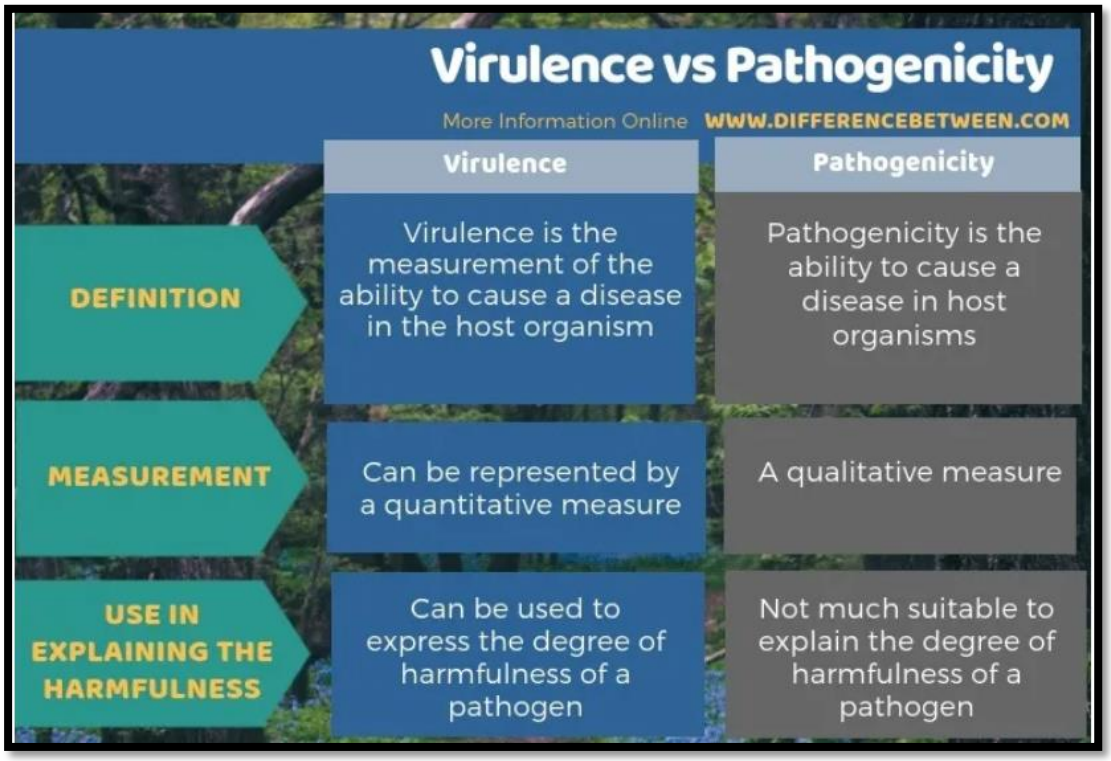

Figure (1-2): Difference between Virulence and Pathogenicity (Cattoir, 2022)

This bacterium's pathogenicity stems from many mechanisms, including adhesion factors, immune system evasion, and the release of cytolysins and other toxins that cause cellular membrane permeability (Ghalavand *et al*., 2020). Even though *E. faecalis* is ubiquitous in the human population, it has been linked to serious illnesses such as endocarditis, sepsis, UTIs, and meningitis (Li *et al*., 2020) (Figure 1-3).

The bacterium *Enterococcus* uses a wide range of microbiological strategies to spread throughout a host's digestive system. In addition,

there are a variety of variables that determine whether species succeeds in colonizing the intestines (Banla *et al*., 2019).

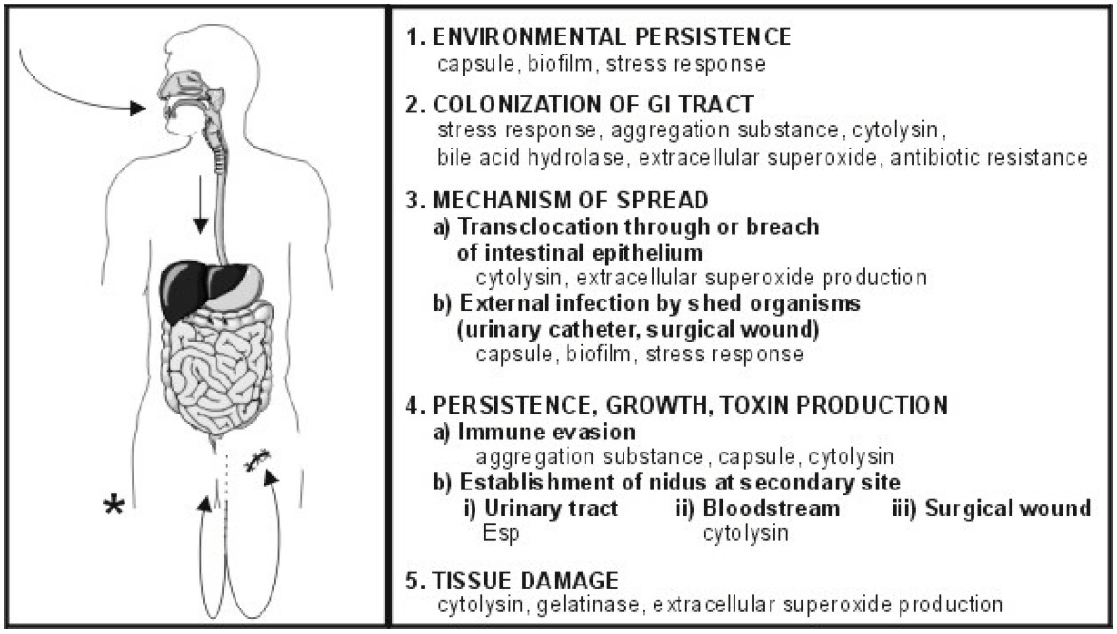

Figure (1-3): Pathogenesis of disseminated enterococcal infection. A stepwise model of enterococcal pathogenesis and potentially relevant factors of *E. faecalis* (Banla *et al*., 2019).

Colonization resistance from competing bacteria and human defenses like stomach acid and bile is a first hurdle that must be cleared before the intestinal tract can be colonized. The bacteria may multiply rapidly, and they can move to new, susceptible hosts. There is a clear correlation between GI colonization and infection risk (Goh *et al*., 2017). (Figure 1-4).

Biofilm production during colonization of abiotic devices has the potential to produce an infection nidus that is resistant to immune clearance and enters the circulation through the exogenous pathway. To spread, enterococci first need to establish a nidus, either in the gastrointestinal system at a spot where transcytosis is heightened, at the site of a surgical incision, or on an abiotic surface. Systemic

dissemination is aided by the organism's ability to evade host defenses, such as by preventing opsonization, impeding phagocytosis, or limiting phagocytic death (Fiore *et al*., 2019).

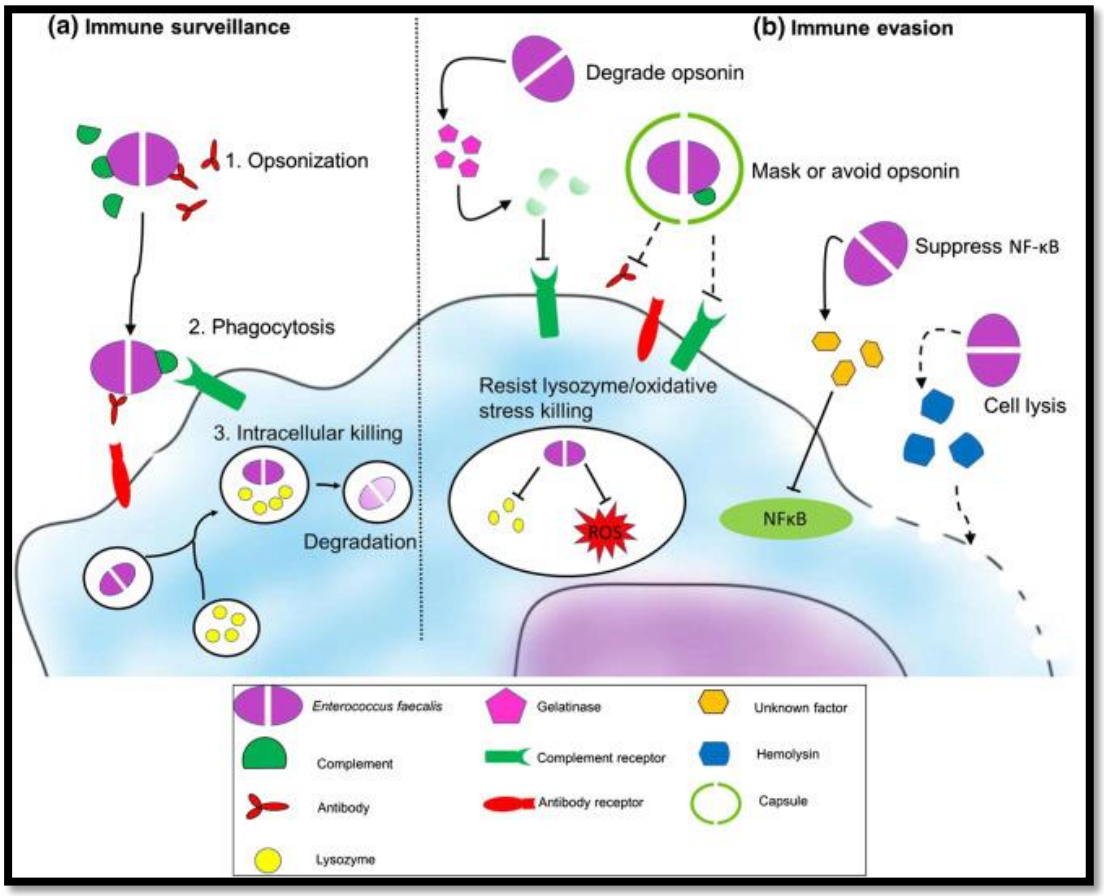

Figure (1-4): Mechanism of *E. faecalis* infection and role of immunity (Goh *et al*., 2017).

There is a wide range of enterococcal characteristics that may either increase the organism's pathogenicity or allow even commensal strains to colonize inappropriate locations in an immunocompromised host (Dubin & Pamer, 2017). There may be additional mediators of pathogenicity that have not been identified, thus it is crucial to remember that the presence of virulent strains among *E. faecalis* alone is not predictive of infection (Ferguson *et al*., 2016).

Virulence is associated with virulent strains' capacity for rapid multiplication in the gut and dissemination to other parts of the body. The capacity of enterococci to initiate infection is also hypothesized to be affected by host variables such predisposing medical conditions, immunological state, and drug exposure (Fiore *et al*., 2019) (Table 1-1).

Table (1-1): Putative virulence factors in *E. faecalis* and *E. faecium*

	Pathophysiology/Virulence	Epidemiology
E. faecium (Efm)		
Esp	Biofilm formation	Specifically linked to HA[b] *Efm*
	Pathogenesis of rat endocarditis	
	Pathogenesis of mouse UTI[a]	
	Antigenic in humans during endocarditis and bacteraemia	
Acm	Binding to collagen type I, IV	Wide spread among *Efm*
	Pathogenesis of rat endocarditis	
	Antigenic in humans during endocarditis	
Pili		
PilA	Not known	Wide spread among *Efm*
PilB		
Scm	Binding to collagen type V	Widespread among *Efm*
EcbA	Binding to collagen type V	Specifically linked to HA *Efm*
	Binding to fibrinogen	
E. faecalis (Efa)		
AS	Promotes conjugation by directing bacterial aggregation	Widespread among *Efa*
	Internalization into intestinal epithetial cells	
Asa1	Binding to renal tubular cells	
	Binding to and survival in macrophages	
Asp1	Binding to ECM	
	Pathogenesis of rabbit endocarditis	
Asc10	Internalization into and survival in PMNs[c]	
	Binding to ECM	
	Pathogenesis of rabbit endocarditis	
Esp	Biofilm formation	Widespread among *Efa*
	Pathogenesis of mouse UTI	
Gelatinase/FSR	Biofilm formation	Widespread among *Efa*
	Pathogenesis of mouse peritonitis	
	Caenorhabditis elegans infection	
	Pathogenesis of rabbit endophthalmitis	
Pili		
ebp locus	Biofilm formation	Widespread among *Efa*
	Pathogenesis of endocarditis	
	Pathogenesis of UTI	
	Antigenic in humans during endocarditis	
bee locus	Biofilm formation	Among 5% of *Efa*
Ace	Binding to collagen type I, IV	Widespread among *Efa*
	Binding to laminin and dentin	
cps locus	Resistance to complement and PMNs-mediated killing	*Efa* serotype C and D strains
epa locus	Biofilm formation	Present in *Efa* cell wall
	Enterocyte translocation	
	Resistance to killing by PMNs	
	Resistance to infection by phages	
	Pathogenesis of mouse peritonitis	
	Pathogenesis of mouse UTI	
LTA	Target of opsonic antibodies	Present in enterococcal cell wall
WTA	Not known	Present in enterococcal cell wall
Glycolipids	Biofilm formation	Present in enterococcal cell membrane
	Binding to colonic epithelial cells	
	Pathogenesis of mouse sepsis	

Pathologic alterations in the host are the last stage of the pathogenesis of infections. Inflammation on the part of the host, or direct tissue

damage caused by released toxins or proteases, are two possible triggers for these alterations (Newton *et al*., 2016).

The pathogenesis of an enterococcal infection might vary somewhat depending on the kind of infection. Enhanced enterococcal transcytosis into the circulation may be involved in bacteremia that does not seem to come from a direct gastrointestinal tract (GI) tract breach (Goh *et al*., 2017).

The ability of *Enterococcus faecalis* to adhere to and be internalized by intestinal epithelial cells in vitro has been linked to aggregation substance, a surface protein involved in the transfer of pheromone-responsive plasmids and clumping, and that this effect is mediated by the functional domain of aggregation substance (Todokoro *et al*., 2017).

1.2.4 Sources of infection and transmission

Enterococcal infections were formerly assumed to originate within the host's natural flora. However, due to the dramatic increase in healthcare-associated enterococcal infections in the 1980s and 1990s, investigations were conducted that definitively established the spread of pathogenic enterococci among hospitalized patients. Healthcare professionals' hands are a major vector for patient-to-patient transmission (Higuita & Huycke, 2014).

In the absence of routine cleaning, enterococci may live on hands for up to 60 minutes after inoculation and on inanimate surfaces for up to 4 months, serving as a reservoir for continuous transmission (Wißmann *et al*., 2021).

Direct inoculation into intravenous or urinary catheters is a potential route of transmission of enterococci from a healthcare worker's hands to a

patient. However, the most plausible mechanism is that healthcare-associated bacteria colonize the GI tracts of individuals with weakened colonization resistance, where they may multiply. By integrating into the host's native microbiome in this way, novel strains may more easily spread infection. Selective pressure from broad-spectrum antibiotics often used in hospitals may allow antibiotic-resistant enterococcal strains acquired by patients to remain in the gastrointestinal system (Jackson *et al*., 2018).

Vancomycin-resistant Enterococci (VRE) have been shown to spread not just across hospitals and nursing homes, but, the general population. These transmission patterns are most likely the result of infected patients' and healthcare workers' frequent interaction and mobility between facilities (Brodrick *et al*., 2016).

Investigations into the causes of healthcare-associated infections have focused on environmental factors since the 1990s, when VRE rates skyrocketed. Vancomycin-resistant enterococci (ARE) are also found in the environment, although the reservoirs for these bacteria are not expected to be any different from those for VRE (Kanamori *et al*., 2017).

1.2.5 Clinical manifestation of *E. faecalis* infections

Enterococci-caused urinary tract infections are the most common kind of infection seen in hospitals. Up to 15% of urine isolates include enterococci, making them the second most common kind of bacterium in the human urinary tract after *Escherichia coli* (Vihta *et al*., 2018). Urinary tract instrumentation or catheterization, other genitourinary tract pathologies, and prior use of antibiotics, particularly cephalosporins, have all been recognized as risk factors for enterococcal urinary tract infection (Walsh & Collyns, 2017).

Enterococcal urinary tract infection presents with symptoms that are interchangeable with those of other causes of UTI. Urinary tract infections may be hard to diagnose since enterococci are opportunistic pathogens that can potentially colonize or produce silent bacteriuria (Walsh & Collyns, 2020).

These bacteria can also be found on the skin and in the vaginal canal. A vaginal infection caused by *Enterococcus* can induce vulvo vaginal irritation and burning, as well as erythema and discharge. The proliferation of protective Lactobacilli was observed to be inhibited by *Enterococcus*, which contributed to the establishment and recurrence of bacterial vaginosis (Loveless and Myint, 2018). Moreover, Aerobic vaginitis (AV) is an endogenous opportunistic illness that occurs when the normal vaginal microbiota is disrupted. Its early detection and treatment during pregnancy may lower the chances of a bad pregnancy outcome (Sonthalia *et al*., 2022).

Endocarditis is often linked to the bacterium *Enterococcus faecalis*. About 8% of the isolates in a 2-year analysis of all endocarditis cases in the Netherlands were from *E. faecalis*, making it the third most prevalent species after Staphylococcus aureus and *Streptococcus sanguis*. Gram-positive bacteria are more likely to develop resistance to antibiotics if they are exposed to certain biocidal chemicals (Kampf, 2019). Enterococcal endocarditis is most often spread via the genitourinary system (14–70%), the digestive system (3%–27%), and dental caries (2%–12%). (Watanabe *et al.*, 2020). Both healthy and abnormal valves are at risk of infection by enterococci. Native valve enterococcal endocarditis is often a less severe, more drawn-out illness (Syed, 2018).

It is debatable whether or not enterococci play a substantial role in intra-abdominal infections, despite the fact that they are isolated from a high percentage of these diseases (Fabre *et al*., 2019). In studies of bacterial peritonitis, it was found that enterococci alone did not result in any abscess formation; however, a mixed inoculation of *E. faecalis* with other aerobe or anaerobe bacteria resulted in death and abscess formation, indicating a synergistic effect of *E. faecalis* in the pathogenesis of bacterial peritonitis (Goh *et al*., 2017). Even when enterococci are present as part of the poly microbial flora, antibiotics that lack effectiveness against enterococcus may frequently be used effectively in intra-abdominal infections (Goodlet *et al.*, 2016).

However, the existence of virulence factors may determine the involvement of *E. faecalis* in experimental peritonitis. In addition, intra-abdominal infection is the source of enterococcal bacteremia in some instances, and the exclusive cause of intra-abdominal infections in others (Najafi *et al.*, 2020).

Up to 5% of skin and soft tissue infection isolations are *E. faecalis*. There is little evidence that enterococci are involved for primary cellulites, since they typically only induce infections in tissues that have already been injured. Enterococci are often cultivated in cases of wound infection after abdominal surgery (Grigoropoulou *et al*., 2017). However, the function of enterococci in the pathogenesis of skin and soft tissue infections is unclear since they are often cultivated in conjunction with other pathogens (Heitkamp *et al*., 2018).

A significant number of nosocomial enterococcal infections are linked to medical devices such intravascular or urinary catheters, bile drains, and prosthetic heart valves (Del Pozo, 2018). Infections caused by

biomaterials begin with bacterial adherence and biofilm growth on the infected implant. Because the adhering bacteria are shielded from the host defense and the action of antimicrobial agents in the biofilm development phase, this biofilm may be a source of a chronic infection (Caldara *et al*., 2022). The morbidity caused by infections caused by biomaterials is high, and they are frequently so difficult to cure that the device must be taken out of the patient's body (Nappi *et al*., 2021).

1.2.6 Virulence factors

There are multiple mechanisms by which enterococcal virulence factors contribute to enterococcal disease. These include facilitating colonization, adherence, and invasion of host tissues; regulating host immunity; and inducing pathological changes in the host that are correlated with infection severity (Najafi *et al*., 2020). There is a standard pattern to the pathogenesis of enterococcal infections, and several *E. faecalis* virulence factors may contribute to each stage of this process (Hashem *et al*., 2021). First, the gastrointestinal tract is colonized asymptomatically by enterococci with a wide range of virulence factors. This growing colony of enterococci may eventually penetrate host tissue and cause illness in certain people. Isolates from hospitalized patients are more likely to include factors that increase the virulence of enterococci in this manner than isolates from the general population. Symptomatic illness, in the form of tissue damage or toxicity, may also be caused by enterococci (Guerardel *et al*., 2020).

The virulence factors that raise pathogenicity in this approach may not be more common among different clinical isolates, but they would be linked to more severe disease. Therefore, a greater occurrence in nosocomial isolates and/or enhanced illness severity in people or animals

might demonstrate the contribution of particular enterococcal features to virulence (Espndola *et al*., 2021).

Patients with severe immune suppression are also vulnerable to commensal strains lacking virulence factors, which complicates efforts to determine how much of a role virulence factors play in the pathogenesis of nosocomial infection (Tripathi *et al*., 2016). Accordingly, hospital-derived infection isolate collections may include a wide range of strain types, from purely commensal to those containing several virulence characteristics. Therefore, the capacity to generate outbreaks should be included in the investigation of a potential virulence feature, since strains that cause outbreaks across diverse groups of patients in hospital wards may reflect the actual virulent enterococcal lineages (Oravcova *et al*., 2017).

Several potential virulence features were found to be expressed on the surface of *E. faecalis*, including several enterococcal adhesions. Adhesion to epithelial cells and synthetic biomaterial surfaces like indwelling catheters may both benefit from these adhesions (Strateva *et al*., 2016). These variables may contribute to *Enterococcus* toxicity and the microbe's ability to persist in various settings. Figure (1-5) (Chajcka-Wierzchowska *et al*., 2017) is a schematic illustration of the majority of virulence factors and the method by which they function.

There have been suggestions that some virulence factors for *E. faecalis* may be at play, however the pathogenesis of this bacteria and its connection to symptoms and consequences of the infection remain unknown. Initiation of colonization, biofilm formation, host tissue destruction, and immune system evasion may all be aided by virulence determinants like aggregation substance, gelatinase, cytolysin,

enterococcal surface protein, collagen-binding-protein and PavA-like fibronectin-binding protein (Ghalavand *et al*., 2020).

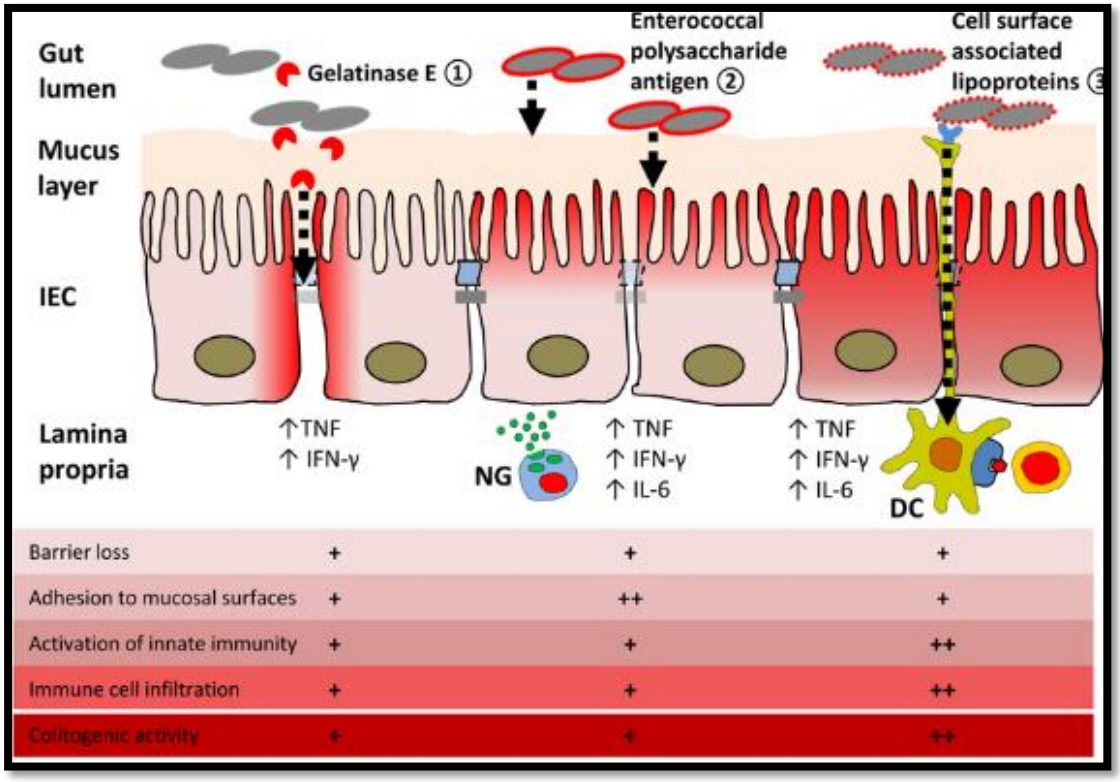

Figure (1-5): *E. faecalis* expressing virulence factors (Chajcka-Wierzchowska *et al*., 2017).

1.2.6.1 Cytolysin/Hemolysin

E. faecalis cytolysin/hemolysin is controlled by a quorum sensing method that employs a two-component regulatory system (Johnson, 2018). Cytolysin lyses human, rabbit, and horse erythrocytes and is active against various Gram-positive bacteria; it is linked to Streptolysin S and the antibiotic bacteriocins (Goossens, 2016).

To produce cytolysin, either a plasmid encoding the pheromone or an island of pathogenicity on the chromosome is integrated. A cytolysin's ability to lyse erythrocytes or kill off other host cells has been proposed as a mechanism by which it might affect the pathogenesis of infection

(Gholizadeh *et al*., 2020). Comparatively, strains with the typical cytolysin phenotype were far more dangerous than the non-cytolytic insertion mutants. The host strains were more dangerous than the wild-type cytolytic enterococci because of a mutant plasmid that amplified the cytolytic phenotype (Ali *et al*., 2017).

Patients with bacteremia caused by a cytolytic strain had a fivefold greater risk of death, demonstrating the pathogenicity of cytolysin in human infections. The cellular toxin cytolysin increases the pathogenicity of enterococci. By killing off immune system cells like macrophages and neutrophils, cytolysin is able to bypass the body's defenses (LaGrow *et al*., 2017). Pathogenic enterococci are mostly characterized by their ability to produce cytolysin. Cytolysin production and activation are multi-step processes. CylM is responsible for the post-translational modification of the ribosomally produced lytic factor precursors CylLL (the long subunit) and CylLS (the short subunit) (Fiore *et al*., 2019).

In addition, bacteriocin activity against streptococcal strains and other Gram-positive bacteria was reported in enterococcal strains that produce hemolysin. An individual molecule was experimentally identified as the cause of both the bactericidal and hemolytic characteristics. After being exposed to UV light, hemolytic and bactericidal activities were eliminated; reestablishing either one of them led to the restoration of the other (Painter, 2016). The molecule's dual bactericidal and cytolytic properties inspired the name "*E. faecalis* cytolysin." As a bacterial aggregation substance (AS), cytolysin helps to control the spread of disease (Hwanhlem *et al*., 2017).

1.2.6.2 Enterococcal Surface adhesion Protein

Cell surface Esp is structurally identical to other streptococcal surface proteins that have been found to be virulence factors and contribute to immune evasion by varying the amount of repeat motifs they contain (Wrobel, 2019). In addition, Esp was shown to be a component of a pathogenicity island that encodes several other genes that are unusual in isolates that did not originate from an infection (Jeyachandran, 2019).

Esp, which may be generated by *E. faecalis*, is a key factor in the pathogenicity of enterococci. Evidence suggests a function for Esp in pathogenesis, since its prevalence is greater in clinical strains of *E. faecalis* (Kiruthiga *et al*., 2020). Moreover, it seems to facilitate the initial process by which Enterococci adhere to and from biofilms on surfaces. The esp may function as a colonization factor that promotes adhesion to the uroepithelium, analogous to the fimbriae of *Escherichia coli* (Armbruster *et al*., 2018).

There is universal conservation in the sequence and structure of Esp proteins in *E. faecalis*. They have an N-terminal region, a core region made up of repeat units, and a signal sequence (Terlizzi *et al*., 2017). Esp proteins are membrane proteins with a cell wall anchor motif and a membrane-spanning hydrophobic domain at their C-termini (Desvaux *et al*., 2018).

Effect of surface protein on *E. faecalis* colonization and persistence in urinary tract infections. It's also linked to an increase in *E. faecalis* biofilm development and primary attachment to inorganic substrates (La Rosa *et al*., 2016). The frequency of *Enterococcus faecalis* isolates containing the enterococcal surface protein, a high-molecular-weight

surface protein with an unclear function, is much higher than that of other types of *Enterococcus faecalis* isolates (Russo, 2019).

1.2.6.3 Collagen-binding adhesion

Infection begins when bacteria attach to proteins in host cells or the extracellular matrix. Research on enterococcal adhesion to extracellular matrix components such fibronectin, collagen(s), and laminin is extensive (Hammerschmidt *et al*., 2019).

The surface hydrophobicity of *E. faecalis* strains from a variety of clinical sources was discovered to account for their potent agglutination with collagen type I and type IV, two ECM proteins (Stinemetz, 2017).

E. faecalis required a protein to bind to extracellular matrix (ECM) proteins after growth in a stressful setting, here defined as growth at 46°C. However, pre-treatment with a protein-digesting enzyme or pre-incubation with soluble forms of collagen, or subsequent digestion of the binding substrate with collagenase, hindered the so-called 'conditional adherence' of the bacteria to collagen (Jäger *et al*., 2017).

Proteins from enterococci that have been shown to bind to collagen have been sequenced, and these sequences, together with the amino acid and nucleic acid sequences that regulate the precise collagen-binding domains of these proteins, have been published. Environmental cues such as serum, high temperature, and bile salts promote the expression of the virulence factor adhesion to collagen of *Enterococcus faecalis* (Manias & Dunny, 2018).

1.2.6.4 Aggregation substance

For plasmid transfer to occur, bacteria use an adhesion called aggregation substance (AS), which is encoded on plasmids and is activated by pheromones. In the bacterial conjugation process, the donor cell expresses the antigen presenting (AS), and the recipient cell expresses the corresponding ligand for AS, which is called binding substance (BS) and is encoded by the cell's chromosomes (Breuer *et al.*, 2018).

Moreover, AS was discovered to facilitate binding to collagen type I and other ECM proteins. Since collagen type I is the primary organic component of dentin, bacterial binding to this protein may be especially relevant to endodontic infections (Anshida *et al*., 2020). It has been revealed that AS facilitates the complement receptor-mediated, opsonin-independent binding of *E. faecalis* to human neutrophils. Despite extensive phagocytosis and neutrophil activation, *E. faecalis*-carrying AS was shown to be resistant to death by human neutrophils due to the unique binding it engaged in to survive (Shehab *et al*., 2021). Infection with *E. faecalis* cells expressing AS has been shown to cause tissue damage (Joczyk-Matysiak *et al*., 2017), and this damage may be caused in part by phagosomal oxidant generation by neutrophils.

Cell extracts of *E. faecalis* strains positive for both AS and BS were reported to activate macrophages, leading to the production of tumor necrosis factor alpha, and to stimulate T-cell proliferation, leading to the release of tumor necrosis factor beta and gamma interferon (John *et al*., 2015). Nitric oxide (NO) is a cytotoxic agent, and it increases the production of NO by many different cells, including macrophages and neutrophils, which may lead to unfavorable cell and tissue damage. The

sex-pheromone system, by which *E. faecalis* efficiently collects plasmids, was the first to characterize aggregation material (Hirt *et al*., 2018).

E. faecalis is bound to human neutrophils by AS via a complement receptor-mediated process that does not need the presence of opsonin. The *E. faecalis*-binding aggregation material was shown to be able to withstand death by human neutrophils and to increase the amount of time the bacteria spent within macrophages (Todokoro *et al*., 2017).

The 'older' sections of the cell wall contain proteinaceous aggregation material that appears as a hair-like structure on the cell surface. Serum may trigger its surface expression (Jakubovics *et al*., 2021). In addition to acting as an adhesin during bacterial conjugation, AS also plays a role in the adherence of *E. faecalis* to a wide range of eukaryotic cells *in vitro*, including renal tubular cells and intestinal epithelial cells (Zheng *et al*., 2018).

1.2.6.5 Gelatinase and serine protease production

Gelatinase and serine protease, both of which are secreted by *E. faecalis*, are under the control of the fsr system. Hydrolysis of a variety of proteins and other tiny physiologically active peptides by the gelatinase has been shown. These include gelatin, collagen, casein, lactoglobulin, pig myofibrillar proteins, and porcine sarcoplasmic proteins (Toopcham, 2015).

By degrading targeted host proteins and causing tissue damage, gelatinase may play a role in virulence. Animal studies verified gelatinase's virulence-related function (Vences *et al*., 2017). Among the many bioactive compounds that gelatinase can degrade are the insulin

peptide chain, tissue collagen, hemoglobin, and heme (Shimoda *et al.*, 2021).

The fact that, GelE can cleave fibrin has significant implications for *E. faecalis* pathogenicity. Singh *et al*., (2018) reported that, enterococci in blood infections and vegetations generated during endocarditis were likely to be covered with polymerized fibrin. Through the expression of gelE, the fibrin layer around the bacterium would be degraded, allowing the germs to spread further. In addition, GelE has been found to degrade innate immune system antimicrobial peptides (Mateescu *et al*., 2015).

Gelatinase, as a member of the matrix metalloproteinase (MMP) family, may also be generated by a broad range of mammalian cells, including inflammatory cells, epithelial cells, fibroblasts, osteoclasts. Host gelatinase, which acts on substrates comparable to those of bacterial gelatinase, contributes to proper physiology by degrading the extracellular matrix, for example in the control of tissue development and remodeling (Jaboska-Trypu *et al.*, 2016).

GelE impact on C5a-complement activity modulation. C5a of the complement system is a multifunctional inflammatory peptide that regulates cytokine production and operates as a strong chemoattractant for neutrophils and monocytes (Escamilla-Rivera *et al*., 2019).

A serine protease's characteristic structure consists of two beta-barrel domains that converge at the catalytic active site. Based on the substrates they cleave, these enzymes are further classified as trypsin-like, chymotrypsin-like, or elastase-like (Hartini *et al*., 2021).

Autolysin N-acetylglucosaminidase (AtlA) has a role in fratricide and extracellular DNA release during biofilm development, and its activity is

regulated by serine proteases. Deleting sprE led to a rise in fratricide activity, suggesting that the serine protease was blocking lysis of the cells. Cell lysis may also be modulated by serine protease, which alters AtlA binding capability to the cell wall via a variety of interactions (Ali *et al*., 2017).

1.2.6.6 Lipoteichoic acids

The polyglycerolphosphate backbone of lipoteichoic acids (LTA) (1.3 phosphodiester-linked chains of 25–30 glycerolphosphate residues alternatively modified with glycosyl and Dalanyl ester groups) is covalently attached to a glycolipid moiety, creating a family of similar amphipathic compounds (Dufresne & Paradis-Bleau, 2015).

Platelets, erythrocytes, lymphocytes, Polymorph nuclear leukocytes (PMNs), and epithelial cells have all been shown to bind to the LTA molecule due to its lipidic moiety (Seilie & Wardenburg, 2017). During bacterial infections, LTAs may contribute to tissue damage by making bound erythrocytes vulnerable to lysis in both *in vitro* and *in vivo* settings when exposed to even their own complement system. It's possible that LTA's ability to promote Gram-positive colonization on tooth surfaces will be a game-changer for dental hygiene. Further, *Enterococcus* LTA extracts bind to calcified matrix and cells in newborn rat parietal and long bones (Mashraqi, 2017).

Apoptosis is the controlled cell death that does not affect neighboring cells. In addition to being a continual process in practically all organs throughout life, it has been linked to various disorders, including oral diseases and periradicular lesions (D'Arcy, 2019). Cell death through apoptosis has been seen in cell culture and in investigations of tissue culture, suggesting that streptococcal lipoteichoic acids are toxic. Studies

examining the effects of *E. faecalis* components, especially LTA, on apoptosis in relevant cell lines (osteoblasts, osteoclasts, periodontal ligament fibroblasts, macrophages, and neutrophils), are warranted because they may provide light on the nature of periradicular lesions caused by *E. faecalis* (Ramachandran *et al*., 2021).

It has been shown that lipoteichoic acids isolated from *E. faecalis* or other Gram-positive bacterial strains stimulate leukocytes to produce numerous mediators that are known to have a role in different stages of the inflammatory response (Wang *et al*., 2015). The lipoteichoic acid produced by enterococci is often cited as one of the components that affects the host immune response and, in turn, causes tissue damage. Several research teams discovered that lipoteichoic acid is a powerful inducer of several cytokines, making it as inflammatory as Gram-negative bacterial lipopolysaccharide (Najafi *et al*., 2020).

1.2.6.7 Extracellular superoxide production

In many illnesses, particularly inflammatory conditions, the highly reactive oxygen radical superoxide anion plays a role in causing cell and tissue damage (Bhattacharya, 2015). Many biological components, including lipids, proteins, and nucleic acids, are damaged by oxygen radicals such superoxide anion. While neutrophils and other phagocytic cells' generation of superoxide is crucial for destroying germs, it also damages tissue at the site of inflammation (Ozougwu, 2016).

Researchers hypothesized that, the periapical damage and bone loss seen in chronic apical periodontitis resulted from an imbalance between the creation of oxygen radicals by phagocytic cells in periapical lesions and their clearance. Osteoclasts have been linked to the production of superoxide anion and bone resorption. Also, the superoxide anion in the

plasma may react with a chemotactic for neutrophils precursor to produce that factor (Vengerfeldt *et al*., 2017).

Superoxide anion is produced by both bacteria and host cells. A *Streptococcus* D spp. strain isolated from a clinical setting produced superoxide, which was erythrocyte-lytic. It has been shown that many different *E. faecalis* strains have the ability to generate superoxide radicals outside of their cells (Mohammadian *et al*., 2020).

The highly reactive oxygen radical superoxide anion contributes to cell and tissue damage in a wide range of pathologies, including inflammatory conditions. The destructive effects of superoxide anion and other oxygen radicals may be seen in a variety of natural mixes (Marcelli, 2017). Neutrophils and other phagocytic cells produce superoxide, which is essential for the death of microorganisms but also causes tissue damage at the site of inflammation (Kehrer & Klotz, 2015).

1.2.6.8 Hyaluronidase production

Hyaluronidase is a degradative enzyme that causes tissue injury as a result of its action on hyaluronic acid (hyaluronate, hyaluronan) (Weber *et al*., 2019). Numerous organisms on Earth use it, including mammalian cells like spermatozoa, snake venom, and parasites like leeches and hookworms. Numerous bacteria, including streptococci, generate it in large amounts (Liang *et al*., 2016).

By depolymerizing the mucopolysaccharide component of connective tissues, hyaluronidase makes bacteria more likely to invade. Exogenous hyaluronidase was revealed to be necessary for Streptococcus pneumoniae strains with low or no hyaluronidase to induce brain infections in mice (Passi and Vigetti, 2019). It has been shown that

hyaluronidase plays a crucial role in the spread of Treponema pallidum, the bacterium responsible for syphilis. *Streptococcus intermedius*, an organism isolated from human pus, has been shown to have hyaluronidase activity in its culture supernatants, suggesting it may play a role in tissue deterioration (Khan *et al*., 2018).

Since hyaluronidase's target substrates degrade into disaccharides that may be carried and processed intracellularly by bacteria, hyaluronidase may also play a role in supplying nutrition for the bacteria (Knopf-Marques *et al*., 2016). Dentin has been shown to contain hyaluronic acid, which is the substrate for hyaluronidase. It has been shown that streptococci isolated from carious dentin may thrive in a media consisting only of hyaluronic acid; the bacteria may get the carbon they need for their development by hydrolyzing the substrate (Abbass *et al*., 2020). It is possible that streptococci and an *E. faecalis* strain isolated from carious dentin contribute to tissue damage by producing hyaluronidase (Chenicheri *et al*., 2017).

Apical periodontitis-related root canal-infecting bacteria also produce hyaluronidase, and the enzyme's activity seems to be correlated with the severity (acute vs. sub-acute) of clinical symptoms (Chapple & Gilbert, 2019). 'The spreading factor,' hyaluronidase, is thought to aid in the dissemination of bacteria and their toxins throughout the host's tissues. Hyaluronidase may not only have harmful consequences on its own, but also prepare the way for the adverse effects of other bacterial toxins, so amplifying the damage (Dahlen *et al*., 201).

1.2.7 Sex pheromone system

Plasmid-free *Enterococcus faecalis* excrete peptides (sex pheromones) which specifically induce a mating response in strains harboring certain conjugative plasmids. The response is characterized by the synthesis of a "fuzzy" surface material, visible by electron microscopy, which is believed to facilitate the aggregation of donors and recipients. Transconjugants which receive a specific plasmid shut down the production of endogenous pheromone; however, they continue to produce pheromones specific for donors harboring different classes of plasmids (Lin *et al*., 2021).

Exchange of plasmids among themselves and other genera is unique involved the production of sex pheromone, while other species of enterococci have the capacity to acquire and exchange is not pheromone-dependent. The sex-pheromone system has been limited to *E. faecalis* and has not been observed in other enterococcal species (Sterling *et al*., 2020).

There are more than 47 plasmids in *E. faecalis*, with the three major categories being (1) tiny, cryptic plasmids, (2) big, conjugative plasmids, and (iii) sexual, pheromone-responding plasmids, of which only around 20 have been identified so far (Zou *et al.*, 2020). Clumping induction assays in mating mixes or in donor cell cultures incubated with recipient cultures filtering offered the first evidence of the presence of pheromone; hence, the term clumping-inducing agent (CIA) is used to characterize these compounds (Bhatty *et al.*, 2015).

The sex pheromone system is a kind of chemical communication among bacteria, and it is notably common among gram-positive bacteria. Several crucial activities in Gram-positive bacteria are regulated by cell-

to-cell communication, which is mediated by extracellular signal molecules. Peptides and peptide-like compounds make up the bulk of these substances. Either the signal is transduced across the cytoplasmic membrane or it is imported into the cell and then interacts with intracellular effectors (Slamti & Lereclus, 2019).

In *E. faecalis*, chromosomally encoded pheromones for reproduction are short (7 or 8 amino acids) hydrophobic peptides that act as signaling peptides. *E. faecalis* strains' ability to produce sex pheromones and the resulting bacterial clumping effect. Researchers have shown that the sex pheromone system in *E. faecalis* increases the transfer frequency of particular conjugative plasmids by a factor of several hundred (Segawa *et al*., 2021).

The pheromone response mechanism and its control and function have been shown to be very complicated, playing a dual role in plasmid conjugation and modulating enterococcal pathogenicity. The stabilization/partition mechanisms, along with other functional modules found in pheromone plasmids, are essential for the plasmid molecule to be reliably maintained in the host bacterium (Dunny & Berntsson, 2016).

The pheromone is a short, low-molecular-weight peptide (oligopeptide) released by cells. It typically contains seven or eight amino acids (less than 1000). Cells that get the peptide have it encoded on their chromosomes (plasmid-free cells). Multiple pheromones are produced by *E. faecalis* bacteria. To far, at least five distinct sex pheromones and five distinct peptide inhibitors have been found. Receptor plasmid-encoded peptide inhibitors in donor cells (Lin *et al*., 2021).

The protein (AS) expressed by the plasmid that binds to the receptor (BS) encoded by the chromosome on the surface of the receiving cell.

Divalent cations (Mg+2, Mn+2, Ca+2, Co+2) and phosphate ions are necessary for AS and BS binding. After the mating channel is formed by cell-cell contact and binding, a single strand of plasmid is transmitted to the recipient cell (Bandyopadhyay *et al*., 2016). Once the plasmid has taken hold in the recipient cell, it will cut down the pheromone's action by two encoding processes: a decrease in pheromone production, and the creation of a particular inhibitor peptide encoded by the prg-Q gene (Dunny *et al*., 2016).

By binding to the pheromone receptor, the inhibitors are competitive with exogenous pheromone. Substances on the surface (ES proteins) produced by pheromones have a significant role in blocking plasmid transmission across clumped donor cells. By degrading or inhibiting the function of the pheromone in the cell wall of the donor cell, Prg-Y proteins are expressed to avoid self-induction by endogenous pheromone which is generated from donor cell (Kohler *et al*., 2019).

The technique of this plasmid collecting system's transmission is shown in Figure (1-2). There are three parts to a conjugation: First, strains lacking the corresponding sex-pheromone plasmid excrete small linear peptides known as sex-pheromones; second, cells carrying the corresponding sex-pheromone plasmid respond by expressing a plasmid-encoded surface protein known as the Agg; and third, the Agg facilitates close cell-cell aggregation between the donor and recipient, allowing for the transfer of the sex (Kohler *et al*., 2019).

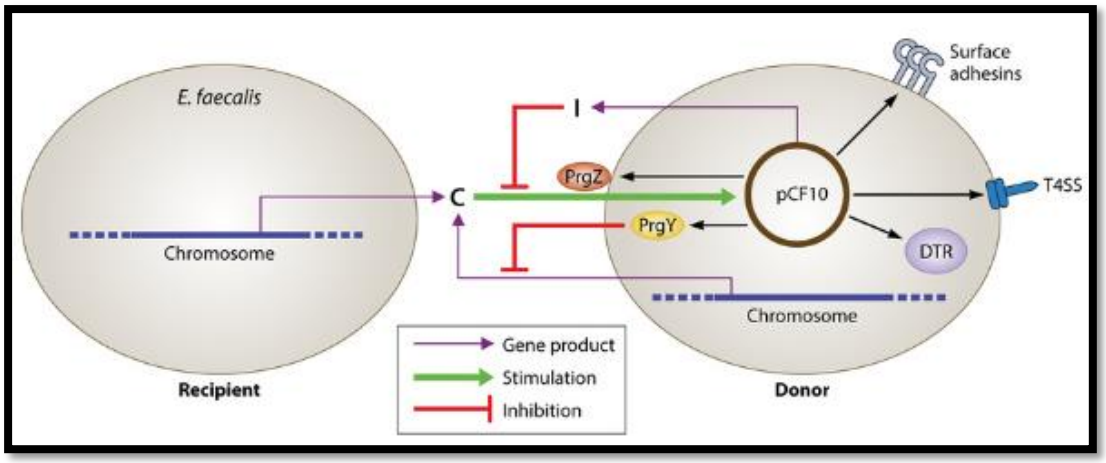

Figure (1-6): Diagram of the signaling circuits in the *E. faecalis* pCF10 conjugation system plasmid (Kohler *et al.*, 2019).

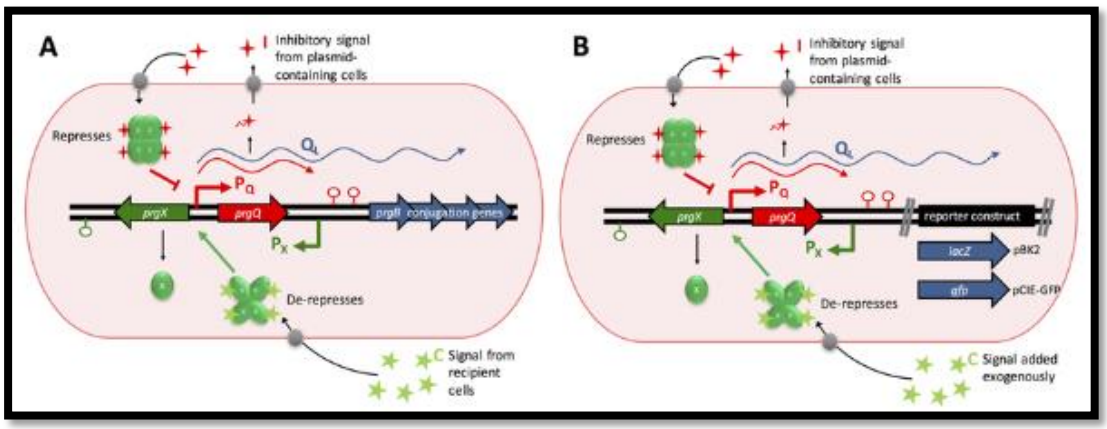

Figure (1-7): Model of induction of the pCF10 conjugative plasmid and reporter systems. (A) The Q L transcript from the pCF10 plasmid encodes the proteins that mediate conjugation and is induced upon signaling by C pheromone (green stars) from potential recipient cells. The I inhibitory peptide (red stars) counteracts C and is produced by plasmid-containing cells from a short transcript from the P Q promoter (red). PrgX complexes (green circles) repress the P Q promoter whereby PrgX-C complexes allow induction of Q L transcription and PrgX or PrgX-I complexes inhibit transcription of Q L. (B) The pBK2 and pCIE-GFP reporter plasmid constructs have the same P Q / Q L regulatory region as pCF10. However, either lacZ for pBK2 or gfp for pCIE-GFP have been inserted in place of the conjugation genes (Kohler *et al.*, 2019).

A wide variety of sex pheromone plasmids that encode for antibiotic resistance and other virulence factors. In contrast to the Agg on plasmid, which does not match the overall homology (Chajcka-Wierzchowska *et al.*, 2017). DNA hybridization experiments have indicated that all sex-pheromone plasmids have a homologous DNA region that encodes the Agg.

E. faecalis sex pheromone production has been the subject of several hypotheses. As you can see, cCF10 is the most researched sex pheromone. The Ccf-A gene encodes a secretory precursor of cCF10 in plasmid-free cells (cCF10 p). Lipoprotein serves as the antecedent. Lipoprotein Ccf-cCF10 A's is located in its signal peptide's carboxy-terminal terminus (Beukers *et al*., 2017).

Signal peptidase I cleaves before the cystein residue inside the conserved lipobox processing site, releasing the signal peptides, allowing the AS lipoprotein to be released across the cytoplasmic membrane and attached to the cell wall. Most likely, Eep (increased expression of pheromone) cleaves at the amino-terminal end of the cCF10 peptide sequence in the cell wall to produce pro.cCF10 (Heywood & Lamont, 2020).

Although some pheromone is released into the medium after being processed by exo-peptidase, which cleaves off the last three c-terminal residue to form mature cCF10, a significant amount is still bound to the cell wall of the organism. This new kind of bacterial conjugation, known as pheromone-inducible plasmid transfer, involves a complex series of interactions between donor and recipient cells (Parthasarathy *et al*., 2016).

In the absence of plasmids, the cells secrete a peptide sex hormone. Peptide acts as chemical signal that is conveyed from recipient cells to donor cells, and pheromone interacts with receptor on surface of donor cells by specific binding by plasmid-encoded lipoproteins (*PrgZ* gene). Once the receptor recognizes the pheromone, the pheromone is imported into the responder cell's cytoplasm by a chromosomal oligopeptide permease (Opp). The Opp function as a membrane translocator and are made up of four different proteins: two transmembrane pore-forming subunits (Opp-B and Opp-C) and two cytoplasmic ATPase (Opp-D and Opp-F) subunits (Bandyopadhyay *et al*., 2016).

Active transport of import peptides via Opp requires the usage of the later subunits, which are powered by the ATP-binding cassette (ABC). Prg-X is a negative regulator that controls how pheromones transported into the cytoplasm interact with intracellular effector molecules. Pheromone binding to the *Prg-X* gene inhibits the gene's ability to regulate the production of two surface proteins, aggregation substance (AS) and exclusion substance (ES), on donor cell surfaces (Hirt e*t al*., 2022). There are two ways for horizontal gene transfer across bacterial genera, one of which involves transposons moving DNA around inside the bacterium. The transposons are DNA segments that may hop across the bacterial chromosome or plasmid with relative ease (Gillings, 2017).

In contrast to their name, transposons do not reproduce independently but rather as a part of the host DNA. They may encode enzymes that are resistant to drugs, produce poisons, or participate in a wide range of metabolic processes. One more method, bacterial horizontal gene transfer. There are three mechanisms for this transfer: conjugation, transduction, and transformation. Among themselves and with other

gram-positive bacterial species, enterococci are capable of horizontal exchange of genetic material (Bello-López *et al*., 2019).

Conjugational gene transfer in *Enterococcus faecalis* was a major research focus of the 20th century. The bacterium *Enterococcus faecalis* has been linked to at least three different conjugative systems (braodhost range plasmids). This kind of transmission relies heavily on a membrane filter and is particularly inefficient in broth since it requires the cells to come into direct touch with each other. Comparatively, plasmid transfer is less efficient than pheromone-induced gene transfer (10-4 10-5 per donor cell). The R-wide gene's dispersal may be explained by (ii) the low-frequency, filter-based transposon mode of plasmid transfer. Conjugative transposons encode not only the capacity to cause conjugation between different bacterial cells, but also to move from one DNA site to another inside the same cell. Plasmid transfer of the third kind is exclusive to strains of *Enterococcus faecalis* (narrow-host range plasmids). Conjugation allows for the rapid spread of antibiotic resistance and virulence factors encoded on plasmids (reaching 10-1 per donor cell). Signaling peptides, sometimes known as sex pheromones, induce the transfer of conjugative plasmids from donor to recipient cells. Thus, they are thought to have a role in the dispersal of antibiotic resistance and virulence factors in the environment (Rebelo *et al.,* 2021).

1.2.8 Biofilm formation

Microorganisms and their extracellular products accumulate to establish a population on a surface in the form of a biofilm. Significant problems might arise when introducing alien objects or equipment into the human body because of biofilm growth (Campoccia *et al*., 2021) (Figure 1-8).

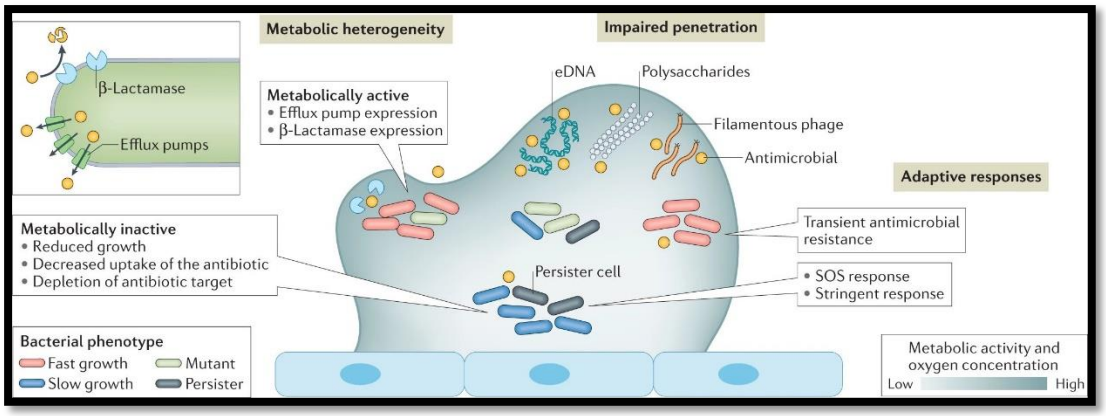

Figure (1-8): Tolerance and resistance of microbial biofilms (Campoccia *et al*., 2021).

One of the many shapes bacteria take on as they multiply is a biofilm. The biofilm growth phenotype refers to the bacterial behavior pattern in which the bacteria form sessile clumps. Whenever bacteria are able to establish a biofilm inside a host, the infection becomes persistent and resistant to treatment (Sauer *et al*., 2022).

Resistance to antibiotics and other traditional antimicrobial treatments, as well as the ability to evade the host's defenses, are two of the most prominent characteristics of persistent biofilm-based illnesses (Khan *et al*., 2021).

Bacterial colonies called biofilms are connected to a surface and are surrounded by a matrix of exopolysaccharides, proteins, and extracellular DNA. Up to 80% of microbial infections in the human body and many hospital-acquired illnesses, especially in instances where in-dwelling medical devices are necessary, are thought to be contributed to by biofilm development, which adds to persistence and severity (Batoni *et al*., 2021).

Compared to planktonic cells, bacteria that form biofilms are extremely resistant to antibiotic treatment, making it very difficult to eradicate any preexisting biofilm infections. Human pathogens rely on

biofilms for survival, transmission, and enhanced infectiousness in the environment (Bekele *et al*., 2018).

Biofilms are a kind of microbial community comprised of cells that are attached to an interface, embedded in a matrix of exopolysaccharides, and exhibit an altered phenotype. This is a growing concern as the number of biomaterial devices used in urology continues to rise. Microorganisms connect to a conditioning coating of host proteins linked with the catheter surface, and studies suggest that biofilm growth starts as soon as the catheter is inserted (Karygianni *et al*., 2020). The vast majority of microorganisms in nature are members of communities attached to surfaces (called biofilms) (Sauer *et al*., 2022). Biofilm bacteria are quite distinct from their planktonic relatives. Biofilm bacteria are distinguished by the fact that their cells develop in multicellular aggregates wrapped in an extracellular matrix (Memariani *et al*., 2019).

Some of the cells in a community of bacteria remain metabolically dormant because their extracellular matrix is a barrier to diffusion for tiny molecules and protects the cells from external assault (Makabenta *et al*., 2021).

Biofilm communities that are sessile may also give birth to individuals that are not sessile, which means they can quickly reproduce and spread. Therefore, biofilms might not only serve as a resistant source of germs during antimicrobial treatment, but also shield bacteria from host-defense processes including phagocytosis (Batoni *et al*., 2021).

Biofilm development is suspected in about 60% of bacterial illnesses treated by doctors in the industrialized world at now (Khatoon *et al*., 2018). In the recent decade, the detection of microbial biofilms has risen to prominence as a major issue in the field of microbiology, with crucial

implications for many areas of today's society. Some of the most dangerous pathogens are also model systems for the study of biofilm-forming organisms. There is a thorough understanding of the biofilm formation processes, bacterial life cycles, and molecular mechanisms of these microorganisms that produce biofilms (Bhowmik *et al*., 2021).

Some characteristics are known to be universal to biofilms, however the processes that bacteria use to create biofilms vary greatly across species and even between strains of the same species. Not many genes with major functions in biofilm formation have been found. At first, physical forces or bacterial appendages, such flagella, bring planktonic microbial cells to the conditioned surfaces (Gebreyohannes *et al*., 2019).

It is well established that, bacterial biofilms play a role in the development of diseases such cystic fibrosis, periodontitis, and nosocomial infections brought on by the insertion of catheters and prosthetic heart valves. It is also known that biofilms may develop in food processing facilities, and that harmful and spoilage bacteria can be isolated from them (Nyenje *et al*., 2013).

Because the microbes in biofilms are so resistant to antimicrobial medicines, they constitute a public health risk for those who rely on indwelling medical devices (Soumia *et al*., 2021). Treatment failure and reinfection may occur because pathogenic biofilms are able to thrive in the face of high doses of antibiotics, a trait of the biofilm lifestyle that is referred to as "recalcitrance." Ultraviolet (UV) radiation, heavy metals, acidity, changes in hydration or salinity, and phagocytosis are only some of the physical and chemical stresses that bacteria in a biofilm may withstand. Some treatment challenges seen in clinical settings may be

traced back to the fact that biofilm bacteria have a particular capacity to resist antibiotic-mediated death (Lorenz, 2018).

The capacity of biofilms to persist in the presence of antibiotics is only marginally influenced by mechanisms implicated in traditional antibiotic resistance, such as efflux or antibiotic-modifying enzymes. Even though bacteria are totally sensitive to antibiotics in vitro under planktonic circumstances, they may be somewhat resistant to such medications when entrenched in a biofilm. Recalcitrance of biofilm bacteria against antibiotics is a multifaceted phenomenon that results from a combination of tolerance and resistance mechanisms (Iglesias, 2020).

Biofilm bacteria, also known as sessile bacteria, are distinguished from planktonic bacteria by their stationary or quiescent development phase and unique morphologies. Bacteria in biofilms are highly resistant to drugs and other environmental stressors. It has been hypothesized that the phenotypic shift that occurs during this transition is the product of a complicated and highly controlled process (Ballén *et al*., 2022).

1.2.9 Antibiotics Profile

Antibiotic drugs are often used for both treating and avoiding these illnesses. Bacterial growth may be stopped or slowed down by these substances (Colomer-Winter *et al*., 2018). Multiple-drug resistance is widespread among enterococci strains recovered in different settings. Most occurrences of antibiotic resistance stem from a mixture of innate and acquired mechanisms (Torres *et al*., 2018). (Figure1-9).

The epidemiology of enterococcal infections has shown many patterns, including an increase in the number of infections among the most critically sick hospitalized patients. Resistance to the most frequently used anti-enterococcal drugs is prevalent. Some examples of

these antibiotics are listed below: Ampicillin, Linezolid, Daptomycin, Quinupristin-Dalfopristin, Vancomycin, and there is also a significant degree of resistance to aminoglycosides (Aamodt *et al.*, 2015).

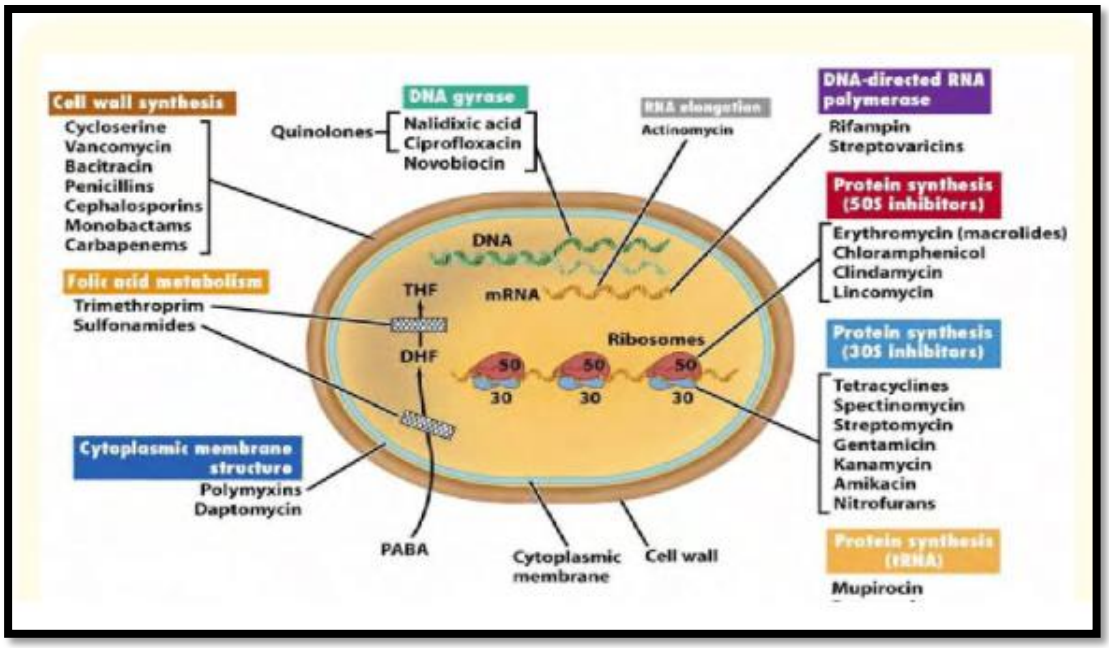

Figure (1-9): Antibiotic target sites (Torres *et al.*, 2018)

Enterococci are naturally resistant to several antibiotics, including those indicated above (cephalosporins, sulfonamides, oxacillins, ertapenems, and perfloxacins). In particular, *E. faecalis* have innate resistance to cephalosporin, aminoglycosides, clindamycin, fusidic acid, trimethoprim, and sulfamethoxazole, and once again, *E. faecalis* have innate resistance to quinupristin. Additional resistance mechanisms to important antibiotics, such as Tetracycline, Erythromycin, Fluoroquinolones, Rifampicin, Chloramphenicol, Nitrofurantoin, Fusidic acid, glycopeptides (Vancomycin & Teicoplanin), and high concentrations of aminoglycosides and β-lactams, can be acquired by enterococci through mutation and horizontal gene transfer (HGT) processes (Kateete *et al.*, 2019) (Figure 1-10).

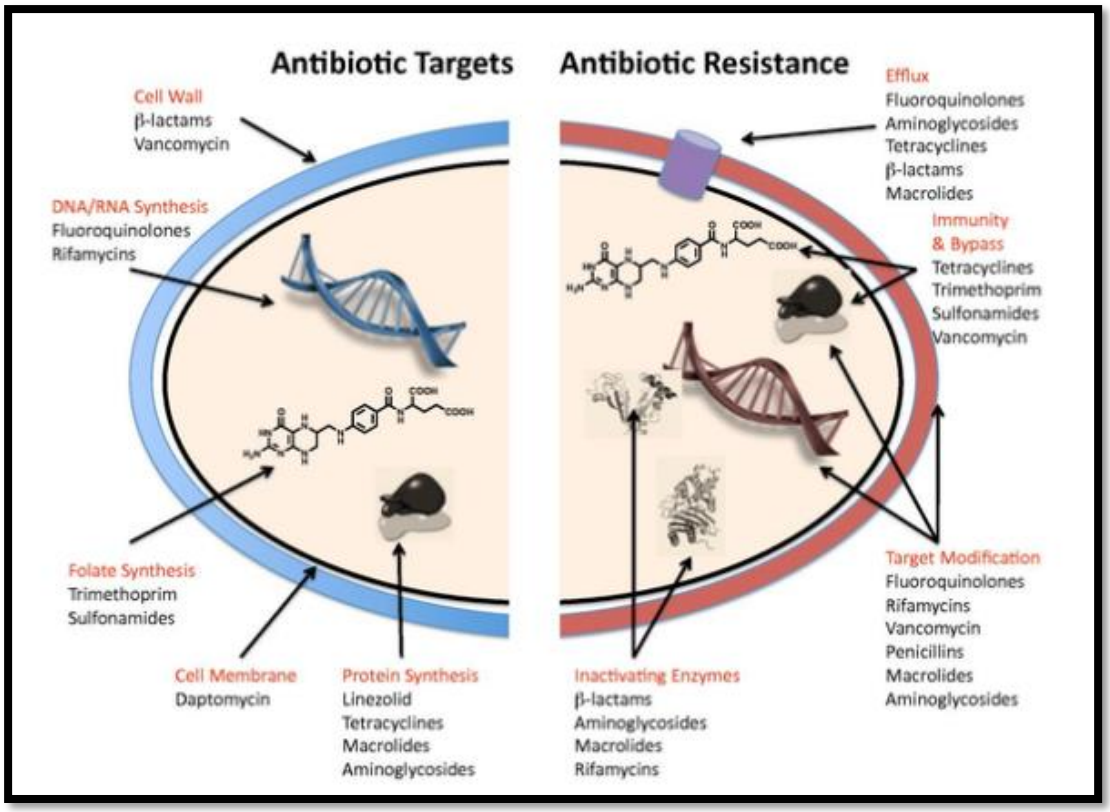

Figure (1-10): Antibiotic targets and mechanisms of resistance (Kateete *et al.*, 2019).

In addition, there has been a rise in infections caused by multidrug-resistant (MDR) enterococci and Vancomycin-resistant (VRE) strains because of the rising prevalence of hospitalization, advances in medical technology and treatment, and the widespread use of antibiotics (Satlin & Walsh, 2017) (Figure 1-11).

The limited options for treating infections caused by vancomycin-resistant enterococci (VRE) have caused worry in the worldwide infectious diseases community. Infection and nosocomial dissemination are important problems in hospitals, and drug-resistant enterococci that have colonized the intestines of patients are a major source of both (Regasa *et al.*, 2020).

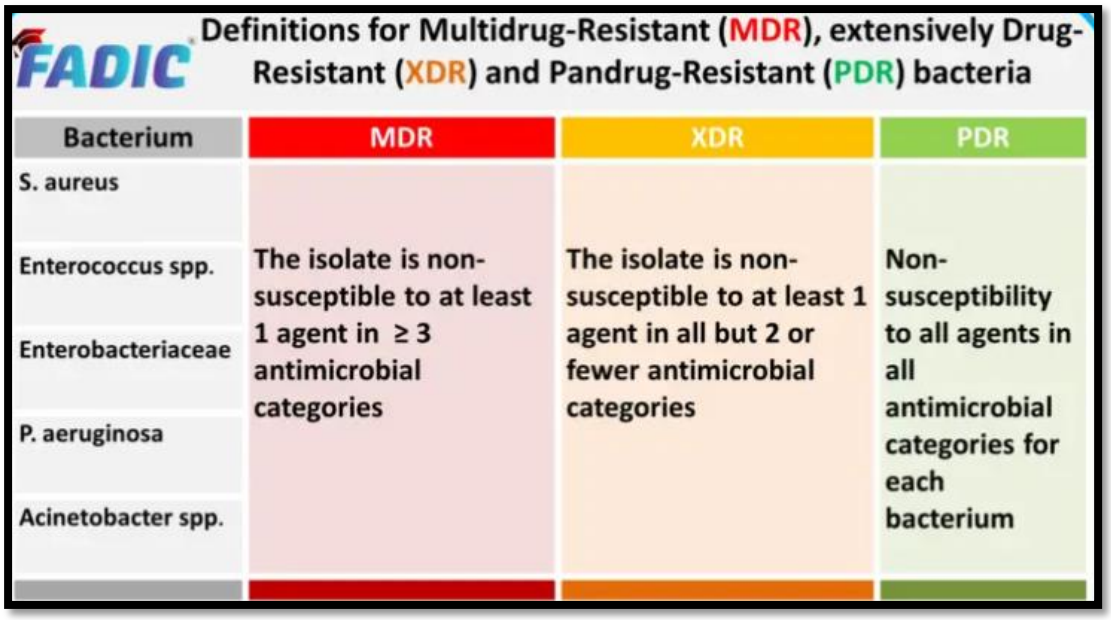

Figure (1-11): Comparison between the MDR, XDR, and PDR (Satlin & Walsh, 2017).

Since vancomycin is a potent antibiotic used to treat infections caused by Gram-positive bacteria, the emergence of vancomycin-resistant enterococci is a major public health problem. Clinical microbiology labs are urged to speciate enterococcal isolates from hospitals and test them for Vancomycin resistance since VRE have emerged as a major source of dangerous invasive infections across the world (Satlin & Walsh, 2017).

Because of insufficient response to therapy and rising resistance among enterococcal strains to β-lactam antibiotics, aminoglycosides, and glycopeptides, the death rate associated with enterococcal infections is disproportionately high. Constant monitoring and early identification of VRE and Minimum Inhibitory Concentrations are necessary due to the pandemic spread of Vancomycin-resistant enterococci (VRE) and the acquisition of resistance to newer antimicrobials (MIC). Penicillin, amoxicillin, and imipenim are all examples of beta-lactam antibiotics that

are effective against most strains of *Enterococcus* (Santajit & Indrawattana, 2016) (Figure 1-12).

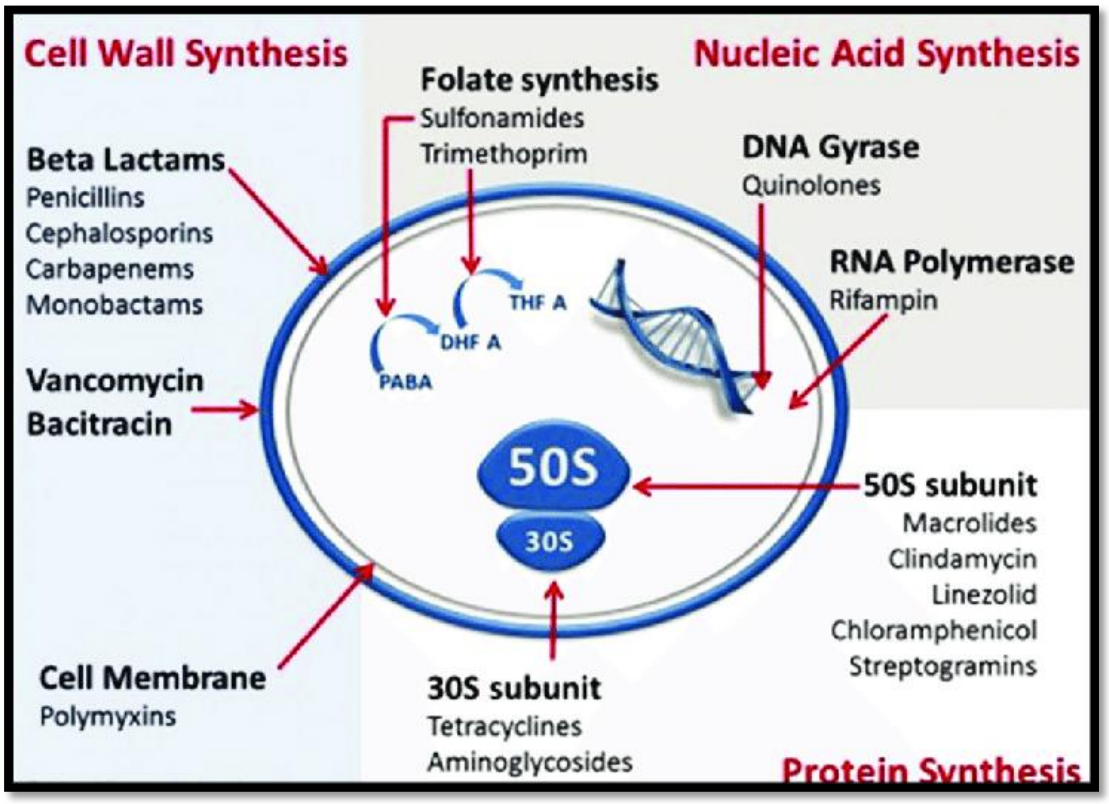

Figure (1-12): Mechanism of action of antibiotics (Santajit & Indrawattana, 2016).

Essential for supplying high-fidelity translation of genetic information, aminoglycoside antibiotics prevent the ribosome from carrying out translation, leading to cell death (Garofalo, 2017). Except for simple UTIs, aminoglycosides aren't often used as a stand-alone treatment for infections. For severe infections such septicemia, nosocomial respiratory tract infections, complex intra-abdominal infections, and enterococcal endocarditis, a combination of cell wall synthesis inhibitors and beta-lactam antibiotics is indicated (Morrill *et al.*, 2015).

The quinolones, a class of antibiotics that includes the drugs Ciprofloxacin, Ofloxacin, Levofloxacin, Trovafloxacin, and Grepafloxacin, inhibit DNA gyrase activity, stopping both DNA replication and transcription. The quinolone resistance is due to changes in the genes gyrA (gyrase) and qnrS (quinone oxidoreductase), since 44% of *E. faecalis* strains are susceptible to these drugs (topoisomerase). Since many enterococcal strains are resistant and the majority are just intermediately susceptible (Ezelarab *et al.*, 2018).

However, Cephalosporins antibiotics (such as Cefixime, Cefotaxime, and Ceftriaxone) are a poor option for therapy due to the high degree of resistance enterococci have to these drugs compared to Ampicillin and Penicillin (Maloo *et al*., 2017).

Antibiotics having beta-lactam action against *Enterococcus*, such as penicillin, amoxicillin, and imipenem, are often used with beta-lactamase inhibitors because they are effective against most *Enterococcus* strains. There are many different therapeutic uses for beta-lactam antibiotics, making them one of the most often prescribed medication groups. Their effects on *Enterococcus* spp. (Balsalobre *et al*., 2019).

1.2.10 *Enterococcus* genome

The first Vancomycin-resistant strain in the United States was isolated from *E. faecalis*, and its genome was analyzed in detail (Yang *et al*., 2015). More than a quarter of the genome was made up of MGEs such transposons, insertion sequence elements, genomic islands, and pathogenicity islands (Jiang *et al.*, 2019).

Genomic analysis reveals that enterococci have a remarkable capacity for acquiring and spreading mobile DNA, the vast majority of

whose components encode for multidrug resistance. The examination of the genome indicates the existence of sugar absorption systems and the encoding for many energy generation processes, including glycolysis and the pentose phosphate pathway. Because of this, enterococci are able to persist in the gastrointestinal system (Dobrindt *et al*., 2015).

The genomic size of an average *Enterococcus* organism is 3.20 Mb, and its average GC content is 37.99%. There are 605 genes in the core-genome, the vast majority of which are involved in cellular processes including glucose, protein, nucleic acid, and lipid metabolism. Comparative genome research of several *Enterococcus* strains from various origins demonstrates that environmental factors play a significant role in the development of *Enterococcus* species, with genetic similarities being more strongly shared across strains that originated in the same environment (Zhong *et al*., 2017). The topology of the time tree also revealed that humans and animals could have been the first hosts of enterococci, and that afterwards, other species of enterococci migrated from their former hosts to plants, birds, food, and other places (Dennehy, 2017).

Through genome comparison, we learned that commensal and pathogenic enterococci strains had different levels of genetic diversity. A complete genome analysis of *E. faecalis* isolated from a healthy infant revealed that this strain had adapted to a commensal lifestyle by retaining genes for the expression of gelatinase E (*gelE*), serine protease, and enterococcal surface protein while losing those responsible for producing some major virulence factors. The intricacy of explaining the genetic changes that separate commensal and pathogenic *E. faecalis* isolates was made clear by this study's investigation of this specific commensal's genome (Zischka *et al.*, 2015).

Enterococci may be more reliably and accurately identified to the genus and species level by molecular characterisation utilizing polymerase chain reaction (PCR) and sequencing. In place of phenotypic identification, multiplex PCR has been developed as a faster method for determining which species of enterococci are present. Currently, the most reliable method for identifying enterococci is by *16S rRNA* gene sequencing (Ganda *et al*., 2016). Better discriminative methods for separating enterococci into species may be found in their corresponding *16S rDNA*, RNA polymerase subunit beta (*rpoB*), and superoxide dismutase (*sodA*) genes (Savas *et al*., 2019).

Table (1-2): The Difference between *16s rRNA* and *16s rDNA*

16S RRNA	16S RDNA
A component of the small (30S) subunit of prokaryotic ribosome	The gene, which encodes the 16S rRNA in prokaryotes
Made up of RNA nucleotides	Made up of DNA nucleotides
Consists of four domains	Organized in an operon along with 23S and 5S rRNA genes
Takes parts in the binding to the Shine-Dalgarno sequence on mRNA to be translated	Undergo transcription in order to produce its gene product, which is the 16S rRNA
Facilitates the binding of small and large subunits by interacting with the 23S rRNA subunit	Important for the identification of prokaryotes

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1.2.11 Molecular aspects of *E. faecalis* virulence

1.2.11.1 Cytolysin gene

In the past, *E. faecalis* (back then known as *Streptococcus faecalis*) was thought to be pseudo-hemolytic because hemolytic activity was difficult to detect in liquid broth but was easily recognized on blood agar when exposed to the cytolysin toxin of *E. faecalis* (Stoneham *et al*., 2021).

Large pheromone-responsive plasmids or the Pathogenicity Island region of the chromosome may encode the cytolysin. *cylR1*, *cylR2*, *cylLL*, *cylLS*, *cylM*, *cylB*, *cylA*, and *cylI* are the byproducts of eight genes involved in its production. The genes for cytolysin toxin, maturation, and regulation are separated into two transcripts: a structural transcript and a regulatory transcript, cylR1R2. cylLL" and cylLS ", the active cytolysin subunits, are produced as non-identical peptides on the ribosome, changed after translation, secreted, and activated (Fiore *et al*., 2019).

Two proteins, cylR1 and cylR2, are responsible for repressing the cytolysin operon, whereas a quorum-sensing mechanism involving the secreted auto inducer cylLS is responsible for derepressing it. Other virulence factors, such as the aggregation substance and the enterococcal surface protein, esp, are linked to the cytolysin operon inside the *E. faecalis* pathogenicity island (Weaver, 2019).

As an added bonus, certain *E. faecalis* strains have the cytolysin operon encoded on mobile elements. Neither haemolytic toxins nor bacteriocins have ever combined their two functions into a single molecule before (Araya *et al*., 2022).

There are two overlapping promoters that control expression of the genes in the cytolysin cluster. In one transcriptional unit are the structural genes for the cytolysin subunits (cylLL and cylLS), the genes for post-translational modification and secretion (cylM, cylB, and cylA), and the gene for producing cell immunity (cylI), and in another transcriptional unit are the regulatory genes (cylR1 and cylR2) that are transcribed in the opposite direction (Fiore *et al*., 2019).

Pheromone-responsive plasmids carry the genes for the generation of cylA, a bacterial toxin produced by many strains of *E. faecalis* that causes lysis of horse, rabbit, and human blood cells. cylA is bactericidal against other Gram-positive bacteria and exhibits -haemolytic effects in humans. Large and small subunit oligopeptides, encoded by the genes *cyllL* and *cyllS*, respectively, make up the functional cytolysin toxin. CyllL, the larger component, has a higher affinity for the target cell membrane than the smaller cyllL and cyllS subunits, which compromise the membranes of the target cells and cause their lysis via the development of pores. In order to make the hemolytic toxin, the *cylA* gene first processes the peptides and then activates the other genes in the cytolysin operon (Das *et al*., 2020).

1.2.11.2 Surface Adhesions genes

The esp enterococcal gene, which codes for the high-molecular-weight surface protein Esp, has been found in considerable numbers in bacterial blood and heart valve infections but very rarely in healthy people's stool samples (Lee *et al*., 2020). Primary adhesion and biofilm development by *E. faecalis* on abiotic surfaces are aided by Esp. Infected root canals are often treated with calcium hydroxide, although *E. faecalis*

has been shown to build biofilms on medicated dentinal walls, which may help the bacteria survive the treatment (Taglialegna *et al*., 2020).

Antiserum from a patient with *E. faecalis* endocarditis was used to locate the efaA gene. When compared to the sequence of other streptococcal proteins, the related protein, EfaA, showed 55-60% homology to the adhesions. So it was proposed that *EfaA* has a role in endocarditis as an adhesion. *E. faecalis* strains often produce EfaA. Mutants with the *efaA* gene showed prolonged survival, compared with E. faecalis strains bearing no *efaA* gene, suggesting a role for the efaA gene in disease. The *efaA* gene was detected in all medical (blood, pus, urine, feces, hospital environment) and almost all food (milk, cheese, meat) isolates of *E. faecalis* (Najafi *et al.,* 2020).

One of the manganese transport systems in *E. faecalis* uses a solute-binding protein called EfaA as a receptor. EfaA is highly expressed in a manganese-ion-depleted environment, likely to regulate the cation's cytoplasmic homeostasis, despite the fact that manganese is essential for the development and survival of most microorganisms. The limited supply of and in dentin may promote EfaA *in vivo* expression (Colomer-Winter et al., 2018).

When the ace gene was deleted, *E. faecalis* was unable to interact to ECM proteins under certain conditions. Patients with enterococcal infections, and notably those with *E. faecalis* endocarditis, have been shown to have antibodies against Ace, suggesting that this gene is widely expressed *in vivo* during human infections by a variety of strains, and not simply at 46°C *in vitro* (Cao *et al.,* 2021).

1.2.11.3 Aggregation Substance gene

The *asa1* gene encodes the aggregation substance, a pheromone-inducible surface protein that may enhance cell aggregation and conjugation (Schmitt *et al*., 2020). Pheromone-inducible aggregation substance (asa1), expressed on a plasmid, and facilitates the conjugative transfer of sex pheromone gene-containing plasmids by causing one *Enterococcus* to clump with another (Molale, 2016).

Antibiotic resistance and the development of enterococcal infections may both involve the surface protein aggregation substance (asa1). *E. faecalis* sex pheromones are short, 7–8 amino acid peptides that stimulate the expression of the asa1 gene found on sex pheromone plasmids (Breuer *et al*., 2018).

Several roles for asa1 that might contribute to pathogenicity have been discovered. Host cell adhesion is an important role for asa1. Colon and duodenal, but not ileal, epithelial cells have been shown to have enhanced uptake of Asa1 through Asc10-mediated adhesion. In an ex vivo model of the colonic mucosa, Asa1 has been shown to promote invasion without affecting translocation (Chajcka-Wierzchowska *et al.*, 2017).

Asa1 has the typical N-terminal signal sequence and C-terminal LPXTG cell wall anchor motif seen in gram-positive surface proteins. The analysis of sequenced genes for related proteins indicates substantial conservation of their encoding sequences. The N-terminal half of Asa1 has 90% identity with its counterpart, with the exception of a variable region of 30–50% identity spanning amino acids 266–559. (Breuer *et al.*, 2018).

E. faecalis binds to human neutrophils by a complement receptor-mediated pathway that is independent of opsonin. As previously mentioned, Stpie-Pyniak *et al*. (2019) shown that a binding, E. faecalis-containing aggregating material is resistant to death by human neutrophils and promotes intracellular survival time in macrophages.

When compared to Enterococci without asa1 with neutrophils, the survival rate of un opsonized *E. faecalis* carrying asa1 following phagocytization by polymorph nuclear leuckocyte (PMNs) and macrophages was higher. They demonstrated that PMNs' inability to eradicate a*sa1+ E. faecalis* was not attributable to a deficiency in PMN activation but rather likely resulted from an alteration in phagosomal maturation (Fiore *et al*., 2019).

Analysis of the *asa1* gene revealed that the adhesion molecule had two RGD (arg/gly/asp) motifs, which are also found in fibronectin and mediate binding to integrin receptors on eukaryotic cells (Bin-Asif & Ali, 2019).

1.2.11.4 *E. faecalis* gelatinase and serine protease genes

gelE on the *E. faecalis* chromosome codes for gelatinase, a zinc-containing metalloproteinase that is released. Gelatinase is manufactured as a 509-amino-acid pre-properly peptide, and then its pre-sequence or signal sequence and pro-sequence are cleaved off at the amino-terminal end. Macrophages were discovered to respond to collagen peptides by releasing a cascade of inflammatory molecules, including hydrogen peroxide, superoxide anion, elastase, and gelatinase (Glennon-Alty *et al.*, 2018).

A virulence factor called serine protease (sprE) is hypothesized to contribute to systemic illness in mammals. *sprE*, a gene that encodes a serine protease, is located directly downstream of gelE and is co-transcribed with it. This gene produces a 26-kDa serine protease that is secreted and has similarity with *S. aureus* V8 protease (Yong, 2019).

This quorum-sensing mechanism is controlled by an auto inducing peptide termed gelatinase biosynthesis-activating pheromone, and it positively regulates the expression of pathogenicity-related extracellular proteases in *Enterococcus faecalis* (GBAP). GBAP is a lactone-linked 11-amino-acid cyclic peptide (Singh *et al.*, 2016).

Sequencing the areas downstream of *gelE*, the gene coding for the gelatinase, allowed the serine protease to be identified. Protein kinase E (gelE) and serine protease E (sprE) were shown to be cotranscribed in a Northern blot (Yousuf *et al*., 2018). The fsr (*E. faecalis* regulator) locus was discovered after sequencing the areas upstream of the *gelE* gene, and it has three open reading frames. Gelatinase and serine protease expression is controlled by the quorum-sensing Fsr system (He *et al.*, 2016). The gelatinase gene, *gelE*, is part of an operon with the serine protease gene, *sprE*. Direct and indirect harm to host tissues is caused by the action of these proteases, which also nourish bacteria with peptide nutrition (Ali *et al*., 2017).

The fsr quorum-sensing system positively regulates serinase and gelatinase via a gene called *sprE* that is situated next to the fsr genes and shares a promoter with them (Deepika & Bramhachari, 2018). The fsr locus, which is very comparable to the agr regulatory locus in *S. aureus*, positively regulates the transcription of the sprE- gelE operon at different stages of growth. Located upstream of the sprE operon is the fsr locus,

which contains the three regulatory genes *fsrA*, *fsrB*, and *fsrC*. Sequence similarity between *agrA* and *agrC* suggests that *fsrA* and *fsrC* make up a conventional two-component system, with fsrC serving as a histidine kinase sensor and *fsrA* as a response regulator. A glutamyl endopeptidase I of 25 kDa, encoded by the *sprE* gene, has been identified as a virulence factor of *Enterococcus faecalis*. This protease has a role in pathogenesis in a wide range of organismal models (including humans, animals, and plants) (Graham, 2017).

1.2.11.5 Endocarditis biofilm associated pilli gene

Surface-associated filamentous structures called pili (ebp) are thought to be crucial to *Enterococcus faecalis'* ability to form biofilms and cause endocarditis (La Rosa *et al*., 2016). The three components of an Ebp molecule are denoted by the letters ebpA, ebpB, and ebpC; ebpC is the main subunit of the pilus, ebpB is located at the pilus's base, and ebpA is located at the pilus's tip. The transcription of the three subunits occurs at the ebpABC locus and is positively controlled by ebpR, a transcriptional regulator that is encoded upstream of ebpABC (Floyd *et al*., 2017).

The length of the polymerized ebp pilus may approach 10 m, and it exists as high-molecular-weight polymers (>200 kDa). Although pilus expression is restricted to a small percentage of cells and may fluctuate with the passage of time, it is regulated by environmental cues such as serum, glucose, and bicarbonate. Attachment to host fibrinogen and collagen is mediated by ebpA, an adhesion protein found at the cell's tip (Afonina *et al.*, 2018).

A cell wall sorting signal (CWSS) is found at the C terminus of each pilus subunit. This signal is composed of a sortase recognition motif (Leu-Pro-Xaa-Thr-Gly or LPXTG-like peptide sequence), a hydrophobic

transmembrane domain, and a positively charged C-terminal tail. Sortase enzymes found in the pilus are essential for major pilin polymerization and minor pilin incorporation into the fiber. Only srtC is required for *E. faecalis* ebp pilus construction and integration of the ebpA and ebpB minor subunits, in contrast to other pilus systems that use numerous pilus-associated sortases with redundant and distinct roles (Yong, 2019).

Multiple disease models and biofilm experiments have shown the importance of cell surface proteins in *E. faecalis* pathogenicity. Important for virulence in animal models of ascending UTI in *E. faecalis* and *E. faecium*, and for infective endocarditis in *E. faecalis*, is the sortase-assembled endocarditis-and biofilm-associated pilus (ebp pilus). The minor component of these pili, ebpA, has been shown to mediate colonization of the bladder and intra bladder implants by *E. faecalis* (Yong, 2019).

Also, enterococcal-infected patients' sera react with pili on the surface of *E. faecalis* cells and recombinant ebp structural proteins, providing further evidence for an *in vivo* function for ebp pili in nosocomial enterococcal infection (Gao *et al.,* 2018). Numerous Gram-positive bacteria species have pili that are built by sortase, including the ebp pili. The interpilin isopeptide bonds that connect the structural components of these pili and their covalent connection to the peptidoglycan cell wall are both unique characteristics made possible by the biochemistry of the sortase transpeptidase enzymes (Ramirez *et al.,* 2020).

Patients with *E. faecalis* endocarditis have been shown to have antibodies against at least some of these proteins, proving that they are expressed in vivo during infection; most *E. faecalis* patient sera showed

especially high titers against 3 of these proteins, namely ebpA, ebpB, and ebpC (endocarditis and biofilm-associated pili) (Brown *et al*., 2021).

1.2.11.6 Sex pheromone-responsive plasmids (*cpd*) gene

Multiple plasmids in *E. faecalis* are sensitive to sex pheromones and encode bacteriocins, aggregation chemicals, and a wide variety of antibiotic resistance determinants (Rodrigues *et al*., 2019). Peptide pheromones (auto inducers) are carried through the ATP-binding cassette transport mechanism. These auto inducers accumulate in the extracellular environment, where their receivers may detect them and use this information to control mating and conjugation processes (Jaafar *et al*., 2022).

There was a significant amount of intricacy and a double role for pheromone response in plasmid conjugation and in the control of enterococcal virulence that was uncovered by studying its regulation and function. The stabilization/partition mechanisms, along with other functional modules found in pheromone plasmids, are essential for the plasmid molecule to be maintained reliably inside the host bacterium (Ferchichi *et al*., 2021).

The enterococcal phenotype is a major vector of antibiotic resistance in this species, and pheromone-responsive plasmids play a role in this. It was discovered that these plasmids included not just vanA and vanB, but also several additional resistant phenotypes. Both aggregation substance (AS) and cytolysin, two fundamental agents of enterococcal pathogenicity, are encoded by these genes. AS not only helps enterococci cling to human tissues during infection, but it also plays a role in mating-pair formation during conjugation. Cytolysin, the second protein, may

cleave red blood cells and aid in the invasion of eukaryotic cells (Erickson *et al*., 2020).

The *cpd* gene encodes a peptide pheromone that is produced by recipient enterococcal cells to activate the conjugative apparatus of donor enterococcal cells. The pheromone-responsive plasmids they mediate may include virulence genes that aid in biofilm formation or regulate biofilm development (Ferchichi *et al*., 2021).

Because the particular mechanism or processes by which a plasmid limits synthesis of its pheromone remain unclear, the event has been dubbed "pheromone shutdown." However, the elaboration of pheromones specific for other plasmids continues unabated. Another peptide, dubbed inhibitor, is secreted by plasmid-containing cells; it acts as a competitive inhibitor of the related pheromone (Lassinantti *et al*., 2021).

1.2.12 Immunity

In order to induce infection, *E. faecalis* must first avoid being eliminated by the host's clearance mechanisms. PMNs, or polymorph nuclear leukocytes, play an important role in the immune response of humans against bacterial infections (Kao & Kline, 2019). Invading germs may be engulfed and destroyed by PMNs if they are first coated with complement proteins or particular antibodies. Opsonization is the process of covering bacteria with complement proteins or antibodies to improve phagocytosis (Weiss & Schaible, 2015).

When studying the phagocytic death of enterococci, researchers found that PMN mediated killing was reliant on complement activation through either the classical or alternative route, rather than antibodies (Ali *et al.*, 2019). Antibodies against *E. faecalis* improved PMN-mediated death, but

they were not necessary, since many trials demonstrated effective killing even in the absence of gamma globulins in the serum (Zeng *et al*., 2016).

In order to colonize and remain inside a host, pathogens must be able to tolerate, alter, or escape immune-mediated clearance processes. *E. faecalis* may avoid being eaten by the host's immune cells by forming biofilms, and it can hide out within macrophages and neutrophils for long periods of time (Goh *et al*., 2017). Cells in mammals use pattern recognition receptors (PRRs) to identify PAMPs, which then activate nuclear factor kappa B (NF-B)-dependent host defenses. Some of the genes regulated by NF-B include those encoding cytokines and chemokines, which are involved in the recruitment and activation of immune cells in response to infection (Zhu *et al*., 2018).

The infection of macrophages with *E. faecalis* at a low multiplicity of infection (MOI = 10) activates mitogen-activated protein kinases (MAPKs) and NF-B, which in turn causes the production of pro-inflammatory cytokines (Zhao *et al*., 2019). Some *E. faecalis* strains isolated from the intestines of healthy human neonates, however, are able to inhibit MAPK and NF-B signaling and interleukin-8 (IL-8) production in intestinal epithelial cells in vitro (Li *et al*., 2020).

Aggregation substance (AS), gelatinase, and TcpF are only a few of the *E. faecalis* virulence factors that modify immunity during infection (Chan *et al.*, 2019). TcpF, which contains the Toll-interleukin-1 receptor (TIR) domain, blocks the TLR-MyD88 interactions that are itself mediated by the MyD88 TIR domain. To conclude, expressing TcpF from *E. faecalis* inhibits NF-kappaB p65 translocation in RAW macrophages (Carlsson *et al*., 2016). AS expressed on a plasmid stimulates phagocytosis and internalization by macrophages via contact with

complement receptor type 3. When within the body, AS increases macrophage survival through promoting resistance to death by superoxide. The cleavage of complement components C3, C3a, and C5 by the secreted quorum-responsive gene product gelatinase inhibits opsonization and reduces neutrophil recruitment, all of which contribute to innate immune evasion (Bhunia, 2018).

The cellular response to *E. faecalis* infection in urinary tract infection (UTI) is mostly monocytic and does not need TLR2. In the bladder, neutrophils and monocyte-derived cells are activated into a robust pro-inflammatory response just by the presence of a urinary catheter (Tien *et al.*, 2017). Despite the robust inflammatory response elicited by catheterization, *E. faecalis* infection of catheterized bladders leads to the establishment of high-titer catheter-associated biofilms and bladder infection (Acharya, 2019). IL-23, or interleukin-23, is a cytokine whose discovery has far-reaching consequences for our understanding of chronic inflammation and autoimmune. The cytokine IL-23 is a member of the IL-12 family, which is itself a subset of the IL-6 superfamily. To present, four sequence-identical heterodimeric cytokines have been identified as members of the IL-12 family (Druszczyska *et al.*, 2022). The interleukin (IL)-23 protein is similar to the interleukin (IL-6) and granulocyte colony-stimulating factor (G-CSF). To form the IL-23 receptor (IL-23R), IL-12R 1 is fused with a separate chain, IL-23R that is structurally similar to IL-6gp130. A major function for STAT3 is shared both IL-12 and IL-23's signaling pathways (Ushach, 2015).

Antigen presentation by DCs is stimulated by IL-23, and Th17 cells are differentiated from T cells and interferon-gama (IFN-Ɣ) is produced. By directly affecting macrophages, IL-23 also functions as a terminal effector cytokine. That's consistent with an IL-23 autocrine loop acting on

macrophages, at least in part. Also, peritoneal macrophages express mRNA for IL-1 and IFN-ϒ after receiving intraperitoneal dose of recombinant IL-23 (Li *et al.*, 2019). A key function for IL-23 in autoimmune illness was first indicated by studies of central nervous system autoimmunity. Both the central nervous system and inflammatory macrophages contained IL-23. Consequently, it was shown that myeloid cells in addition to T cells express their receptor, IL-23R (Bridgewood *et al.*, 2020).

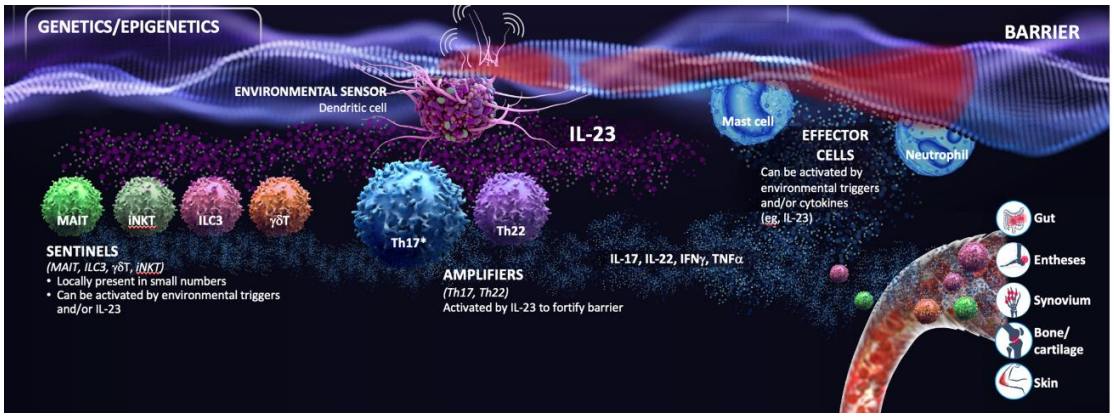

Figure (1-13): Cytokines across the autoimmune axis (Seledtsov *et al.*, 2015)

Interleukin-23 merits close study because of its crucial function in chronic inflammation and its efficient inhibition. To accurately interpret findings obtained employing inhibitors of varying specificities, it is crucial to keep in mind the method in which IL-23 interacts with cells (including its heterodimeric structure). To treat chronic inflammatory joint disease, IL-23 is one of the most promising targets (Zhong *et al.*, 2021). Chronic and acute inflammatory responses are induced by pro-inflammatory cytokines such IL-1, IL-18, IL-17, IL-6, and IFN-ϒ, all of which have a role in autoimmunity by acting on the innate or cognate stages of the process. All of these cytokines are now being studied as possible targets for autoimmune disorders (Seledtsov *et al.*, 2015).

Chapter Two

Materials and Methods

2.1 Materials

2.1.1 Laboratory Instruments and Equipment

The main scientific apparatus, and technical instruments with disposable materials were listed in Table (2-1) and Table (2-2).

Table (2-1): Laboratory instruments and equipment

Instruments	Company	Country
Applied Biosystems™ ProFlex™ PCR System	Fisher Scientific	USA
Autoclave	Herayama	Japan
Bensen burner	Satorins	Germany
Cabinet hood	BioLAB	Korea
Centrifuge	Hettich	Germany
Cooling box	Ningbo	China
Digital camera	Samsung	Japan
Distillator	GFL	Germany
Electrophoreses	Clarivate	UK
Hood	Labogene	Danemark
Light microscope, digital camera	Olympus	Japan
Micropipettes 5-50 µl, 100-1000 µl, 0.5 – 10 µl, 2.20 µl.	Eppendorf	Germany
Microwave oven	Memmert	Germany
Nano drop	Memmert	Germany
Platinum wire loop	Himedia	India
Power Supply	Biorad	USA
Refrigerator	Al Balsan	Turkey
Sensitive electron balance	A & D	Japan
Thermostatic Incubator	Zxinstrument	Chain
UV- transilluminator PCR	Vilber Lourmat Sté	Farance
Vortex	Fisher Scientific	USA

Table (2-2): Technical Instruments and Disposable Materials

Item	Company	Country
96.well flat bottomed polystyrene micro plate	Spektar	Serbia
Glass slides	Sail brand	China
Medical cotton, Medical gloves, Para film	Medicare Hygiene Limited	India
Screw capped bottles (30ml), Beakers	Hirschmann	Germany
Microscopic Cover slide	Gitoglas	China
Millipore filters (0.45mm)	Sigem	Spain
PCR Eppendrof tubes 1.5ml, PCR tubes Eppendrof 200µl	Biobasic	Canada
Wooden sticks, Pettri dishes, Sterile swabs	Blastilab	Lebanon
Plastic test tubes 10ml, Syringes	Dolphin	Syria
Tips (Different sizes)	Jippo	Japan

2.1.2 Chemical and Biological Materials

2.1.2.1 Chemical Materials

The chemical material used in this study were listed in Table (2-3).

Table (2-3) Chemical Materials

N0.	Chemicals	Company/country
1.	NaCl, Na_2HPO_4 , KH_2PO_4 , NH_4Cl, $MgSO_4$, $CaCl_2$, NaOH, phosphate buffer	Merk Darmstadt/ Germany
2.	Gelatin, Barium chloride, Sulfonic acid	B.D.H / England
3.	Hydrogen peroxide	Fluka /England
4.	Nuclease free water (1.25) ml	Promega(USA)
5.	Voges-Proskauer reagent	England
6.	Glycerol	Fluka /England
7.	Alcohol (Ethanol) 70% and 95%.	Fluka/ Germany
8.	Gram Stain kit	Crescent/ KSA
9.	Agarose	Carl Roth/Germany
10.	Tris-Borate-EDTA (TBEx10) buffer	Bio Basic/ Canada
11.	Tris EDTA (TE)	Bio basic Canada
12.	Catalase reagent	Schuchariot/ Germany
13	Oxidase reagent	Himedia / India
14.	Red safe staining solution	Intron / Korea

2.1.2.2 Biological materials

2.1.2.2.1 Culture media

Several types of culture media are used in the current study listed in Table (2-4). All media were prepared according to the manufactures instructions, after sterilized by autoclaving (121C˚-15 pound /inch² for 15 min). Culture media incubation was done for (24) hrs. After pouring it in sterile plates. It was done to avoid any contamination if present, and then stored at (4) C˚ until use.

Table (2-4) Culture media used in identification and isolation of *Enterococcus faecalis*.

No.	Media	Purpose	Company/Origin
1.	Blood agar medium	Enrichment medium	Accumix^{TM}/India
2.	MacConkey agar medium	Differential medium	Accumix^{TM}/India
3.	Brain heart infusion agar	Activation medium	BIOMARK/India
4.	Brain heart infusion broth	Activation medium	HIMEDIA/India
5.	Nutrient agar medium	Cultivation medium	HIMEDIA/India
6.	Nutrient broth	Grow and preserve bacterial isolates	Accumix^{TM}/India
7.	CHROM Agar Medium	Selective medium	HIMEDIA/India
8.	Urea agar medium	Ability of bacteria to produce urease enzyme	HIMEDIA/India
9.	M_9 agar medium	Ability of bacteria to protolytic activity	HIMEDIA/India
10.	Motility agar medium	Ability of bacteria to motility	HIMEDIA/India
11.	Brain heart infusion-glycerol agar medium	preserve bacterial isolates	AccumixTM/India
12.	Tween 80 agar medium	Ability of bacteria to lipase production	AccumixTM/India
13.	Bile esculin sodium	used for rapid detection of Esculin hydrolysis	HIMEDIA/India
14.	Columbia Agar Base	Used for Hydrophobicity test	OXOID, Hampshire UK)
15.	gelatin medium	Used for gelatinase production	HIMEDIA/India

2.1.3 Molecular materials

Table (2-5): Commercial kits used in the present study

Type of kits	Company/country
DNA extraction kit	Geneaid / Korea
Green master mix 2X Kit	Promega/ USA
DNA ladder 100bp-1500bp	South Africa
Human IL-23 ELISA Kit	Elabscience/ China
6X Loading dye	Intron / Korea

Table (2-6): DNA extraction kit (Geneaid / Korea)

DNA extraction kit
Materials: GT Buffer 30 ml GB Buffer 40ml W1 Buffer 45 ml Wash Buffer 25 ml+100 ml Ethanol Elution Buffer 30ml In addition to proteinase K, absolute ethanol and RNase A The kit contains were stored at 22.25ºC
DNA ladder
Materials: 1. Ladder consist of 11 double stranded DNA with size 100.1500 bp.

Table (2-7): Master Mix Used in PCR (promega/USA)

Materials
1. DNA polymerase enzyme (Taq) **2.** dNTPs (400 µm dATP, 400 µm dGTP, 400 µm dCTP, 400 µm dTTP) **3.** MgCl2 (3mM) **4.** Reaction buffer (pH 8.3)

2.1.4 Antibiotics Disks

Antibiotic disks were listed in Table (2-8).

Table (2-8) Antibiotics Disks (Bioanalyze / Turkey)

No.	Antibiotics	Sync	Potency (µg per disk)
1.	Ciprofloxacin	CIP	5µg
2.	Erythromycin	ERT	15µg
3.	Gentamicin	CN	10µg
4.	levofloxacin	LEV	30µg
5.	Linezolid	LIN	30µg
6.	Nitrofurantoin	NIT	300µg
7.	Norfloxacin	NOR	10µg
8.	Synercid	SYN	15µg
9.	Teicoplanin	TEC	30µg
10.	Vancomycin	VAN	30µg

2.2 Methods

2.2.1 Patients

This study were included 200 infected patients, with age range (8-56 years old). The samples were collected from urine, vagina and blood, from patients who were visited to Al-Karama Hospital and Medical City Hospital in the Capital, Baghdad, during a period of three months from May to end of July, 2022.

2.2.2 Ethical approval

The necessary ethical approval from ethical committee of the hospitals and patients and their followers must obtained. Moreover, all subjects involved in this work are verbal informed and the agreement required for doing the experiments and publication of this work are obtained from each one prior the collection of samples.

2.2.3 Collection of samples

The proper samples collected for bacteriological analysis. Those specimens were collected in proper ways to avoid any possible contamination.

2.2.3.1 Urine samples

The specimen were generally collected from patients suffering from UTIs. Mid-stream urine samples were collected in sterilized screw-cap containers, then the urine samples were inoculated on selective media (CHROM agar) and incubated aerobically at 37°C for 24h (Ali, 2017).

2.2.3.2 Vaginal swabs

The samples were generally collected from women (pregnant and non-pregnant) suffering from vaginitis. The swabs were inserted into the posterior fornix, upper part of the vagina and rotated there before withdrawing them. A vaginal speculum was also used to provide a clear sight of the cervix and the swabs were rubbed in and around the introits of the cervix and withdrawn without contamination of the vaginal wall. Swab for culture should be placed in tubes containing normal saline to maintain the swab moist until taken to laboratory. The swab was inoculated on selective media (CHROM agar) and incubated aerobically at 37°C for 24 hrs. (Ames *et al*., 1983).

2.2.3.3 Blood samples

Blood sample was collected from all 200 infected patients and 44 healthy patients as control. 5ml of fresh venous blood samples were putted in gel containing tubes was allowed to clot at room temperature for 30 minutes and then centrifuged at 3000×g for approximately 20 minutes. Then the sera were obtained and stored at (-20˚C) till it would be used (Ali, 2017).

2.2.4 Study protocol

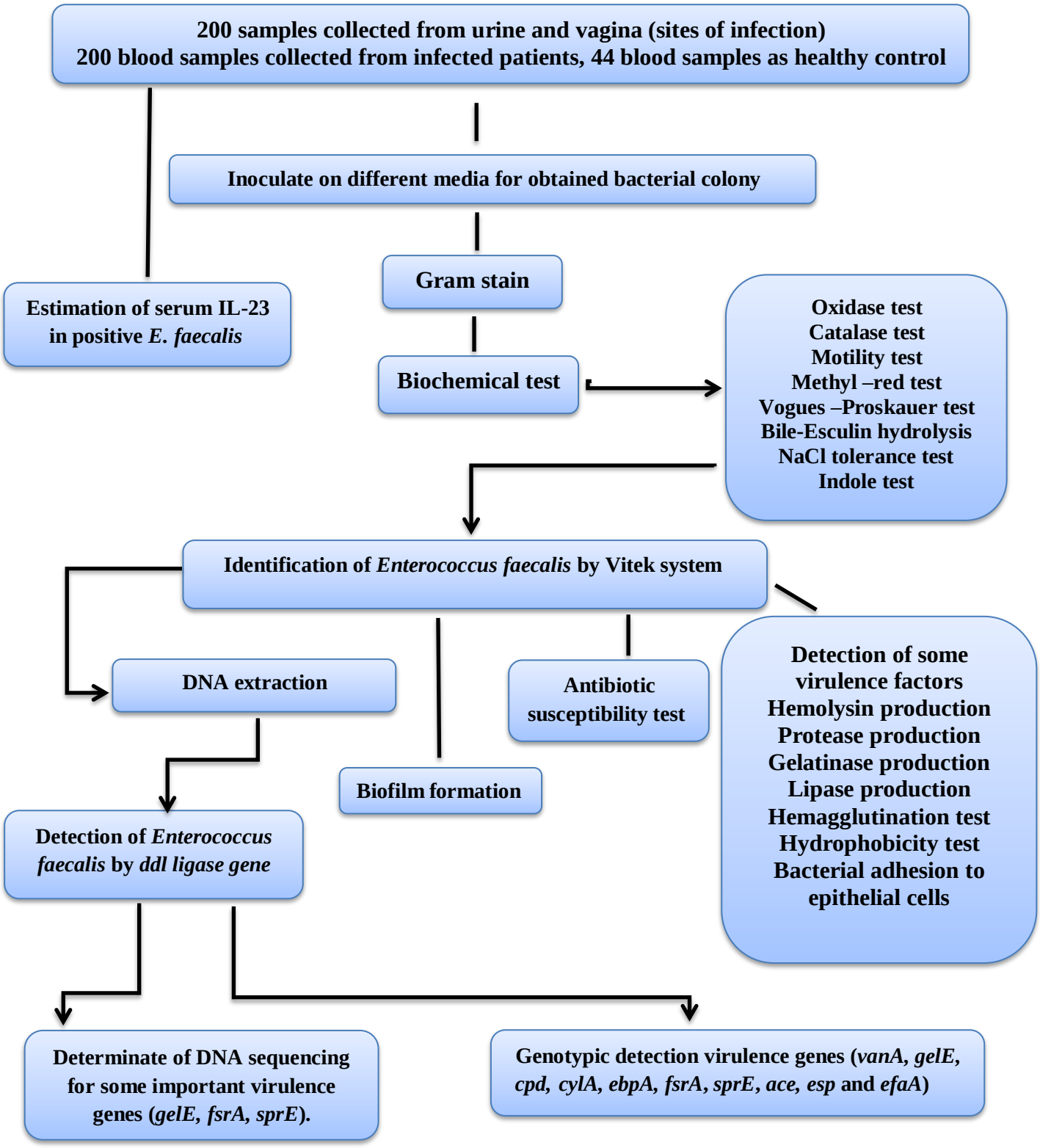

Figure (2-1): Scheme of study protocol

2.3 Preparation of Stains, Solutions, Buffers and Reagents

The stains, solutions, buffers and reagents were used in this study are listed in Table (2-9).

Table (2-9): The stains, solution and reagents used in this study

No.	Subject	Purpose	Reference
1.	Agarose Gel	Gel electrophoresis	Sambrook and Rusell (2001)
2.	Catalase reagent	used to identify bacterial ability to produce catalase enzyme	McFadden, (2000)
3.	EDTA Solution	DNA extraction	Sambrook and Rusell (2001)
4.	Gram's stain solutions	Morphology and its arrangement of bacterial cells	Forbes *et al*., (2007)
5.	Hydrogen peroxide solution (H_2O_2)	Detection of ability of bacteria to produce catalase	Collee *et al*., (2006)
6.	Kovacs reagents	to detect the indole production	McFadden, (2000)
7.	McFarland Standard Solution	results in turbidity approximately equal to bacterial cells density of 1.5× 108 cell/ml	Baron *et al*., (1994)
8.	Methyl red reagent	It was used to detect complete glucose hydrolysis	McFadden, (2000)
9.	One X TBE Buffer	DNA extraction	Sambrook and Rusell (2001)
10.	Oxidase reagent	used as an indicator in oxidase test	McFadden, (2000)
11.	Trichloroacetic Acid (TCA) Solution	Used in extracellular protease production test	Piret *et al*., (1983)
12.	Tris-borate EDTA Buffer	DNA extraction	Sambrook and Rusell (2001)
13.	Voges – Proskauer reagent	It was used to detect partial glucose hydrolysis	Collee *et al*., (1996)

2.4 Identification of *Enterococcus faecalis*

According to (Collee *et al*. 1996 and McFadden, 2000), the isolation and identification of *E. faecalis* were performed as follows:

2.4.1 Morphological tests

2.4.1.1Colonies morphology and microscopic examination

A single colony was taken from each primary positive culture. Its identification depended on the morphology properties (colony size, shape, colour, and translucency, edge, and elevation of texture). The colonies were then investigated by gram stain to observe bacterial cells. Specific biochemical tests and Vitek system were done to reach the final identification.

2.4.2 Biochemical tests

The biochemical tests used to identify *E. faecalis* were listed in Table (2-10).

Table (2-10): The biochemical tests used to identify *E. faecalis*.

No.	biochemical test	Purpose	Reference
1.	Bile-Esculin Hydrolysis Test	It is used for rapid detection of Esculin hydrolysis	McFadden, (2000)
2.	Catalase test	Ability of *E. faecalis* to produce catalase enzyme	Winstedt *et al*., (2000)
3.	Growth at 10ºC and 45ºC	Ability of *E. faecalis* to growth at 10ºC and 45ºC	McFadden, (2000)
4.	Growth at alkaline pH (9.6)	Ability of *E. faecalis* to growth at alkaline pH (9.6)	McFadden, (2000)
5.	Motility test	Ability of *E. faecalis* to motile	McFadden, (2000)
6.	NaCl Tolerance Test	To check the ability of *E. faecalis* to tolerate 6.5% NaCl	Collee *et al*., (1996)
7.	Oxidase test	Ability of *E. faecalis* to produce oxidase enzyme	Winstedt *et al*., (2000)
8.	Sugar fermentation test	Ability of *E. faecalis* to ferment different types of carbohydrate	McFadden, (2000)
9.	Voges –Proskauer test	Ability of *E. faecalis* to partial hydrolysis of glucose	McFadden, (2000)

2.5 Identification of *E. faecalis* by GP-ID with VITEK-2 Compact

This system consists of personal computer, reader/incubator made up of multiple internal components including: card cassette, card filler mechanism, cassette loading processing mechanism, card sealer, bar code reader, cassette carousel and incubator, in addition to transmittance optics, waste processing, instruments control electronics and firmware.

The system was equipped with an extended identification database for all routine identification tests that provide an improved efficiency in microbial diagnosis, which reduces the need to perform any additional tests, so that safety for both test and user will be improved. All the following steps are prepared according to the manufacturer's instructions. Three ml of normal saline were placed in plane test tube and inoculated with a lopefull-isolated colony. Insert the test tube into dens check machine for standardization of colony to McFarland is standard solution (1.5×10^8 cell/ml) (Figure 2-2). The standardized inoculums were placed into the cassette and a sample identification number entered into the computer software via barcode. The VITEK-2 card type then is read from barcode placed on the card during manufacture, and the card, thus, connected to the sample ID number. Then the cassette was placed in the filler module. When the cards were filled, the cassette was transferred to the reader/ incubator module. All subsequent steps were handled by the instrument, the instrument; controls the incubation temperature, optical reading of the cards and continually monitors and transfers test data to the computer for analysis.

2.5.1 Inoculum Preparation

Suspension was prepared according to the manufacturer's recommendations of BioMérieux Company by transferring sufficient number of colonies from overnight pure culture by swab and suspending

the microorganism in 3.0ml of sterile saline in a (12 x 75) mm clear plastic (polystyrene) test tube. The turbidity was adjusted to become equivalent to a McFarland No. (0.5) using a turbidity meter called Densi Chek. The same suspension was used in antibiogram testing with VITEK-2 compact system.

MFU	Approximate cell density
0.5 MFU	1. 5x10^8 Cells/ ml
1 MFU	3.0 x 10^8 Cells/ ml
2 MFU	6.0 x 10^8 Cells/ ml
3 MFU	9.0 x 10^8 Cells/ ml
4 MFU	12 x 10^8 Cells/ ml
5 MFU	15 x 10^8 Cells/ ml
6 MFU	18 x 10^8 Cells/ ml
7 MFU	21 x 10^8 Cells/ ml
8 MFU	24 x 10^8 Cells/ ml
9 MFU	27 x 10^8 Cells/ ml
10 MFU	30 x 10^8 Cells/ ml

*MFU- McFarland Standard

Figure (2-2): McFarland standard scale value (McFadden, 2000)

2.6 Detection of virulence factors

2.6.1 Hemolysin production

Hemolysin production was carried out by inoculating bacterial isolate on blood agar medium at (37˚C) for (24-48) hrs. An appearance of clear zone around the colonies referred to complete hemolysis (β-hemolysis), greenish zone around the colonies referred to partial hemolysis (α-hemolysis), while no zone referred to non-hemolysis (γ- hemolysis) (Baron *et al.*, 1994).

2.6.2 Gelatinase production

Inoculate the tubes of gelatin medium with bacteria by stabbing, and then the tubes were incubated at 37°C for 24 hrs. This test was performed to demonstrate the ability of bacteria to hydrolyze gelatin (Baron *et al*., 1994).

2.6.3 Extracellular protease production

M_9 media was used for the detection of protease enzyme. After sterilization in autoclave and cooling at (50°C), and (0.25) gm/L glucose (sterilized by filtration) was added, the medium was then supplemented by (1%) casein. Pores was made in agar medium and inoculation of this media with (20µl) from bacterial broth in each pores and incubation at (37°C) for (24) hrs; (3ml) of (5%) trichloroacetic acid was added to precipitate the protein. The formation of transparent area around the colony indicated a positive result (Piret *et al.*, 1983).

2.6.4 Lipase production

According to Collee *et al*., (1996), isolates were streaked individually on tween 80 agar (1%). After a week of incubation at 37∘C, lipase producing isolates form an opaque precipitation zones.

2.6.5 Haemagglutination test

A slide method was adapted for detection of erythrocytes clumping by bacterial fimbriae as described by Mayumi *et al*. (1971). The test was done using human blood (type "O"). After three times of washing steps of red blood cells with saline, 3% RBCs suspension in fresh saline was prepared. A drop of this suspension was added to one drop of the tested bacterial culture. Then the slide was rolled for 5 min at room temperature. Clumping was considered as a positive haemagglutination result.

2.6.6 Bacterial adhesion

One of the key virulence qualities of E. *faecalis* is their capacity to attach to epithelial cells, which may be identified in the following ways; Incubate the brain-heart infusion broth in a non-oxygenated environment for seventy-two hours. Use phosphate buffer (PBS) to dilute bacterial broth, and then measure out 1.5 x 10^8 CFU/ml. Urine epithelial cells were obtained by collecting a urine sample, centrifuging it, and then transferred the sediment to a sterile tube containing phosphate-buffered saline (pH 7). The epithelial cells were washed three times with PBS while being centrifuged at 5000 rpm for 10 minutes. Using filter paper, and removed the epithelial cells from the PBS, and then transfered them to a cover slide by pressing the cover into the filter paper and lifting it when dry. It was Putted the epithelial cells and the bacterial broth on a sterile glass plate, apply the cover slides, and putted the plate in an incubator at 37 degrees Celsius for an hour. It was Scrubed the cover slides with phosphate-buffered saline (PBS) to killed any germs that didn't stick. Seventeen minutes of ethanol fixation ensured that the epithelial cells would remained in place. To stain the cover slides, it was soaked them in Giemsa stain (30%) for twenty minutes, and washed them in D.W., and let them to dry in the air. Tested the cover slides under a light microscope by placed them on glass slides in an upside-down orientation (Furumura *et al.,* 2006).

2.6.7 Hydrophobicity test

The hydrophobicity of cell surfaces was evaluated using a technique developed by Dec and colleagues, (2016). Subcultures of each isolate were grown at 37 degrees Celsius in 5% defibrinated blood on Columbia Agar Base. Once the 24-hour culture was ready, it was collected and

suspended in 5 ml of 0.9% NaCl at an OD600 of 0.8-1.0. (A0). After adding xylene (1.7ml) to glass test tubes, the liquids were violently vortexed for 90 seconds. After a period of phase separation (about 15 minutes), the optical density of the aqueous phase (A) was remeasured and compared to the starting value. Hydrophobicity (%) of the xylene-adhering strain's cell surface was determined using the formula: %H = [(A0 A)/A0] 100. Hydrophobic strains were defined as those having a hydrophobicity of 50% or above.

2.7 Biofilm production: Tissue culture plate method (TCP)

The TCP assay described by (Christensen *et al*, 1985) is most widely used and was considered as a standard test for detection of biofilm formation.

In the present study, all isolates were screened for their ability to form biofilm by TCP method as described by (Christensen *et al*, 1985) with a modification in duration of incubation which was extended to (24) hours according to (Mathur and Mathur, 2006).

Isolates from fresh agar plates were inoculated in tryptic soy broth and incubated for (18 hrs.) at (37°C) in stationary condition and diluted (1) in (100) with fresh medium. Individual wells of sterile polystyrene (96) well-flat bottom tissue culture plates were filled with (0.2 ml) aliquots of the diluted cultures and only broth served as control to check sterility and non-specific binding of media. The tissue culture plates were incubated for (18 hours) and (24 hours) at (37°C).

After incubation, content of each well was gently removed by tapping the plates. The wells were washed four times with (0.2 ml) of phosphate buffer saline (pH 7.2) to remove free-floating ‘planktonic’ bacteria. Biofilms formed by adherent ‘sessile’ organisms in plate were fixed with

sodium acetate (2%) and stained with crystal violet (0.1% w/v). Excess stain was rinsed off by thorough washing with deionized water and plates were kept for drying. Adherent bacterial cells usually formed biofilm on all side wells and were uniformly stained with crystal violet. Optical density (OD) of stained adherent bacteria was determined with a micro ELISA auto reader at wavelength of 570 nm ($OD_{570\ nm}$).

Experiment was performed in triplicate and repeated three times, the data was then averaged, and the results were interpreted according to Table (2-11) (Mathur and Mathur, 2006).

Table (2-11) Classification of bacterial adherence by TCP method

Mean of OD value at 630nm	Biofilm formation
$<OD_C$	Non-adherent
$OD_C < OD \leq 2 \times OD_C$	weakly adherent
$2 \times OD_C < OD \leq 4 \times OD_C$	Moderately adherent
$4 \times OD_C < OD$	Strongly adherent

2.8 The Diffusion of Antibiotics Test (the Kirby-Bauer susceptibility test) (DDT)

1. A pure culture of a bacterium that has been previously identified was used.
2. The inoculum for this test was made by adding the growth of five isolated colonies cultured on brain heart infusion plates to five milliliters of BHI broth and incubating the mixture for two hours to create a bacterial suspension of moderate turbidity.
3. Inoculum was taken from the normal culture using a sterile swab, and the inoculum was afterwards transferred onto a Mueller-Hinton plate and allowed to dry.

4. Using flamed forceps or a disc applicator, antibiotic discs were equally spaced over the surface of the medium before being incubated at 37 degrees Celsius, often overnight.
5. Measurements of the inhibition zones of the antibiotics were compared to the CLSI-2021 standard inhibition zones to establish the organism's sensitivity or resistance to the antibiotics (CLSI, 2021).

2.9 Molecular study

2.9.1 DNA Extraction

This method was made according to the genomic DNA purification Kit supplemented by the manufacturing company Geneaid, (Korea).

Chromosomal DNAs obtained were used as templates for all PCR experiment. The PCR reaction were carried out in a thermal cycler. Before PCR assay, DNA profile was performed by using bacterial DNA and loading buffer without thermal cycling condition, and according to the following step:

1. Cultured bacterial cells were transferred to 1.5µl micro-centrifuge tube, centrifuged for 1 minutes at 14-16,000 rpm and the supernatant was discarded.
2. A volume of 200µl of Gram Buffer was added to 1.5µl micro centrifuge tube then 200µl of lysozyme buffer was added to the Gram Buffer then vortex to completely dissolve the Lysozyme.
3. A volume of 200µl of Gram Buffer in the 1.5µl micro-centrifuge tube, incubated at 37ºC for 30 minutes. During incubation the tube was inverted every 10 minutes.
4. A volume of 20µl of proteinase K was added then mixed by vortex, incubated at 60ºC for at least 10 minutes. During incubation the tube was inverted every 3 minutes.

5. A volume of 200µl of GB Buffer was added to the sample and mix by vortex for 10 minutes.
6. The sample lysate was incubated at 70ºC for at least 10 minutes. During incubation, the tube was inverted every 3 minutes. At this time, the required Elution Buffer (200µl per sample) was pre heated to 70ºC (for step 5 DNA Elution).
7. Following 70ºC incubation, 5µl of RNase A (10mg ml) was added to the clear lysate and mixed by shaking vigorously.
8. The lysate was incubated at room temperature for 5 minutes.
9. A volume of 200µl of absolute ethanol was added to the clear lysate and immediately mixed by shaking vigorously, the precipitate was broken up by pipetting.
10. A GD Column was placed in a 2µl collection tube.
11. All of the mixture was transferred (including any precipitate) to the GD column, centrifuged at 14000-16000 rpm for 2 minutes.
12. The 2µl collection tube was discarded containing the flow through and the GD column was placed in a new 2 ml collection tube.
13. A volume of 400µl of W1 buffer was added to the GD Column, Centrifuged at 14000-16000 rpm for 30 second.
14. The flow through was discarded and placed the GD column back in the 2ml collection tube.
15. A volume of 600µl of wash buffer (ethanol added) was added to the GD column, centrifuged at 14000-16000 rpm for 30 seconds.
16. The Flow through was discarded and placed the GD column back in the 2µl collection tube, Centrifuged again for 3 minutes at 14000-16000 rpm to dry the column matrix.
17. The dried GD column was transferred to a clean 1.5 µl centrifuge tube.

18. A volume of 100µl of preheated elution buffer or TE was added to the center of the matrix, centrifuged at 14000-16000 rpm for 30 second to elute the purified DNA.

2.9.2 Estimation of DNA Concentration

The extracted genomic DNA is checked by using Nano drop spectrophotometer (scan drop, Germany) measures DNA concentration (ng/µl) and checks the DNA purity by reading the absorbance at (260 /280 nm).

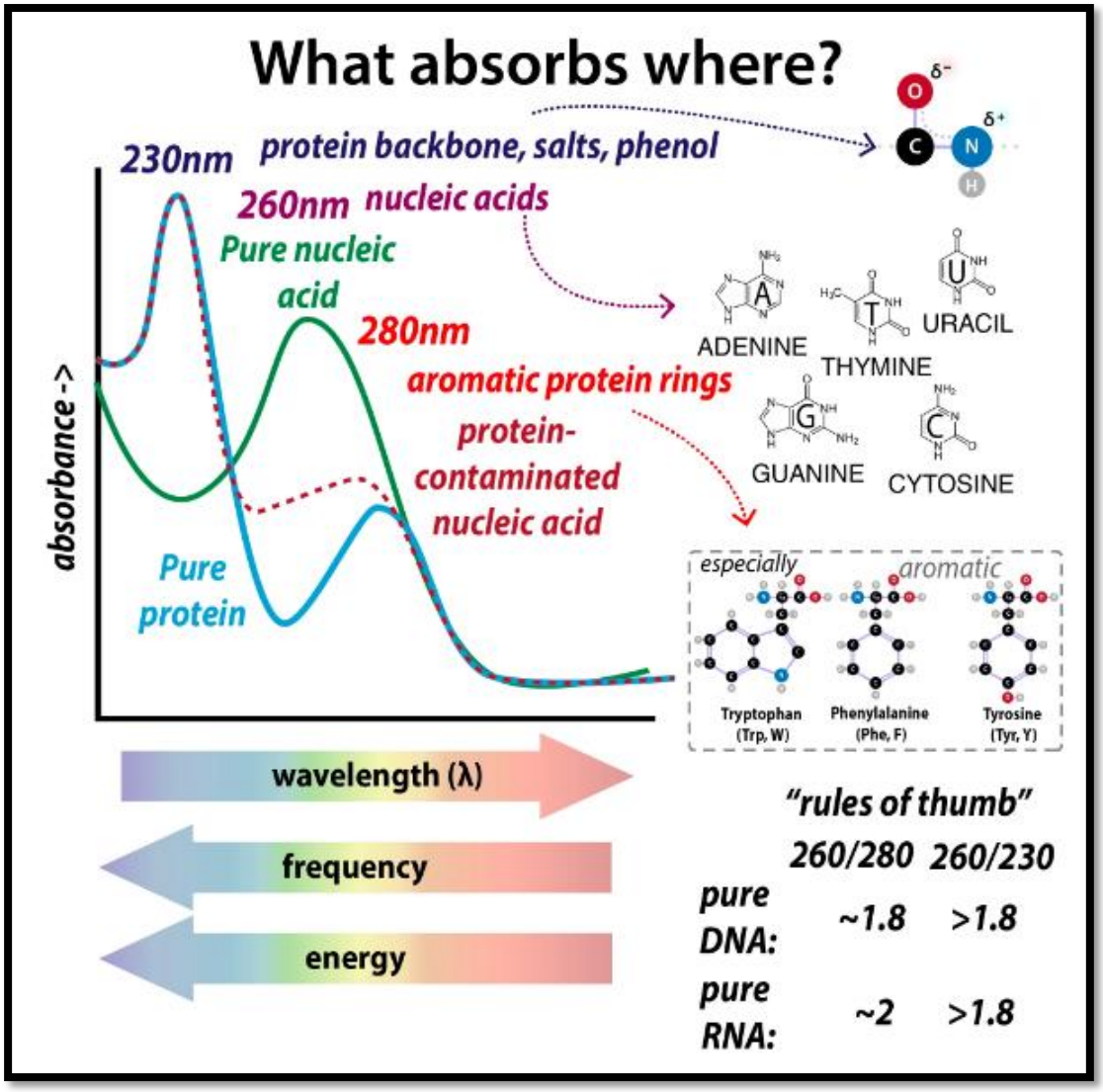

Figure (2-3): Quantification Methods for Extracted DNA & RNA

Table (2-12): DNA concentration and size resolution

Concentration (%)	DNA Size Resolution (bp)
0.5	1,000 – 25,000
0.75	800 – 12,000
1.0	500 – 10,000
1.2	400 – 7,500
1.5	200 – 3,000
2.0	50 – 1,500

2.9.3 The Mixture of PCR Reaction

Amplification of DNA was carried out in final volume of 20µl containing the following as mentioned in Table (2-13).

Table (2-13): Contents of the Reaction Mixture

Component	25 µL (Final volume)
Master Mix or GoTaq® Green Master Mix	12.5µl
Forward primer	10 picomols/µl (1µl)
Reverse primer	10 picomols/µl (1µl)
DNA	1.5µl
Distill water	9 µl
Total	25 µl

2.9.4 Gel Electrophoresis to Analyze DNA Quality (Barril & Nates, 2012).

1. An agarose solution was prepared by dissolve 1g of agarose powder in 100ml of 1x TBE in the (100ml) flask, agarose was melted in hot block until the solution became clear.
2. The agarose solution was made cool to about (50-55^{0}C), swirling the flask occasionally to cool evenly.
3. Red stain (3µl) was added to the warm gel then sealed the ends of the casting tray with two layer of tape.
4. The combs were placed in the gel-casting tray.
5. Melted agarose solution was poured into the casting tray.

6. The agarose was allowed to solidify at room temperature, the comb pulled out carefully and the tape was removed. The gel was placed onto the electrophoresis chamber that was filled with TBE (1x) buffer.
7. DNA samples (5µl) were mixed with (3µl) DNA loading buffer and loaded in agarose gel wells.
8. The agarose gel electrophoresis was completed at 70V, 65Amp for 1hour. The DNA was observed by viewed under UV trans illuminator.

2.9.5 Detection of *d-Ala:d-Ala ligases (ddl) E. faecalis* gene

DNA (extracted from bacterial cells) was used as a template in specific PCR for the detection of *d-Ala:d-Ala ligases E. faecalis* gene. DNA was purified from bacterial cells by using the Geneaid DNA extraction Kit.

The primers used for the amplification of a fragment gene were listed in Table (2-14).

Table (2-14): Primers sequences and PCR conditions for detection of *E. faecalis*

Genes	Primer sequence (5′.3′)	Size of product bp	PCR condition	Reference
ddl of *E. faecalis*	F:ATCAAGTACAGTTAGTCTTTATTAG R: ACGATTCAAAGCTAACTGAATCAGT	941	95ºC, 5 min. 95ºC, 1 min. 56ºC, 1 min. 72ºC, 1 min. 72ºC, 10 min.	Fines *et al*., (1999)

2.9.6 Detection of some of *E. faecalis* virulence genes

DNA (extract from bacterial cells) was used as a template in specific PCRs for the detection of some of *E. faecalis* virulence genes. DNA was purified from bacterial cells by using the Geneaid DNA extraction Kit.

The primers used for the amplification of a fragment gene were listed in Table (2-15).

Table (2-15) the primers, sequences, and PCR conditions for detection of some virulence gene

Gene name	Primer sequence (5´- 3´)	Size of Bp	Conditions	References
vanA	F: GCGCGGTCCACTTGTAGATA R:TGAGCAACCCCCAAACAGTA	314	95ºC, 5 min. 95ºC, 45sec. 50ºC, 45 sec. 72ºC, 1min. 72ºC, 10 min.	Ahmed *et al*., (2020)
gelE	F: TATGACAATGCTTTTTGGGAT R: AGATGCACCCGAAATAATATA	213	95ºC, 5 min. 95ºC, 1 min. 56ºC, 1 min. 72ºC, 1 min. 72ºC, 10 min.	Aghdam *et al*., (2017)
cpd	F: TGGTGGGTTATTTTTCAATTC R: TACGGCTCTGGCTTACTA	782	95ºC, 5 min. 95ºC, 1 min. 56ºC, 1 min. 72ºC, 1 min. 72ºC, 10 min.	Poeta *et al*., (2005)
cylA	F: ACTCGGGGATTGATAGGC R: GCTGCTAAAGCTGCGCTT	668	95ºC, 5 min. 95ºC, 1 min. 56ºC, 1 min. 72ºC, 1 min. 72ºC, 10 min.	Poeta *et al*., (2005)
ebpA	F: AACTAACAAAAATGATTCGGCTCCAG R: CATCTCACGCATTTTATCTTCAACT	517	95ºC, 5 min. 95ºC, 1 min. 52ºC, 1 min. 72ºC, 1 min. 72ºC, 10 min.	Poeta *et al*., (2005)
sprE	F: TTGAGCTCCGTTCCTGCCGAAAGTCATTC R:TTGGTACCGATTGGGGAACCAGATTGACC	591	95ºC, 5 min. 95ºC, 1 min. 58ºC, 1 min. 72ºC, 1 min. 72ºC, 10 min.	Poeta *et al*., (2005)
fsrA	F:CGTTCCGTCTCTCCATAGTTA R:GCAGGATTTGAGGTTGCTAA	474	95ºC, 5 min. 95ºC, 30 sec. 58ºC, 30 sec. 72ºC, 30.0 sec. 72ºC, 10 min.	Aghdam *et al*., (2017)
ace	F: AAAGTAGAATTAGATCACAC R: TCTATCACATTCGGTTGCG	320	92 ºC-30s 56 ºC-30s 72 ºC-60s 72 ºC-2min	Lebreton *et al*., (2009)
esp	F TTGCTAATGCTAGTCCACGACCC	933	94 ºC-2 min	Toledo-Arana *et al*.,

	R: GCTGCGTCAACACTTGCATTGCCGAA		92 ºC-30s 52 ºC-30s 72 ºC-60s 72 ºC-2min	(2001)
efaA	F: CCAATTGGGACAGACCCTC R: CGCCTTCTGTTCCTTCTTTGGC	688	95ºC, 5 min. 95ºC, 1 min. 53ºC, 10 min. 70ºC, 1 min. 70ºC, 10 min	Preethee *et al*., (2012)

2.10 Determinate of DNA sequencing for some important virulence genes (*gelE, fsrA, sprE*)

One specific PCR fragment partially covering the coding regions of the *gelE*, *fsrA*, *sprE* proteins were selected in this study. The amplified fragments were directly exposed to direct sequencing experiments to assess the pattern of genetic polymorphism in the collected bacterial samples. Then, specific comprehensive trees were built to assess the accurate discrimination of the observed variants and their phylogenetic distribution.

2.10.1 Nucleic acids sequencing of PCR amplicons

The resolved PCR amplicons were commercially sequenced from both (forward and reverse) directions, following to instruction manuals of the sequencing company (Macrogen Inc. Geumchen, Seoul, South Korea). Only clear chromatographs obtained from ABI (Applied Biosystem) sequence files were further analyzed, ensuring that the annotation and variations are not because of PCR or sequencing artifacts. By comparing the observed nucleic acid sequences of local samples with the retrieved nucleic acid sequences, the virtual positions, and other details of the retrieved PCR fragments were identified.

2.10.2 Interpretation of sequencing data

The sequencing results of the PCR products of the targeted samples were edited, aligned, and analyzed as long as with the respective sequences in the reference database using BioEdit Sequence Alignment Editor Software Version 7.1 (DNASTAR, Madison, WI, USA). The observed variations in each sequenced sample were numbered in PCR amplicons as well as in its corresponding position within the referring genome. The observed nucleic acids were numbered in PCR amplicons as well as in their corresponding positions within the referring genome. Each detected variant within the bacterial sequences was annotated by SnapGene Viewer ver. 4.0.4 (https://www.snapg ene.com).

2.10.3 Translation of nucleic acid variations into amino acid residues

The amino acid sequences of the targeted protein were retrieved online from the protein data bank (http://www.ncbi.nlm.nih.gov). The observed nucleic acid variants in the coding portions of the analyzed genetic loci were translated into a reading frame corresponding to the referring amino acid residues in the encoded protein using the *Expasy* online program (http://web.expasy.org/translate/). Multiple amino acid sequence alignment was conducted between the referring amino acid sequences and their observed mutated counterpart using the “align” script of the BioEdit server.

2.11 Human IL-23 ELISA kit

2.11.1 Principles

This ELISA kit was created using the Sandwich - ELISA principle. Each kit comes with a micro ELISA plate that has been pre-coated with an antibody specific to Human IL-23 (interleukin 23). Samples (or

standards) are mixed with the appropriate antibody and added to wells on a micro ELISA plate to get a positive result. After adding a biotinylated antibody against Human IL-23 to each microplate well, the presence of the antigen is detected using Avidin-Horseradish Peroxidase (HRP). In order to get rid of the extraneous parts, a good old-fashioned rinse is used. Each well is injected with the substrate solution. All of the wells will have a clear color except for the ones that contain Human IL - 23, the biotinylated detection antibody, and the Avidin - HRP conjugate. After adding the stop solution, the blue color fades and the yellow hue remains, indicating that the enzyme-substrate reaction has been stopped. Optical density (OD) at 450 + 2 nm is estimated using spectrophotometric techniques. Human interleukin (IL-23) concentration is directly correlated with the OD value. Comparing the samples' optical densities (ODs) to the standard curve allows you to determine the concentration of Human IL - 23 in the samples.

2.11.2 ELISA components

Table (2-16): ELISA kit components

Reagent	Quantity
Micro ELISA coated Plate	8 wells ×12 strips
Reference Standard	2 vials
Reference Standard & Sample Diluent	1vial 20mL
Concentrated Biotinylated Detection Ab	1vial 120µL
Biotinylated Detection Ab Diluent	1vial 10mL
Concentrated HRP Conjugate	1vial 120µL
HRP Conjugate Diluent	1vial 10mL
Concentrated Wash Buffer (25×)	1vial 30mL
Substrate Reagent	1vial 10mL
Stop Solution	1vial 10mL

2.11.3 Sample Preparation

All blood collection tubes must be single-use and endotoxin-free. Samples with high levels of hemolysis or fat are unsuitable for the ELISA test. Samples kept between 2 and 8 ºC should be tested within 7 days; otherwise, they should be frozen at either -20 degrees Celsius for one month or -80 degrees Celsius for an indefinite amount of time (3 months). Avoiding several freeze-thaw cycles is recommended. Careful thawing and centrifugation of frozen samples was required to remove precipitates prior to analysis. It was maked a rough estimate of the concentration before submitting it for analysis. If the sample concentration was beyond the range of the standard curve, the user will be needed to determine the optimal sample dilutions for the study. However, a pilot study was advised to prove validity if the sample type was not specified in the manual. Tissue homogenates and cell lysates may be altered by the presence of an extra chemical if a lysis buffer was used to make them. Some recombinant proteins may not be detected because their coated antibody and the detecting antibody aren't a good match.

2.11.4 Reagent preparation

1. The reagents was brought to room temperature (between 18 and 25 degrees Celsius) before usage.
2. Wash Buffer: 30 mL was diluted of Concentrated Wash Buffer with 720 mL of deionized or distilled water to create 750 mL of Wash Buffer. In the event that crystals have formed in the concentrate, reheat it in a water bath at 40°C and stirred it gently until the crystals have dissolved.
3. The working standard was centrifuged for 1 minute at 10,000 x g. After inverting several times and setting aside for 10 minutes, 1 mL of the reference standard was added and the Sample Diluent. After it has

dissolved all, stirred it very well with a pipette. A 2500 pg/mL working solution may be obtained after reconstitution (or add 1 mL of Reference Standard & Sample Diluent, let it sit for 1-2 minutes, and then thoroughly mixed it with a low-speed vortex meter). Centrifuged at a low speed has the potential to remove the bubbles created by the vortex. Then, if more dilutions was required, it was make them. The recommended dilution series is as follows: (pg/mL) 2500, 1250, 625, 312.50, 156.25, 78.13, 39.06, and 0.

4. The Water Diluting Technique: 500 mL of the Reference Standard was putted and Sample Diluent into each of the seven EP tubes. Half-fill one tube with a 2500 pg/mL worked solution, then 500 L of the solution was transfered to the second tube and stir until uniform. After this is done, 500 mL of the liquid was transferred from the first tube to the second using a pipette. The last tube was treated like a blank.
5. Working solution was Determined of Biotinylated Detection Ab (100 L/well) and will be needed before beginning the experiment. After centrifuged, the Concentrated Biotinylated was Detected Ab for 1 minute at 800xg, it was diluted the 100x Concentrated Biotinylated Ab with the Biotinylated Detection Ab Diluent (1x working solution) was the next step
6. HRP Conjugate working solution was Determined, it will be needed ahead of time (100 L well). A good rule of thumb was make somewhat. The Concentrated HRP Conjugate may be diluted to 1:99 used the HRP Conjugate diluted and centrifuged at 800xg for 1 minute to produce a 1x working solution (Concentrated HRP Conjugate: HRP Conjugate Diluent).

2.11.5 Assay procedure

1. Created three distinct wells: one for the sample, one for the blank, and one for the diluted standard. As was previously indicated, 100 L of each dilution of the standard, the blank, and the sample should be placed in the corresponding wells (It is recommended that all samples and standards be assayed in duplicate). It was used the sealant that included with the order to make sure the plate doesn't leak. It was maintain a temperature of 37 degrees Celsius for the incubator for 90 minutes.
2. The wells did not rinse with water after they have carefully removed the liquid and decanted it. After thoroughly mixing the samples, immediately it was added 100 L of the Biotinylated Detection Ab working solution to each well. It should be used a new plate sealer. Incubated for one hour at 37 degrees Celsius.
3. They have drained the solution from each well, filled it with 350 mL of wash buffer. After letting the solution sit for one minute, it was removed it with an aspirator or decanter and wipe dry with absorbent paper. Washing in this manner should be repeated thrice. It was possible to utilize a microplate washer for this and other washing procedures.
4. 100 mL of the HRP Conjugate working solution was putted into each of the wells.
5. The containers was taken out of the fridge. It was should be used a new plate sealer. The optimal incubation temperature and duration is 37 degrees Celsius for 30 minutes.
6. After the solution has been drained from each well, the steps 3 was repeated and 5 four more times.
7. 90 mL of the Substrate Reagent was added to each well and stir to combine. It should be used a new plate sealer. The incubator was

putted on the stove at a temperature of 37 degrees Celsius for 15 minutes. Sunlight might damage the plate.

8. 50 mL of Stop Solution was poured into each well and putted the dish aside to cool. The stop solution must be applied after the substrate solution in the same sequence for both to have any effect.
9. The optical density (OD) was determined of each well simultaneously by using a microplate reader with a laser wavelength of 400 nm.

2.11.6 Calculation of results

Average optical density of the zero standard may be determined by subtracting the duplicate values for each standard and sample from the original ones. A log-log graph was make of the data, with standard concentration along the x-axis and OD readings along the y-axis, to represent a four-parameter logistic curve. If the optical density (OD) of the sample was more than the upper limit of the standard curve, the sample should be retested after being appropriately diluted. To get the actual concentration, multiply the computed concentration by the dilution factor.

2.11.7 Typical data

Since the OD values of the standard curve might vary depending on the actual assay performance (for example, operator, pipetting method, washing technique, or temperature changes), it was important for the operator to create a new standard curve for each test (e.g., operator, pipetting technique, washing technique, or temperature impacts). The following data and standard curve examples were provided for illustrative purposes only.

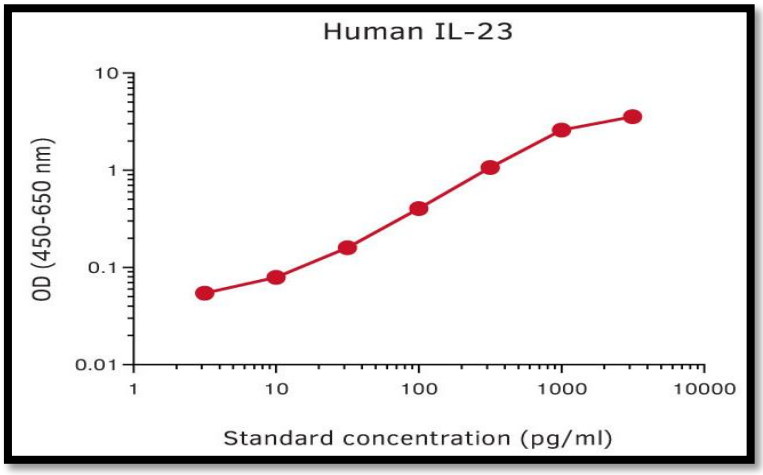

Figure (2-4): Human IL-23 ELISA Standard Curve

2.12 Statistical analysis

Statistical analysis was carried out using SPSS version 25. Categorical variables were presented as frequencies and percentages. Continuous variables were presented as (Means ± SD). Student t-test was used to compare means between two groups. A p-value of ≤ 0.05 was considered as significant (George & Mallery, 2019).

Chapter Three

Results and Discussion

3. Results and Discussion

3.1 Isolation and identification of *E. faecalis* isolates

Patients who suffering from UTIs and vaginitis were visited to Baghdad's Al-Karama Hospital and Medical City Hospital throughout a three-month period (May to July 2022) provided the study's 200 participants with urine and vaginal specimens. At (37ºC), the samples were cultured for (18-24) hours in various mediums. Then, they were incubated at (37ºC) for (18-24) hours on a number of different selective media (Chromo Agar Medium).

When first trying to identify *E. faecalis*, scientists relied on colony morphology, microscopic examinations, and biochemical assays. Out of 200 clinical samples cultured positive, however only 44(22%) of the isolates were identified with *E. faecalis*. As can be seen in Figure (3-1), the automated Vitek 2 system employed GP-ID cards containing 64 biochemical tests to ensure the isolates were really *E. faecalis*. Using this method, it was able to quickly identify 44 different bacterial isolates, with a confidence level ranging from excellent (probability percentage of (94 to 99.7%). The results were shown in Table (3-1).

Table (3-1) Isolation and identification of *E. faecalis* by specific media, biochemical test and automated Vitek 2 system

No. of isolates	selective media		biochemical test		automated Vitek 2 system	
	positive results	Negative results	positive results	Negative results	positive results	Negative results
200	44(22%)	-	44(22%)	-	44(22%)	-

Patient Name: medical, noor
Location:
Lab ID: 183202111

Patient ID: 183202111
Physician:
Isolate Number: 1

Organism Quantity:
Selected Organism : Enterococcus faecalis

Source: **Collected:**

Comments:	

Identification Information	**Analysis Time:**	2.85 hours	**Status:**	Final
Selected Organism	99% Probability **Bionumber:**	**Enterococcus faecalis** 156012765773471		
ID Analysis Messages				

Biochemical Details

2	AMY	+	4	PIPLC	-	5	dXYL	-	8	ADH1	+	9	BGAL	-	11	AGLU	+
13	APPA	-	14	CDEX	+	15	AspA	+	16	BGAR	-	17	AMAN	-	19	PHOS	-
20	LeuA	+	23	ProA	-	24	BGURr	-	25	AGAL	-	26	PyrA	+	27	BGUR	-
28	AlaA	+	29	TyrA	+	30	dSOR	+	31	URE	-	32	POLYB	+	37	dGAL	+
38	dRIB	+	39	lLATk	-	42	LAC	+	44	NAG	+	45	dMAL	+	46	BACI	+
47	NOVO	+	50	NC6.5	+	52	dMAN	+	53	dMNE	+	54	MBdG	+	56	PUL	-
57	dRAF	-	58	O129R	-	59	SAL	+	60	SAC	+	62	dTRE	+	63	ADH2s	+
64	OPTO	+															

Figure (3-1): Identification of *Enterococcus faecalis* by using automated Vitek 2 system

3.2 Distribution of *E. faecalis* isolated from clinical samples

Forty four *E. faecalis*-related isolates were identified in this investigation. As can be seen in Figure (3-2), 32(72.7%) of the isolates were from urine, whereas 12 (27.3%) from the vagina.

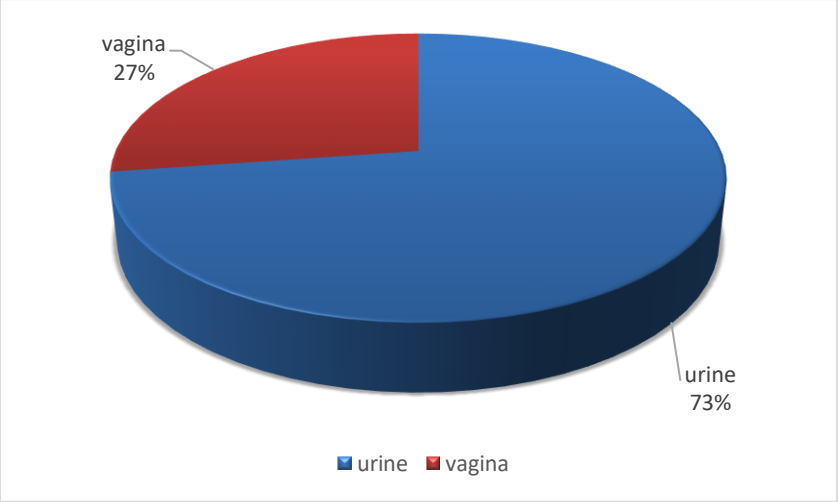

Figure (3-2): Distribution of *E. faecalis* isolated from clinical samples

Hashem *et al*., (2021), whose findings were corroborated by these findings, showed that 45 of the 58 isolates of *Enterococcus faecalis* from clinical specimens were able to grow on selected *Enterococcus* medium, with the greatest number of isolates detected in urine samples. *E. faecalis* was shown to be a pathogenic bacterium by Stpie-Pyniak *et al*., (2021), after being isolated from several sites of infection, most often UTIs. In line with the findings of Jahansepas *et al*., (2020), who discovered that, the majority of *E. faecalis* isolates (22.6%) were detected in urine samples. Results are also agreement with those reported by Wójkowska-Mach *et al.*, (2021), who discovered that, the prevalence of *E. faecalis* was around 21.1% in urine and 30% from vaginal swabs.

Enterococci have gained more attention in recent years due to their rising resistance to several antimicrobial drugs and their capacity to produce severe infections (Mancuso *et al.,* 2021). Most cases of illness caused by enterococci are urinary tract infections, and this is true both in and out of hospitals (Giannakopoulos *et al.*, 2019).

Urinary tract infections and infections of sutured surgical wounds in patients with indwelling devices were due to external contamination, possibly by organisms that had multiplied in the gastrointestinal system and become closely linked to the patient during hospitalization (Majumder *et al*., 2018). Toc *et al*., (2022) analyzed 50 isolates of *Enterococcus faecalis* and found that 32% were from feces, 32% from urine, 12% from wounds, and 10% from the vaginal canal.

Ahmed and Hafidh, (2021) observed that, 75% of *E. faecalis* isolates from urine. Studies found that the rate at which *E. faecalis* was isolated from patients varied depending on a number of variables, including the size of the samples used, the regions in which they were conducted, the

isolation and identification methods employed, the effect of environmental conditions on patients' health, and the social and cultural background of the patients themselves. These bacterial isolates seemed competent to infiltrate a bladder cell line, as evaluated by an antibiotic protection experiment, and their tight contact with epithelial cells shed from patients was indicative of intracellular colonization (da Silva *et al*., 2022).

Most cases of bacterial vaginitis (pH>4.0, *E. faecalis* often resides asymptomatically in the gut) were linked to the presence of En*terococcus faecalis*. However, the infection may become far more dangerous if it spreads to other regions of the body. There are a number of ways for these germs to enter the body after surgery, including through the blood, the urine, or via an incision. Further spread to other areas increases the risk of more severe illnesses (Kao & Kline, 2019). White, watery discharge with a fishy odor and an increased vaginal pH, all of which are symptoms of the most prevalent cause of infectious vaginitis, *Enterococcus faecalis* (Javed & Manzoor, 2020).

3.3 Detection of *E. faecalis* by *ddl* gene

All previously identified isolates' DNA was taken and used in conventional PCR to amplify the *ddl* gene. Figure (3-3) displays the results of gel electrophoresis, which demonstrated that, each of the 44 isolates (100%) had, in fact, generated the same 941bp DNA fragment when run alongside a ladder.

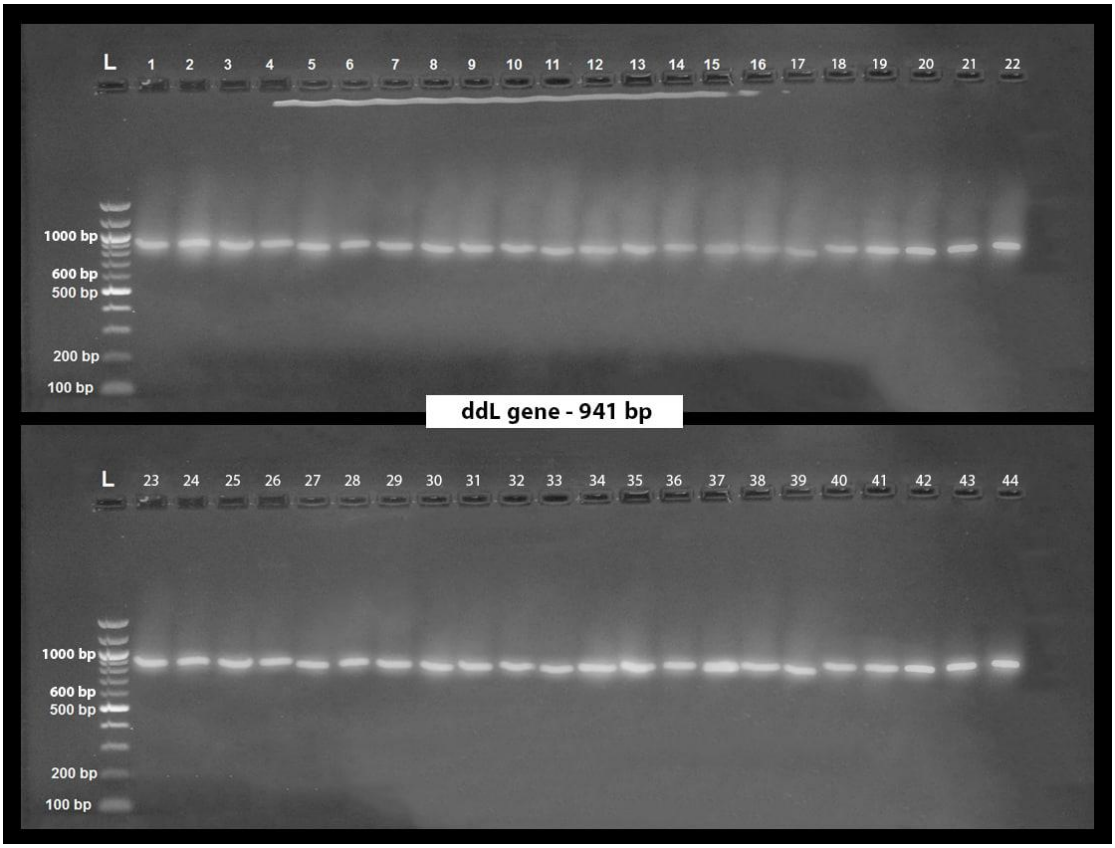

Figure (3-3): 1% Agarose gel electrophoresis image at 75 V, (20 mA) for 1 hour that showed the PCR product analysis of *ddl gene* in *Enterococcus faecalis* isolated from clinical samples. Where L: marker (100-1500bp) and Lane (1-44) showed positive *ddl gene in E. faecalis* isolates were showed (1-32 urine, 33-44 vagina) at (941bp) PCR product.

Results from this investigation corroborated those by Lim, (2018), who utilized species-specific primers to confirm the presence of *E. faecalis* in 28 urine samples. Results from this study conflict with those from studies by Supotngarmkul *et al*., (2020) who found that, of 100 clinical isolates, only 34 were found to be *E. faecalis* when tested with a specific primer, and by Zaheer *et al*., (2020), who found that, of clinical isolates related to *E. faecalis*, 90% were detected.

The results of this research show that, conventional PCR using a selected specific target is faster, easier, and more accurate than earlier approaches for identifying *E. faecalis*. The use of PCR showed that the comparative genomics technique correctly identified the target. When identifying species that are difficult to discriminate using phenotypic methods, PCR using species-specific primers is a helpful tool that may substitute sophisticated molecular clustering techniques and standard microbiological testing (Rubin *et al*., 2022).

The identification of *E. faecalis* by molecular means involves the use of PCR primers based on particular gene sequences. Ghalavand *et al.*, (2020) observed amplification of a particular *ddl* sequence of the *E. faecalis* genome in urine sample from individuals with UTIs. All *E. faecalis* strains could be reliably identified by a single, highly unique marker: the *ddl* gene (Almahdawy *et al*., 2019).

Because *ddl* genes are the most widely shared housekeeping genetic markers, researchers have utilized them to examine the identification, taxonomy, and phylogeny of bacteria. Their ubiquitous existence in bacteria, where they often occur as multi-gene families or operons, and the fact that their function has not altered through time imply that random sequence changes are a more accurate indicator of temporal evolution. Because of their size, these genes are suitable for use in informatics (Kim *et al*., 2020).

Occasionally, it might be difficult or impossible to phenotypically identify a certain *Enterococcus* species because that species lacks defining traits. Ideally, labs that study microorganisms would have access to more efficient and reliable techniques (Rajapaksha *et al*., 2019). In contrast to the culture technique, PCR is the most reliable method for

identifying *E. faecalis* infections because of its superior accuracy, sensitivity, specificity, uniqueness, and cost-effectiveness. Results from a molecular approach may be obtained in 24 hours, but those from a convective method, such as normal culture followed by a biochemical test, take between 24 and 48 hours.

PCR has enabled the rapid, culture-independent identification of enterococci in a wide range of clinical specimens. Results may be obtained in as little as a few hours. However, all existing PCR-based tests are exclusively species-specific for enterococci, which are of clinical importance (Vasala *et al*., 2020).

3.4 Phenotypic detection of some virulence factors of *E. faecalis*

3.4.1 Hemolysin production

The resent study looked at whether or not certain *E. faecalis* isolates may produce virulence factors. Out of 44 isolates tested, 14(31.8%) were found to be hemolytic, as shown by a clear zone around the colonies on a blood agar plate after just 24 hours of incubation at 37 degrees Celsius. These results were shown in Figure (3-4).

Researchers have found that *E. faecalis* isolated from people with urinary tract infections produces hemolysin at a rate of 47.4% (Abasiubong *et al*., 2019), while *E. faecalis* isolated from urine samples produces hemolysin at a rate of 25%, and *E. faecalis* isolated from the vagina produces hemolysin at a rate of 82% (Banerjee & Anupurba, 2015).

Hemolysin is one of two iron-acquisition mechanisms used by *E. faecalis*; these bacteria create two hemolysin; these hemolysin seem to be

cytotoxic for most eukaryotic cells; these hemolysin help to invasion by killing off host cells. Hemolysin may be used by bacteria as a method of parasitism in order to steal resources from host cells (Yip *et al*., 2021).

Strains of *E. faecalis* that produce the hemolysin toxin are highly infectious and contribute to more severe infections in both animals and people (Bulanik & Ozer, 2020). Stpie-Pyniak *et al*., (2021) found that 75% of clinical *E. faecalis* isolates caused the hemolytic halo around colonies on sheep blood agar plates. In a study conducted on horse blood, cell-free hemolysin was detected, suggesting that 11% of *E. faecalis* were capable of producing it (Cirrincione *et al*., 2019). When cultivated in BHI-GA, all of the *E. faecalis* isolates examined by Patidar *et al*., (2013) demonstrated extracellular hemolytic activity against sheep erythrocytes.

The hemolytic activity was maintained even after being heated to 100 degrees Celsius, proving the stability of the hemolysin. In addition, proteolytic enzymes had no effect on its action (Rushdy & Gomaa, 2013). Hemolytic activity on sheep blood was not seen in BHI culture supernatants, indicating that glucose and arginine are necessary for the synthesis of heat-stable hemolysin.

Production of hemolysin, which acts to release iron complex to intracellular heme and hemoglobin, is one mechanism by which iron is taken into the body. Siderophores, which bind iron with a very high affinity and efficiently compete with transferrine and lactoferrin to mobilize iron for microbial use, are a second route for iron acquisition (Klebba *et al*., 2021). That's in line with the current findings, too.

Because of its prevalence, hemolysin production was identified as a virulence factor isolated from *E. faecalis*. Similar to the current findings, it has been hypothesized that, hemolytic strains of *E. faecalis* are more

prone to emerge in urinary tract infections, in particular acute pyelonephritis (Hashem *et al*., 2021).

Previous research has shown that not all instances of toxin binding to the bilayers result in membrane lysis. Because of this, the precise method by which hemolysin inserts itself into lipid bilayers remains unknown. On the other hand, certain cells may have hemolysin receptors, pure lipid bilayers, and newly reported techniques for assessing hemolysin binding to membranes and hemolysin induced lysis, and it was observed that the binding is not always accompanied by membrane damage (Sathyanarayana *et al*., 2020).

Under iron-limited environments, organisms with high-efficiency iron acquisition systems secrete and internalize extracellular ferric chelators to scavenge iron from the environment (Marchetti *et al*., 2020). As a virulence factor, hemolysin is often associated with Gram-positive bacteria; nevertheless, its role in illness may vary widely across microorganisms and host species (Bin-Asif & Ali, 2019).

In order to survive, pathogenic bacteria have developed a variety of strategies for scavenging iron from their natural habitats. The release of iron complex from intracellular heme and hemoglobin is one such mechanism. One of the major virulence factors in bacteria is haemolysins. Haemolysins are a kind of bacterial cytolysin that belongs to a broad family of pore-forming cytolysins that may rupture the cell membrane, releasing cytoplasmic material and killing the cell (Nairz & Weiss, 2020).

3.4.2 Extracellular protease production

The protein was hydrolyzed by 12(27.2%) of the isolates, indicating that they were capable of producing protease. After (24) hours of aerobic

incubation, the findings showed that, *E. faecalis* isolates were able to create extracellular protease, which hydrolyzed the protein by 1% casein and 20% glucose. After adding 3ml of 5% trichloroacetic acid, the region surrounding the colony became transparent and glowed in a clear hallo, these results were shown in Figure (3-4).

E. faecalis may secrete extracellular protease at a high rate (25%) as discovered by Colomer-Winter *et al.*, (2018). The detection of extracellular protease from microorganism was achieved by inserting gelatin as substrates into agar and changing the composition of the culture medium, as was also observed by da Silva-López *et al.*, (2022).

Proteinases that are secreted outside of cells are crucial for cell survival and intercellular communication (Noriega-Guerra & Freitas, 2018). Protease synthesis was also stimulated by the presence of an amino acid-rich growth media (Toe *et al.*, 2019). This result agreed with that mentioned by Hammers *et al.*, (2022) who found that, *E. faecalis* is resistant for phagocytosis and opsonization because it can produce of large number of extracellular proteases like alkaline protease, elastases, and exotoxin A, which can cleave IgA and lead to inhibit the function of the cells of the immune system.

3.4.3 Gelatinase production

In this study, detection of gelatinase production were investigated in all 44 *E. faecalis* isolates, the results showed that, only 14(31%) of the 44 isolates were positive for gelatinase production as shown in Figure (3-4).

According to research by Hashem *et al.*, (2021), gelatinase synthesis is one of the virulence factors that may be connected with *E. faecalis* survival in UTIs, gelatinase, a kind of zinc-containing endopeptidase, is

able to hydrolyze not just gelatin and collagen and fibrin but also other peptides. Furthermore, Shahi *et al*., (2020) discovered that, *E. faecalis* gelatinase synthesis may play a significant role in bacterial adhesion.

Hydrolysis of gelatin, collagen, and casein by the extracellular metalloprotease gelatinase secreted by *Enterococcus faecalis* has been associated to virulence in animal models. Due to its ability to hydrolyze collagens and certain bioactive peptides (Kadhem, 2018), this enzyme is likely involved in *E. faecalis*-related inflammatory processes at both the initiation and maintenance stages. Despite the fact that, 64% of *E. faecalis* isolates from patients with bacteremia produced gelatinase (Rotta *et al.*, 2020), no activity was found in the *E. faecalis* isolates presented here. While 75% of *E. faecalis* isolates were found to have caseinase activity, the data suggests that the gelatin hydrolyzing activity is separate from caseinase (Tsankova *et al*., 2019).

Gelatinase expression has been linked to increased biofilm formation in vitro, according to research by Zheng *et al*., (2020). *E. faecalis* gelatinase synthesis is crucial to its adhesion is yet unclear.

The significance of gelatinase synthesis was confirmed among *E. faecalis* human isolates, as shown by translocation by all gelatinase-positive isolates and little to no translocation for gelatinase-nonproducing isolates (Willett *et al*., 2022).

3.4.4 Lipase production

In this study, lipase production were investigated in 44 *E. faecalis* isolates, the results showed that, 32(72.7%) were able to produce lipase as shown in Figure (3-4).

These findings were agreement with those of Topatan & Hatice, (2022), who tested the capacity of *E. faecalis* isolates to manufacture lipases enzyme on Tween 80 medium at 37oC and discovered that 10% of *E. faecalis* could create lipases. Lipase, or triacylglycerol acylhydrolase, is a kind of hydrolase that catalyzes the hydrolysis of triglycerides to glycerol and free fatty acids at the oil-water interface (Anand *et al*., 2021). In addition to hydrolysis and esterification, lipases may catalyze the transesterification, acidolysis, and ammonolysis of esters. Microbial lipases have attracted the attention of the food, detergent, cosmetic, organic synthesis, and pharmaceutical sectors (Fatima *et al*., 2021).

As a result of their versatility and usefulness in both in situ lipid metabolism and ex situ multifaceted industrial applications, lipases have quickly risen to prominence as one of the leading biocatalysts with proven potential to contribute to the multibillion dollar underexploited lipid technology bio-industry (Zhang *et al*., 2019). Most lipases function at a particular point on the glycerol backbone of lipid substrate, making the lipid substrate water-soluble (Chandra *et al*., 2020).

Important in the development of urinary tract infections, this virulence factor operates on the membrane of animal cells by inserting into the membrane to create a whole and cleaving phospholipids. It also lyses red blood cells, phagocytes, and their granules (Praetorius, 2021). Medium composition, notably carbon and nitrogen sources, together with physicochemical parameters like temperature, PH, and dissolved oxygen, has a significant impact on bacterial extracellular lipase production (Hasan *et al*., 2018).

Microorganisms generate lipases, and due to their wide range of enzymatic characteristics and substrate specificity, only microbial lipases

are considered to be of economic significance (Pascoal *et al*., 2018). A wide range of lipases, enzymes that may hydrolyze or manufacture triacylglycerols, are produced by bacteria, however there is little information connecting these lipases to virulence factors in bacteria (Coelho & Orlandelli, 2021). It has been hypothesized that a microbe's lipase improves adhesion by degrading host surface molecules, freeing up new receptors (Thorn *et al*., 2021). This is because the microbe's primary function of secreting extracellular lipases is to digest the cellular lipids of its host in order to acquire nutrients. It is hypothesized that for Propionic bacterium acnes, the production of free fatty acids (FFAs) increases nonspecific hydrophobic interactions. Lipases have a crucial biological function in bacterial infections and may perhaps be the single most crucial stage in the infection process for many different types of microbes (Pascoal *et al*., 2018).

3.4.5 Attach to epithelial cells hemagglutination and hydrophobicity

The results showed that, 22(50.0%) of the isolates were able to attach to epithelial cells, 18(40.9%) were able to create hemagglutination, and 16(36.3%) were able to raise hydrophobicity, All of these results were shown in Figure (3-4).

The capacity to agglutinate human erythrocytes was evaluated in a study by Nisha *et al*., (2022), and the results were positive. Al-Rammahi *et al*., (2020) discovered that 97% of *E. faecalis* were agglutinated human erythrocytes, and that 86% of the isolates examined did react with the erythrocytes used in the study. Hemagglutination qualities are prevalent across the many enterococcal species studied, as discovered by Abutaleb & Seleem (2020). They also imply that enterococci have both proteic and

non-proteic hemagglutinins, which play a role in the attachment to sialic acid-containing receptors on the surface of erythrocytes.

The ability of the strains to express type 1 and type 3 fimbriae was determined by testing them for their ability to cause a hemagglutination of erythrocytes in a mannose-sensitive and a mannose-resistant environment. Many of the clinical isolates were able to create both adhesions, and the vast majority were able to produce at least one. Adhesion evaluation through hemagglutination is qualitative, therefore it cannot tell us if both type 1 and type 3 fimbriae must be produced during invasion, or whether expression of only one fimbrial adhesion per cell is sufficient (Zadeh *et al*., 2021).

Epithelial cell adhesion by bacteria is a crucial stage in the development of most human illnesses. A urinary tract infection may be symptomatic or asymptomatic depending on the bacteria's ability to attach to the urinary system's mucosal surfaces. It is likely that bladder infection begins with the adherence of bacteria to the bladder epithelial cells and the subsequent development of those bacteria in the urine (Das, 2020). In a study conducted by Hussen *et al*., (2020), they discovered that the majority of *E. faecalis* isolates (75%) were able to attach to epithelial cells.

Pili and fimbrial-like structures are used by *E. faecalis* to stick to and infiltrate human urinary tract epithelial cells; it has been hypothesized that this structure-mediated adherence contributes to inflammation induction and hence increases *E. faecalis* urinary tract pathogenicity (Ranjith *et al*., 2021).

Furthermore, adhesions facilitate bacterial adherence to and penetration of host cells, and contribute to the development of

intracellular bacterial biofilms by uropathogens. Many infections begin with bacteria adhering to host cells (Mirzaei *et al*., 2020).

However, there is a lack of knowledge on the mechanisms that facilitate *E. faecalis* adherence to host tissues. It was found by Montalbán-López *et al*., (2020) that 27.6% of *E. faecalis* strains are able to attach to human urinary tract epithelial cells. The ability of bacteria to stick to host epithelial cells is a crucial stage in the pathogenesis of infections, as proven by Klein & Hultgren, (2020).

Clinical isolates of *E. faecalis* had a stronger capability to adhere to urinary tract epithelial cells. Sixteen of the *E. faecalis* isolates (50%) and thirteen of the isolates (40.6%; Klein & Hultgren, 2020) were able to attach to HeLa cells. Colonization and infection are processes that are often facilitated by bacteria due to their propensity to cling to biomaterials.

Compared to other bacteria, *E. faecalis* isolates were far more hydrophobic. Twenty strains with hydrophobicities more than 50% were observed in an in vitro hydrophobicity test conducted by Colombo *et al*., (2018), eighteen of which were identified as *E. faecalis*. The hydrophobicity of a bacterium's cell surface is crucial to its interactions with host epithelial cells, as Krausova *et al*., (2019) have out. Enterococcal aggregation compounds enhance the hydrophobicity of the cell surface (Stpie-Pyniak *et al*., 2019).

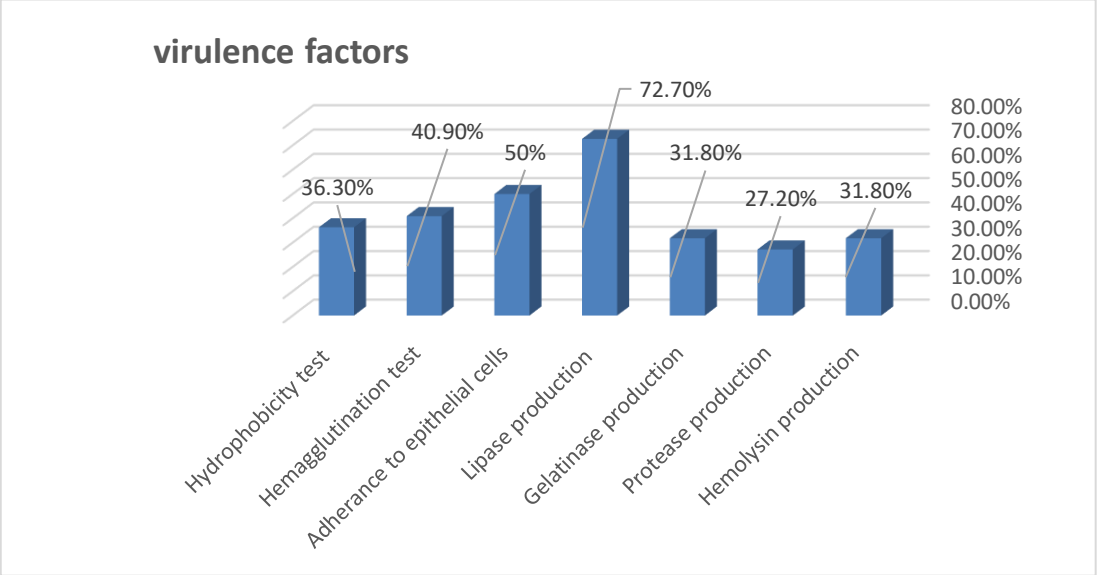

Figure (3-4): Detection of some virulence factors among *E. faecalis* isolates

3.5 Biofilm formation

In the present study, it was used ELISA to distinguish between *E. faecalis* strains that produced biofilms and those that did not, based on the median values of optical density (OD) at 590 nm (> 0.240, 0.120, and 0.120), as previous research has shown that, bacterial biofilms cause chronic diseases that are difficult to treat.

The capacity to form biofilm was evaluated in 44 different isolates, as can be shown in Table (3-2), 31(70.4%) of these isolates were strong biofilm producers, 3(6.6%) were moderate biofilm producers, 4(9%) were weak biofilm producers, and 6(13.6%) were non-biofilm producers.

Table (3-2): Biofilm formation of *E. faecalis* isolates

Bacterial isolates (no.)	Biofilm formation			
	Strong	Moderate	Weak	no biofilm formation
E. faecalis (44)	31(70.4%)	3(6.8%)	4(9%)	6(13.6%)

This finding is agreement with that of El-Atrees *et al*., (2022), who discovered that 75% of *E. faecalis* isolates create biofilm owing to their capacity to attach to any collection of microorganisms in which cells cling to each other and frequently adhere to a surface. The extracellular polymeric matrix that these adhering cells create themselves is a common occurrence. Ozma *et al*., (2021) observed that 35% of 100 clinical isolates of enterococci from the urinary tract formed a robust biofilm, highlighting the need of developing an effective therapy. Findings from a study by Zheng *et al*., (2018), who analyzed 113 UTI patient isolates, showed that 26.5% exhibited high biofilm formation.

According to the results of this study, *E. faecalis* is responsible for about (85%) of all human infections (Grenda *et al*., 2022). The production of biofilms in their hosts is one of the pathogenicity mechanisms of these bacteria, which contributes to an increase in virulence and in resistance to antibiotics, and hence in the survival of these microorganisms (Gebreyohannes *et al*., 2019).

When cultivated on solid or semisolid surfaces, roughly 69% of *E. faecalis* strains are able to produce additional flagella (called lateral). These strains' polar and lateral flagella contributed to their ability to cling to biotic and abiotic surfaces and form biofilms (Taylor *et al*., 2019).

Polymers such as extracellular DNA, proteins, and polysaccharides congregate to form biofilms, often known as slime. Biofilms are ubiquitous in natural, industrial, and healthcare contexts, and may develop on both living and nonliving surfaces (Izadi *et al*., 2022).

Biofilm-forming microbes differ physiologically from their single-celled, liquid-dwelling counterparts, called planktonic cells (Thurnheer & Paqué, 2021). Polysaccharides, proteins, nucleic acids, lipids, and other

macromolecules and chemicals make up the bulk of the extracellular matrix that encloses biofilms (Karygianni *et al*., 2020).

Biofilm formation by microorganisms may be triggered by a variety of stimuli, such as the detection by individual cells of attachment sites on a surface, the presence of nutrients, or even the exposure of planktonic cells to sub-inhibitory concentrations of antibiotics. Phenotypic changes occur in cells when they convert to the biofilm development mode, and this involves the differential regulation of huge groups of genes (Sustr *et al*., 2020). Bacterial adhesion to a surface, the creation of microcolonies, biofilm maturity, and bacterial removal (called dispersion) to potentially colonize other regions are the four steps generally accepted as constituting biofilm formation. Sessile bacteria, those found in biofilms, are distinguished from planktonic bacteria by their inactive growth state and unique morphologies (Muhammad *et al*., 2020). Pathogenic bacteria with the potential to form biofilms have been the focus of three worldwide non-microbicidal techniques that aim to (i) prevent microbial adhesion to a surface. (ii) Interfering with biofilm formation and/or altering biofilm architecture to improve antimicrobial penetration; and (iii) influencing biofilm maturity and/or causing its dispersal and disintegration (Bunyan *et al*., 2019).

3.6 Antibiotic resistance among *E. faecalis* isolates

Antibiotic resistance was evaluated *in vitro* using a modified Kirby - Bauer disc diffusion technique for all of the detected *E. faecalis* isolates. Vancomycin, Teicoplanin, Ciprofloxacin, Norfloxacin, Erythromycin, Synercid, levofloxacin, Gentamicin, Nitrofurantoin and Linezolid were used as examples of selective antibiotics to demonstrate their efficacy against *E. faecalis* isolates. Figure (3-5) was displays the obtained data.

The findings are consistent with what would be considered resistant based on the criteria set out by the Clinical Laboratory Standard Institute (CLSI, 2021).

Highest rate of resistant is seen to almost antibiotics used in present study, 42(95.4%) isolates were resistant to Synercid, followed by 25(56.8%) isolates were resistant to Levofloxacin, 20(45.4%) isolates were resistant to Erythromycin and Norfloxacin, 19(56.8%) isolates were resistant to Ciprofloxacin, 18(40.9%) isolates were resistant to Vancomycin, 13(29.5%) isolates were resistant to Gentamicin, 9(20.4%) isolates were resistant to Teicoplanin, while no resistance were occur against Nitrofurantoin and Linezolid.

These findings corroborated those of Gajan *et al*., (2013), who used a variety of antibiotics to test *E. faecalis* isolates and found that the bacteria were resistant to all of them except for Synercid (100%) and Levofloxacin (86.36%) and amoxicillin (68.18%). When tested, *E. faecalis* showed an (84.8%) resistance to gentamicin, according to research by Aşgn & Taşkn (2019).

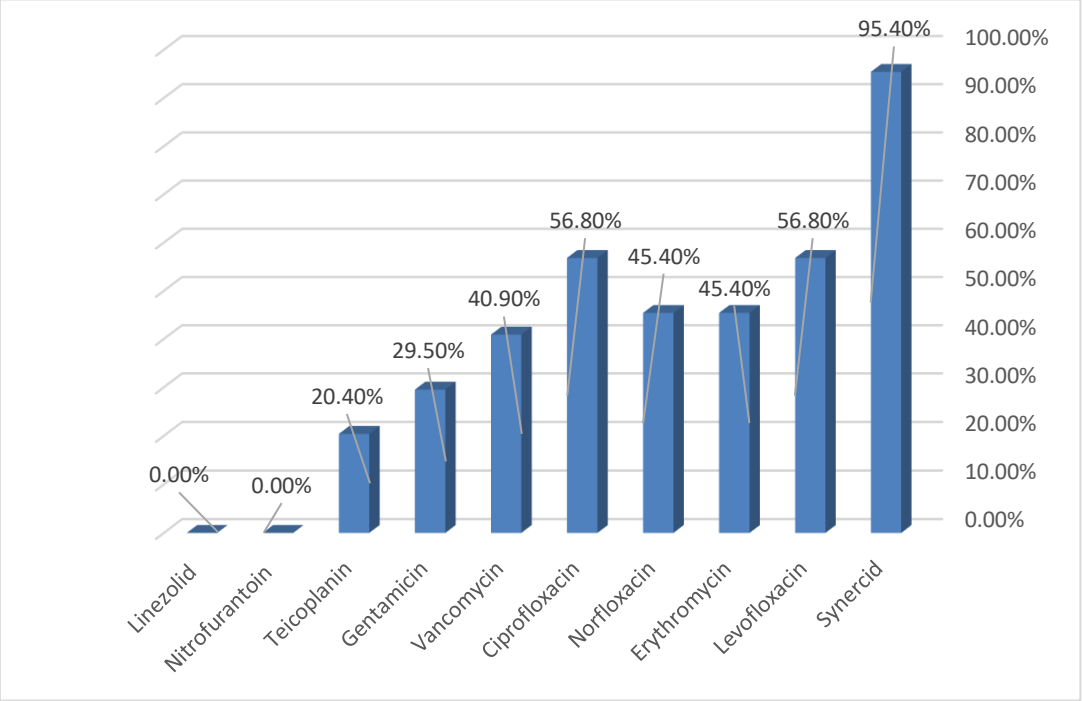

Figure (3-5): Antibiotic resistance among *E. faecalis* isolates

Antibiotics like beta-lactams and aminoglycosides, commonly used to treat infections caused by Gram-positive cocci, are thought to be ineffective against enterococci because of their intrinsic resistance to these drugs (FakhriRavari *et al.*, 2022).

Schell *et al*., (2020) also report a (25.3%) and (45.6%) resistance rate for *E. faecalis* to gentamycin and vancomycin, respectively. Due to its low success rate against *Enterococcus* infections, vancomycin was the only antibiotic Wu *et al*., (2021) discovered to be widely resistant among the strains they tested (64 %). These findings are consistent with those of Khdir, (2020), who showed that (100%) of *E. faecalis* isolates are resistant to erythromycin. In addition, (86%) of isolates showed resistance to tetracycline, and (77.2%) of isolates showed resistance to gentamicin, as found by Regecová *et al*., (2021). All *E. faecalis* isolates tested by Lengliz *et al*., (2022) showed extreme sensitivity to vancomycin, Teicoplanin, and linezolid.

As reported by Coskun, (2019), (8.7%) of *E. faecalis* isolates were resistant to vancomycin. Since no linezolid-resistant *Enterococcus* isolates were found, it may be safely assumed that (Zhou *et al*., 2019). Also, the Nitrofurantoin findings contradicted those of Das *et al*., (2020), who found that all of the *E. faecalis* isolates they tested were resistant to the antibiotic.

Moreover, Vrostková *et al*., (2021) found that all of the *E. faecalis* they collected was sensitive to Nitrofurantoin, a finding corroborated by Bhola *et al*., (2020), who also found that all of the *E. faecalis* isolates they tested were sensitive to the antibiotic. All *E. faecalis* isolates tested by Lengliz *et al*., (2022) showed extreme sensitivity to vancomycin, Teicoplanin, and linezolid. While Sengupta, *et al.*, (2021) reported identifying a linezolid-resistant *Enterococcus* isolate, they could not locate any such strain. All *Enterococcus* isolates were sensitive to gentamicin, ciprofloxacin, vancomycin, linezolid, and erythromycin in a study that sought to determine the antimicrobial resistance pattern of the *Enterococcus* species from vaginal flora.

Pathogenesis of infection by enterococci requires multiple steps beyond antibiotic resistance, including colonization of and adherence to host tissues, invasion of those tissues, and adaptation to and resistance of host defenses (Sora *et al*., 2021).

All *E. faecalis* isolates from a urine sample were sensitive to Vancomycin in a study by Esmail *et al*., (2019), but only 15.6% of isolates from a study by Kim *et al.*, (2021) showed sensitivity to the antibiotic. According to Tollu and Ekin, (2021), all of the *E. faecalis* strains they tested in feces showed a sensitivity to the antibiotic vancomycin.

Among enterococcal isolates from a urinary tract infection, resistance to Vancomycin was highest (90.4%) (Zavaryani *et al*., 2020). All *E. faecalis* isolates were Tetracycline-resistant according to Mengesha (2022), but *E. faecalis* isolates were resistant to Nitrofurantoin in rate (75%), but not Tetracycline, according to Maleki *et al*., (2021).

A study by Noroozi *et al*., (2022) indicated that, all *E. faecalis* strains tested were resistant to nitrates, and 75% were resistant to tetracycline. According to Esfahani *et al*., (2020), 70% of *E. faecalis* isolates exhibited resistance to Ciprofloxacin.

In contrast to the findings of Shiadeh *et al*., (2019), which showed that, *E. faecalis* isolates from UTI were resistant to vancomycin and ciprofloxacin one hundred percent of the time, those of Salm *et al*., (2022) concluded that, ciprofloxacin should no longer be used to treat *E. faecalis* from complicated UTI in men with risk factors because (47%) of those isolates were resistant to the drug.

Over the past 15 years, many different types of resistance mechanisms have emerged, putting intense strain on glycopeptide antibiotics. The main mechanism through which enterococcal resistance has emerged is the upregulation of genes encoding proteins that alter the timing of cell wall production, allowing the bacteria to dodge the antibiotic's effects (Abushaheen *et al*., 2020).

Serious infections caused by bacteria with acquired resistance to vancomycin (caused by transposons or plasmids) can arise in hospital wards that routinely provide the antibiotic. Hospital-acquired infections and infections in intensive care unit (ICU) patients have been linked to vancomycin-resistant enterococci (VRE) (Nellore *et al*., 2019).

The therapeutic options for treating infections caused by these microorganisms are constrained by the emergence of enterococci resistant to glycopeptides, the increase in high-level resistance of aminoglycosides, the production of lactamase by certain strains, and the intrinsic resistance of enterococci to several antimicrobials (Miller *et al*., 2020).

Antibiotic overuse for the prevention of nosocomial infections caused by *E. faecalis* has led to the development of resistant forms of the bacteria, making treatment more difficult. For complete bacterial elimination, it is advised to take Fluroquinolones such as ciprofloxacin, levofloxacin, and Norfloxacin for a period of 4–12 weeks (Karam *et al*., 2019).

Due to inadequate active transport across the cytoplasmic membrane, enterococci are genetically predisposed to resist low concentrations of aminoglycosides. Therefore, aminoglycosides are typically coupled with inhibitors of cell wall production that may enhance their uptake because they are ineffective against enterococcal infections on their own. High peak blood concentrations of aminoglycosides following once-daily dosing have been advocated for therapeutic use because of their superior efficacy compared to longer-established dosing schedules (Reygaert, 2018).

The widespread distribution of genes encoding aminoglycoside modifying enzymes has contributed greatly to the rise in aminoglycoside resistance. Since aminoglycosides are nephrotoxic, their usage is restricted in the critically ill. Similarly, enterococci are resistant to aminoglycosides, and the bactericidal concentrations in the serum would be far greater than is safe for humans to obtain (Gagetti *et al*., 2019).

However, an intracellular bactericidal aminoglycoside concentration can be achieved by using a cell wall-active drug at the same time. In clinical situations where the illness poses a serious risk to the patient's life, bactericidal antimicrobial action is necessary (Kennewell *et al*., 2019).

Over time, the *E. faecalis* species developed a high level of resistance to aminoglycosides and glycopeptides. Nosocomial infections are associated with a twenty-fold rise in the incidence of vancomycin-resistant Enterococci. However, Rao *et al*., (2021) have shown that vancomycin and ciprofloxacin-susceptible *E. faecalis* strains are present.

Since the existing Fluroquinolones, including ciprofloxacin, only display moderate *in vitro* activity, they are of limited interest in the treatment of infections caused by Enterococci. In treating infections caused by these germs, Fluroquinolones may be a viable option (Terreni *et al*., 2021).

However, there is cause for concern due to the introduction and rapid extension of resistance acquisition to Fluroquinolones by Enterococci; ciprofloxacin resistance increased (from 1% to 15%) among Enterococcus faecalis strains (Bennani *et al*., 2020).

E. faecalis were found to be resistant to Fluroquinolones due to a mutation in the *gyrA* gene, which encodes the subunit of the DNA gyrase enzyme (Solano-Gálvez *et al*., 2020). Resistance to multiple classes of antibiotics, including aminoglycosides, beta-lactams, macrolides, and Fluroquinolones, is a rare feature of enterococci (Mutuku *et al*., 2022). Inherent reduced susceptibility to several commonly used antimicrobial agents, such as aminoglycosides (except for high level resistance), clindamycin, cephalosporins, and trimethoprim/sulfamethoxazole (Garca-

Solache & Rice, 2019), makes antimicrobial therapy of infections caused by enterococci difficult. Also complicating matters is the fact that bacteria can gain resistance to a variety of commonly used antibiotics when their genes are transferred laterally to other organisms. This includes the beta-lactams, aminoglycosides (high level resistance), macrolides, glycopeptides, and oxazolidinones (Torres *et al.*, 2018).

Mutation and *HGT* processes also allow enterococci to acquire resistance mechanisms to other important antibiotics, such as erythromycin, Fluroquinolones, rifampicin, chloramphenicol, Nitrofurantoin, fusidic acid, glycopeptides (vancomycin & Teicoplanin), and high concentrations of aminoglycosides and β-lactams (Kateete *et al.*, 2019; Said & Abdelmegeed, 2019).

Broad-spectrum antibiotics like ciprofloxacin are effective against a wide variety of bacteria, both Gram-positive and Gram-negative. Additionally, they are safe for oral consumption and have little adverse effects. As a result of these benefits, Ciprofloxacin has become the go-to empirical treatment for UTI (Aziz *et al*., 2022). At the same time, and maybe as a result, urinary tract infections. There has been a disturbing rise in the prevalence of bacterial strains that are resistant to the antibiotic ciprofloxacin. *E. faecalis*-related UTIs have been treated with Ciprofloxacin, however this has often been done incorrectly or on an empirical basis (Shiadeh *et al*., 2019). Many investigations have reported the rise of Ciprofloxacin-resistant strains of *E. faecalis* due to these factors. However, risk factor study for resistance to Ciprofloxacin among *E. faecalis* isolates from patients with complex UTIs is lacking. (Farman *et al*., 2019).

Antibiotic resistance is a growing problem, with over (70%) of *E. faecalis* found to be resistant to macrolides, fluoroquinolones, and aminoglycosides (Kim *et a*l., 2021; Yi *et al*., 2022). Such a high level of resistance not only hinders treatment but also permits the microorganism to thrive and grow in the hospital (Behzadi *et al*., 2021).

3.7 Molecular detection of some virulence factors genes among *E. faecalis* isolates

E. faecalis strains have produced a variety of biologically active extracellular products similar to the virulence factors of bacteria and these virulence factors associated with health effects in humans (Wang *et al*., 2021). In the clinical *E. faecalis* isolates, Farman *et al*., (2019) found that, fourteen virulence factor genes patterns were discovered, with *ace-asa1-cylA-frsA-esp-gelE* being the most common.

E. faecalis produce virulence factors such as enterococcal surface protein (*esp*) and aggregation substance (*agg*) which could enhance the colonization process in the host and binding to the host epithelium, respectively. Others such as cytolysin, enterolysin A, gelatinase, hyaluronidase, Znmetalloendopeptidase, and adhesion-associated protein *EfaA* (*E. faecalis* endocarditis antigen A) have been reported to be among the most important virulence factors which are important in the pathogenesis of human disease (Venkateswaran *et al*., 2022).

Numerous factors are associated with a greater risk of acquiring enterococcal infections. These factors, including antimicrobial resistance and expression of virulence factors associated with infection-derived *E. faecalis* strains, may account for the establishment and maintenance of this opportunistic pathogen as major community-acquired and

nosocomial pathogens (Li *et al*., 2022). in the present study is used to detect the presence of virulence factor genes, the virulence gene in this study were *Van*, *gelE*, *cpd*, *cylA*, *ebpA*, *frsA* and *sprE* genes.

3.7.1 Detection of Vancomycin gene (*VanA* gene)

Analysis of the *E. faecalis* vancomycin gene revealed that, out of 44 samples, 16 (36.3%) had the *vanA* gene, as shown by the presence of 314 bp bands when compared with an allelic ladder as shown in (Figure 3-6).

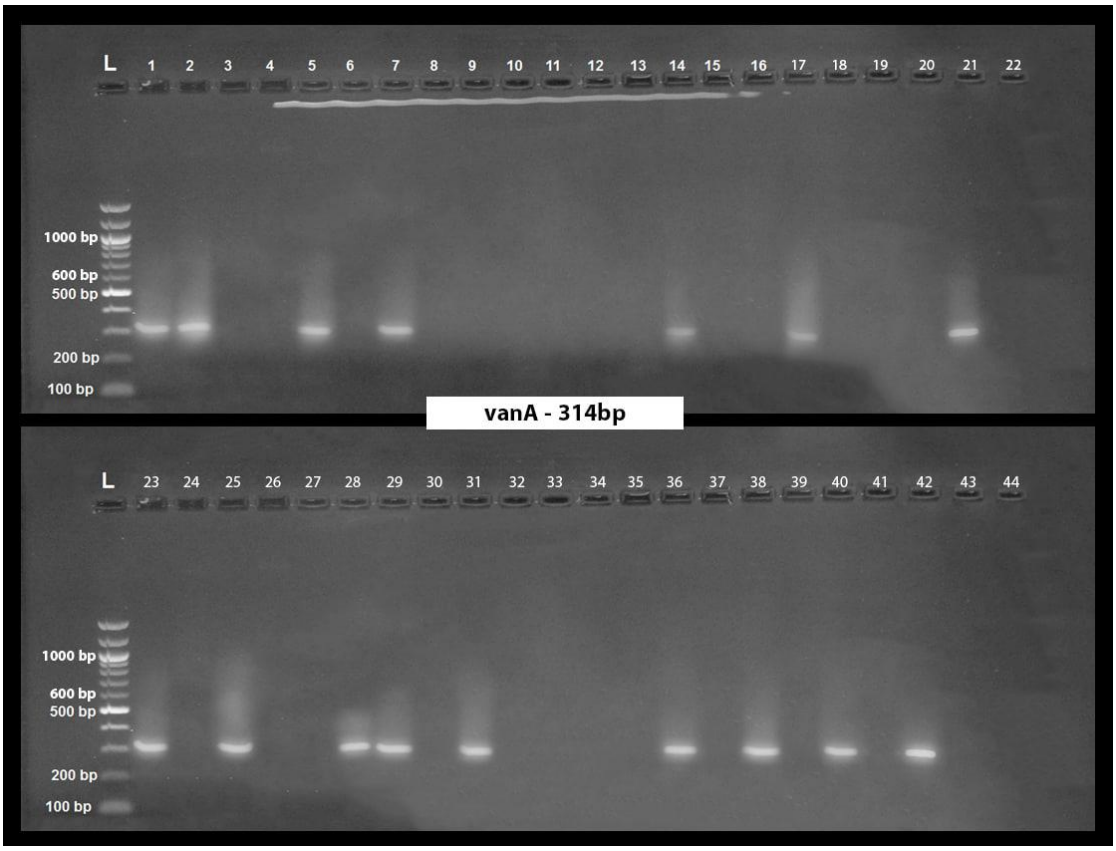

Figure (3-6): Agarose gel electrophoresis (1.5%) of PCR amplified of *vanA* gene at (314bp) of *E. faecalis* for (55) min at 5 volt/cm2. 1x TBE buffer for 1:30 hours.

L: DNA ladder (100). Line (1,2,5,7,14,17,21,23,26,28,2931,36,38,40 and 42) were showed positive results of *vanA* gene.

These findings were consistent with those of Maleki *et al*., (2021), who observed that, the *vanA* gene was discovered in *E. faecalis* at a rate of 95.8%. According to research by Li *et al*., (2022), 99.7 % of *E. faecalis* isolates tested positive for the presence of the *vanA* gene. *vanA* was detected in *E. faecalis* strains isolated from diverse geographic locations and time periods in Europe and the United States, as well as in strains that were resistant to vancomycin.

Results from the study by Zalipour *et al*., (2019) showed that 90.6% of *E. faecalis* tested positive for the vancomycin gene. Among 59 isolates tested for resistance to vancomycin, *vanA* gene detection was observed in 54(91.5%), as reported by Moosavian *et al*., (2018). The two most significant glycopeptide-resistant enterococci genotypes are *vanA* and *vanB*, although there are also *vanC*, *vanD*, *vanE*, and *vanG* (Butiuc-Keul et al., 2022).

Patients admitted to the hospital often have a phenotype associated with the *vanA* or *vanB* gene. Both vancomycin and teicoplanin are ineffective against enterococci carrying the *vanA* gene, however *vanB*-carrying enterococci are extremely resistant to vancomycin but susceptible to teicoplanin (Yan *et al*., 2022).

Trautmannsberger *et al*., (2022) and Hammerum *et al*., (2019) both demonstrated the presence of the *vanA* gene in vancomycin-resistant *Enterococcus* isolates, linking the spread of vancomycin-resistant *Enterococcus* in hospitals to the horizontal transfer of resistance genes across epidemic clones of these bacteria. Additionally, Li *et al*. (2021) found that (91.8%) of Northwest VRE strains tested positive for the *vanA* gene. These findings were compatible to some extent with the current

ones; the minor discrepancies might be due to the different specimen types and time periods used.

Since the *vanA* gene, which codes for a protein essential for cell division, is clustered and takes up a sizable chunk of chromosomal space, the likelihood of its being passed from one strain to another is diminished. The *vanA* gene is also strongly linked to pandemics and food poisoning (Moosavian *et al*., 2018).

Glycopeptide resistance, distinguishable by *vanA* and *vanB* phenotypes of resistance to vancomycin and teicoplanin, is a significant characteristic in clinical and environmental isolates of enterococci (Kutkowska *et al*., 2019).

3.7.2 Detection of gelatinase gene (*gelE* gene)

The virulence factor gelatinase gene in *E. faecalis* was studied in 44 isolates of the bacteria obtained from various environments. Results from our analysis indicated that, only 14 isolates (31.8%) tested positive for this gene, with a molecular length of 213 bp as shown in (Figure 3-7).

This study's findings corroborated those of Esmail *et al.*, (2019), who isolated 15(60%) of 25 *E. faecalis* strains from human urine and discovered that, 15 were connected to this gene. Kaviar *et al*., (2022) observed that, 100% of *E. faecalis* isolates had the *gelE* gene, hence these findings contradict their findings. According to the research by Iseppi *et al*., (2020), the *gelE* gene was the most often discovered of the components it examined, being present in 52.8% of all isolates.

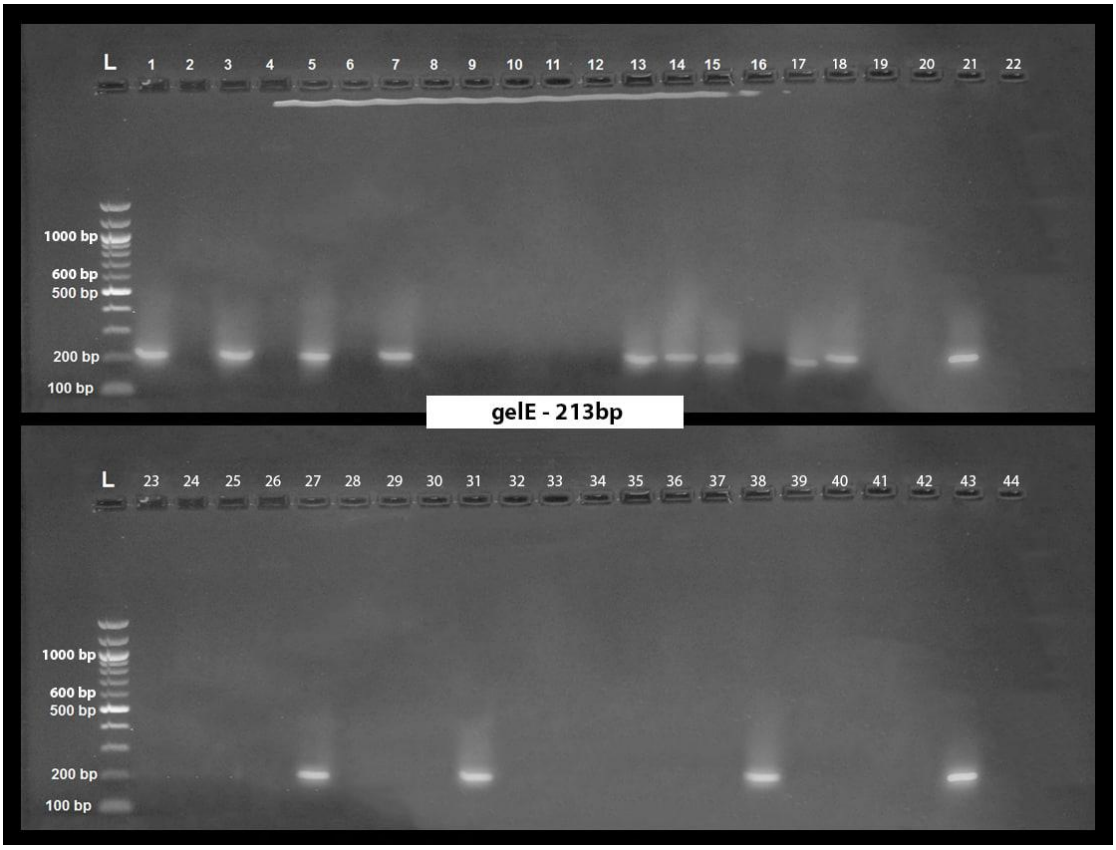

Figure (3-7): Agarose gel electrophoresis (1.5%) of PCR amplified of *gelE* gene at (313bp) of *E. faecalis* for (55) min at 5 volt/cm2. 1x TBE buffer for 1:30 hours. L: DNA ladder (100), line (1,3,5,7,13,15,15,17,18,21,27,31,38,43) were showed positive results of *gelE* gene.

Urinary tract infections (UTIs) and the development of pus reveal a correlation between the presence of the *gelE* gene in *E. faecalis* isolated from urine and wounds. The *E. faecalis* virulence factor gelatinase is an extracellular metalloprotease that hydrolyzes gelatin, collagen, and casein. This enzyme's capacity to hydrolyze collagens and certain bioactive peptides implies it plays a role in *E. faecalis*-related inflammatory processes, both at the outset and throughout their development. It is a hydrophobic metalloprotease having the capability

for cleaving insulin, casein, hemoglobin, collagen, gelatin and fibrin (Olvera *et al*., 2022). (Olvera *et al*., 2022).

There have been attempts to link its proteolytic qualities to an increase in the prevalence of enterococci in a variety of settings, including endocarditis and bacteremia (Gopalasamy, K., & Geetha, R. V. 2018), urinary infections (Liesenborghs *et al*., 2020), and oral infections (Ladjouzi *et al*., 2022). Despite having the *gelE* gene, the microbe could not produce gelatinase because of a 23.9 kb deletion in the locus fsr (Willett *et al*., 2022). In addition, 5 of the 7 gelatinase-producing strains identified in illness cases were moderate to strong biofilm makers, suggesting a probable link between gelatinase production and biofilm-forming abilities, propagation of the infectious process, and bacterial persistence in UTIs. Although the role of gelatinase in enhancing biofilm formation is still unknown,

Ferchichi *et al*., (2021) described possible models: gelatinase might participate in production of an extracellular signaling peptide by proteolytically processing an inactive secreted peptide precursor to a mature component, or it might proteolytically activate another surface protein involved in some aspects of regulation or the process of biofilm development, such as a protein that participates in secretion of extracellular polymeric matrix material. A quorum sensing system controls how much gelatinase is produced.

3.7.3 Detection of sex pheromone gene (*cpd* gene)

All *E. faecalis* isolates were subjected to *cpd* gene molecular investigations utilizing targeted PCR markers. As can be seen in Figure (3-8), 36(81.8%) of the 44 *E. faecalis* isolates tested positive for the presence of a *cpd* gene with a long length (782bp).

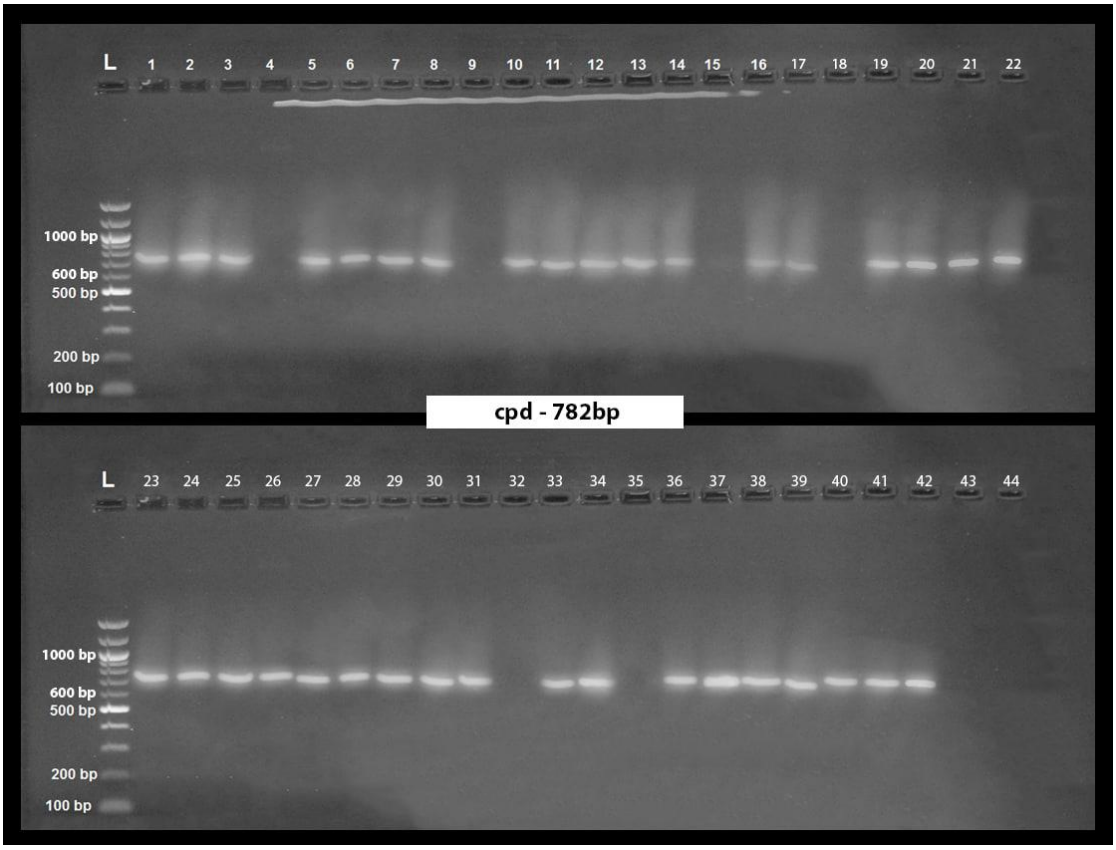

Figure (3-8): Agarose gel electrophoresis (1.5%) of PCR amplified of *cpd* gene at (782bp) of *E. faecalis* for (55) min at 5 volt/cm2. 1x TBE buffer for 1:30 hours. L: DNA ladder (100), line (1,2,3,5,6,7,8,10,11,12,13,14,14,17,16,20,21,22,,23,,24,25,26,27,28,29,30 ,31,33,34,36-42) were showed positive results of *cpd* gene.

Twenty four (96%) of 25 *E. faecalis* isolates from urine samples were confirmed to be linked to the *cpd* gene, as reported by Ferchichi *et al.*, (2021). Gene *cpd* encoding for sex pheromone peptides was more common among *E. faecalis*, according to a research by Stpie-Pyniak *et al.*, (2021). This means that, strains of *Enterococcus* that had and produced virulence components were more likely to result in a severe illness. The *cpd* gene has been shown to be more prevalent in clinical *E. faecalis* isolates, according to another investigation (Jahansepas *et al.*, 2020). Aggregation substance, a sex pheromone plasmid-encoded surface

protein, was shown to have the highest frequency of detection among *E. faecalis* strains by Venkateswaran *et al*., (2022).

All *E. faecalis* strains have the *cpd* genes. Further, *E. faecalis* production of sex pheromones may facilitate the acquisition of antibiotic resistance and virulence from other enterococci, leading to enhanced virulence. One of the most effective conjugative plasmid transfer mechanisms in bacteria is found in *E. faecalis* sex pheromone plasmids, as described by Hirt *et al*., (2018).

Peptide pheromones (such as those encoded by *cpd*) are released by recipient enterococcal cells and act as a signal to activate the conjugative apparatus of donor enterococcal cells. Thus, they serve as a mediator for the transfer of pheromone-responsive plasmids, which may have virulence genes that increase biofilm development or regulation. Key virulence factors in Enterococcus spp. are induced in response to higher cell density (Ferchichi *et al*., 2021). Multiple short peptide sex pheromones are secreted by plasmid-free *Enterococcus faecalis* strains, and these pheromones trigger a mating response in plasmid-containing bacteria. When it comes to plasmids, each pheromone has its own target (Mouton, 2019).

Plasmid-containing cells react to the particular pheromone by producing a proteinaceous "aggregation material," localized to the cell surface, which aids in the formation of mating aggregates; other processes necessary for transfer of plasmid DNA are also activated. When a plasmid is acquired, the pheromone activity that was formerly observable no longer is (Bandyopadhyay, 2018).

3.7.4 Detection of cytolysin gene (*cylA* gene)

The amplified positive samples were compared to an allelic ladder, and the results of the PCR were interpreted as a (688bp) match. As can be shown in Figure (3-9), out of a total of 44 *E. faecalis* isolates, 14 (31.8%) had the cytolycin A gene.

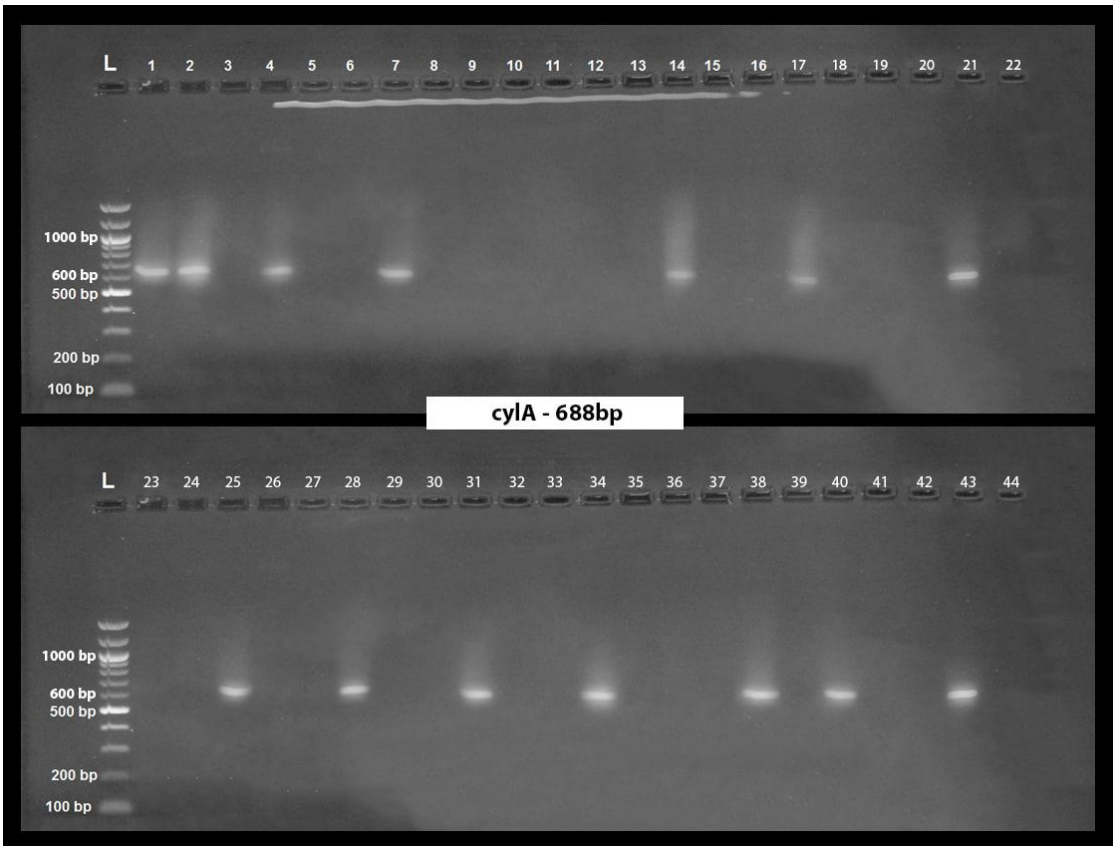

Figure (3-9): Agarose gel electrophoresis (1.5%) of PCR amplified of *cylA* gene at (782bp) of *E. faecalis* for (55) min at 5 volt/cm2. 1x TBE buffer for 1:30 hours. L: DNA ladder (100), line 91,2,4,7,14,17,21,25,28,31,34,38,40,43) were showed positive results of *cylA* gene.

These findings contradict those of Kiruthiga *et al*., (2020), who reported that, 100% of *E. faecalis* isolates had the *cylA* gene. Seventy percent of the clinical strains and only twenty-five percent of the stool isolates were discovered to generate *cylA* by Aladarose *et al*., (2019).

These results highlight the potential significance of this component in infectious diseases affecting humans. *E. faecalis* isolates were found to have a considerably greater frequency of hemolytic genes (47.1%), correlating with the results published by Kouhi *et al*., (2022). The occurrence of the *cylA* gene differed from one *E. faecalis* strain to the next. Some environmental conditions may also affect gene expression.

Some strains of *E. faecalis* that produce the toxin cytolysin have been linked to particularly severe cases of infection in humans (Liu *et al*., 2021). Results from Kiruthiga *et al*., (2021) indicated that 28.6% of human isolates were hemolytic. Among *cylA* positive/hemolysin-negative bacteria, the absence of cytolycin phenotypic/genotypic congruence may indicate the presence of missing genes in the cyl operon.

In this investigation, all of the isolates that carried the *cyl* gene also showed beta hemolytic activity. However, not every isolate that, exhibited hemolytic activity phenotypically also carried the *cyl* gene. Contrarily, it has been found that certain isolates containing the cytolysin gene do not show any phenotypic evidence of hemolytic activity.

Researchers have shown that human infections are more hazardous when the cytolysin is present. Results from a retrospective investigation of (190) clinical *E. faecalis* isolates showed that (45%) were cytolysin positive. Patients infected with cytolytic *E. faecalis* had a five-fold increased risk of an acutely terminal outcome (death within three weeks of diagnosis) compared to patients infected with non-cytolytic strains, and this was true even after accounting for treatment modality and drug resistance (Garca-Solache & Rice, 2019).

According to Aşgn & Taşkn (2019), cyl has a lytic action in macrophages, neutrophils, and cells of the retina and intestine. According

to research by Zou and Xia (2020), 14.1% of all Enterococci have this gene, and *E. faecalis* is especially linked to urinary tract infections. Ayobami *et al*., (2020) also found that, only 39% of isolates had the *cylA* gene. The high mortality and morbidity rates associated with enterococcal infections are due to the fact that these pathogens are able to lyse and kill a wide range of human cell types (Meade *et al*., 2020).

Cytolysin E is a superfamily of toxins and bacteriocins produced by gram-positive bacteria, both pathogenic and nonpathogenic. Since lyses a huge number of gram-positive bacteria in addition to canine, rodent, and horse erythrocytes, which are toxic to a wide variety of prokaryotic and eukaryotic cells, this improves the virulence of Enterococcal infection and correlates with survival in patients (Almeida-Santos *et al*., 2021).

Cyl is bactericidal against other Gram-positive bacteria and has beta haemolytic characteristics in humans. Conjugative hly plasmid may have a significant role in the dissemination (mobilization) of resistance determinants, which may lead to high antibiotic resistance in hemolytic isolates (Sadowy, 2021).

The toxin has been linked to hemolysis, endophthalmitis, lowered immunity, and gastrointestinal illnesses due to its ability to lyse human erythrocytes, polymorphonuclear leukocytes, retinal cells, and intestinal epithelial cells. It was shown that the cytolysin phenotype might be more dangerous than the isogenic non-cytolysin strain (Araya *et al*., 2022).

The cytosolic transport and activation processes are controlled by the *cylA* gene. Post-translational modification is one potential use for the *cylB* and *cylM* genes, while the *esp* gene has been linked to a cell wall protein involved in immune evasion (Molechan, 2019a).

3.7.5 Detection of Endocarditis biofilm associated pili gene (*ebpA* gene)

All 44 *E. faecalis* isolates were tested for the presence of the *ebpA* gene, and the results showed that 34 (77.2%) of the isolates were positive. Gel electrophoresis was used to identify the (517 bp) amplicon, and it was compared to an allelic ladder as shown in Figure (3-10).

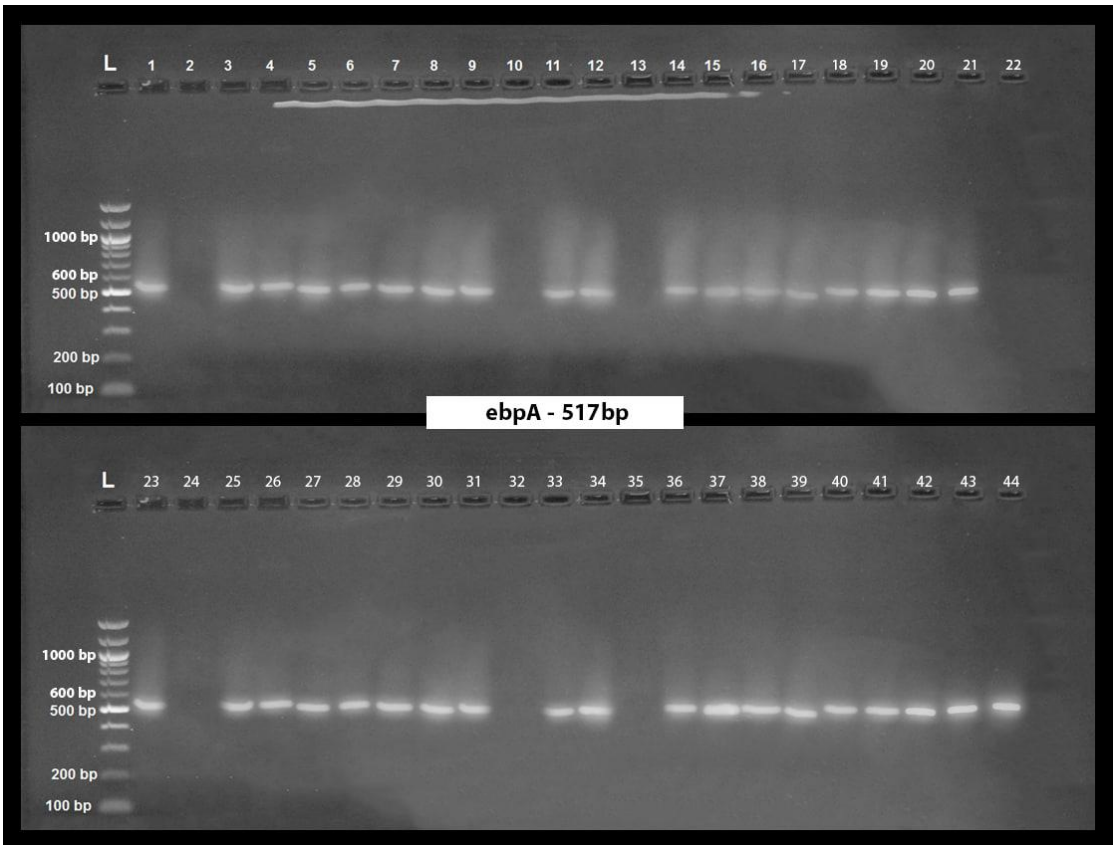

Figure (3-10): Agarose gel electrophoresis (1.5%) of PCR amplified of *ebpA* gene at (517 bp) of *E. faecalis* for (55) min at 5 volt/cm2. 1x TBE buffer for 1:30 hours. L: DNA ladder (100), line (1,3,4,5,6,7,8,9,11,12,14,15,16,17,18,19,20,21,23,25,26,27,28,29,30,31,33,34,36-44) were showed the positive results of *ebpA* gene.

It was revealed in this investigation that, the *ebp* gene was distributed at an 88.1% rate among *Enterococci* isolates, which is consistent with the findings reported by Mouton, (2019b). Based on research by Ramirez *et al*. (2020), it has been shown that the widely distributed *ebp* genes, which code for both expressed and antigenic proteins, are located in a

polycistronic operon that also includes a gene producing sortase. It seems that *E. faecalis* pili are generated by the sequential cross-linking of three distinct *ebp* proteins, each of which has a conserved motif sequence recognized by a specific sortase.

Although only a small percentage of *E. faecalis* cells develop ebp pili, these structures are crucial to the pathogenicity of the bacteria, suggesting that *ebp* proteins might be used as immunotargets (Afonina *et al*., 2018). Endocarditis- and biofilm-associated pili (*ebp*) are adhesions encoded by *Enterococcus faecalis* that have been shown to contribute to biofilm development on abiotic surfaces and in endocarditis (Venkateswaran *et al*., 2022).

Pili inhibit AS-mediated clumping and AS-mediated conjugative plasmid transfer during planktonic development when both genes are expressed on the same set of cells, as is the case with Ebp. Pilus expression initially suppressed horizontal gene transfer rates, but as populations grew, these rates returned to normal. However, at greater cell densities, Ebp synergize to promote maximum biofilm formation by contributing differently to biofilm growth and structure (Khdir, 2020).

3.7.6 Detection of Serine protease gene (*sprE* gene)

For gene identification of *sprE*, a specific PCR primer was utilized. Only 12(27.2%) of the 44 *E. faecalis* isolates tested positive for the *sprE* gene, with a long length of 591 base pairs (bp), as shown in Figure (3-11).

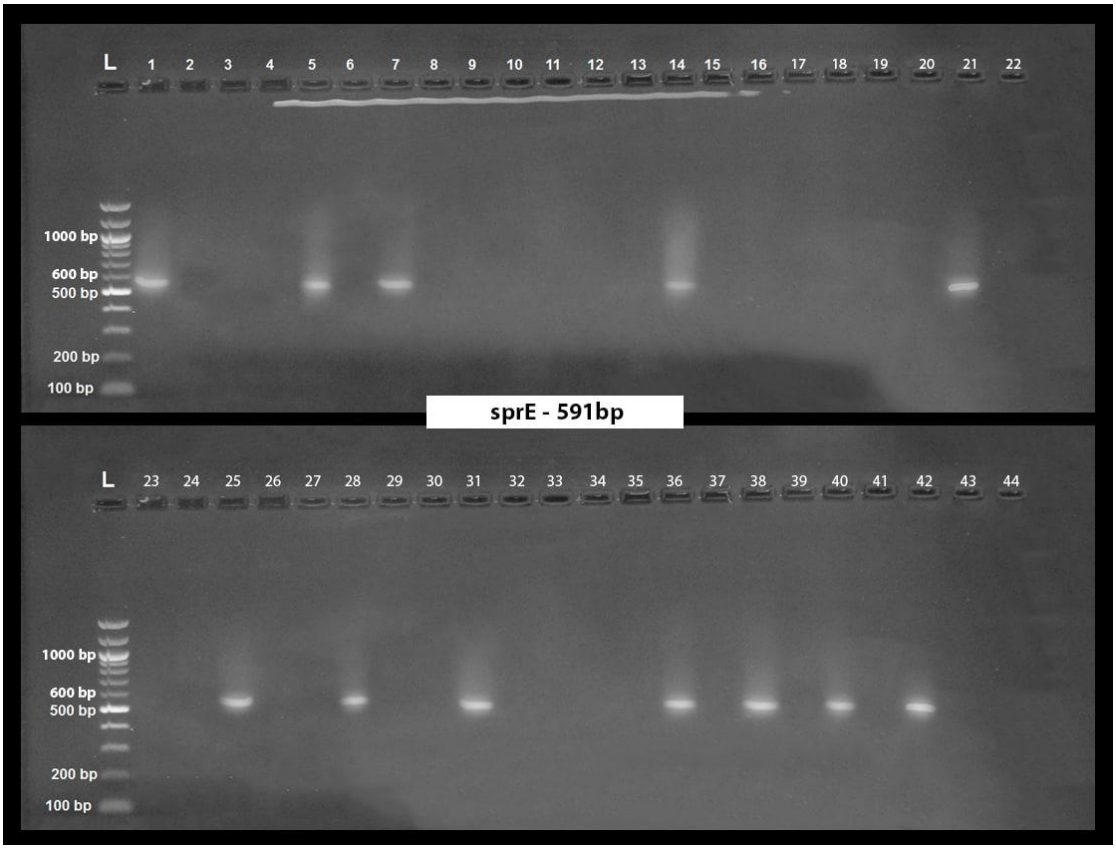

Figure (3-11): Agarose gel electrophoresis (1.5%) of PCR amplified of *sprE* gene at (**591bp) of *E. faecalis* for (55) min at 5 volt/cm2. 1x TBE buffer for 1:30 hours. L: DNA ladder (100), line (1,5,7,14,21,25,28,31,36,38,40,42) were showed the positive results of *sprE* gene.**

In contrast to the findings obtained by Hasan *et al.*, (2018), who reported that, downstream *sprE* gene were identified in all *E. faecalis* isolates (100%) that were discovered by PCR, the results of this investigation showed no such consistency. Based on these findings, *sprE* may have synergistic effects on virulence. Yet another interpretation of the data is that the *sprE* system controls virulence genes.

The quorum-sensing fsr locus controls the transcription of the gelatinase (*gelE*) and serine protease (*sprE*) genes. It has the ability to cleave sex pheromones, which are strong chemo-attractants and may alter

the host's reaction (Ali *et al*., 2022). *GelE* and *sprE* genes were present in *E. faecalis* strains having gelatinase activity, correlating with previous findings by Hashem *et al* (2021).

Kao and Kline, (2019) showed that the proteases separately contribute to *E. faecalis* pathogenicity in distinct infections. As shown by Venkateswaran *et al*., (2022), sprE promotes and represses autolysis in *E. faecalis*. The fsr quorum-sensing system positively regulates the expression of sprE, a serine protease. The *gelE* and *sprE* genes are close to the *fsr* genes and are controlled by the same promoter. Together, the fsr quorum-sensing system and these proteases play a role in pathogenicity, host-tissue destruction, and biofilm development (Willett *et al*., 2022).

3.7.7 Detection of quorum sensing *gene* (*fsrA* gene)

Among 44 *E. faecalis* isolates tested for the presence of the *fsrA* gene, only 20(45.4%) were found to be positive. As can be seen in Figure (3-12), when the (474 bp) band was compared to the allelic ladder, positive findings were obtained for the *fsrA* virulence gene.

Current findings are in accordance with the *fsrA* gene was linked to an *E. faecalis* infection in a study by Najafi *et al*. (2020). The fsr locus of *E. faecalis* was discovered to encode a two-component regulatory mechanism that detects the cell density and modulates virulence by Patil *et al*., (2021). There are four genes that make up the 2.8 kb fsr locus: *fsrA*, fsrB, *fsrD*, and *fsrC*. *E. faecalis*, a bacterium that causes nosocomial infections, relies on quorum sensing mechanisms to regulate key virulence characteristics. The cytolysin operon, which encodes the cytolysin toxin, is a component of the *E. faecalis* quorum-sensing system (Ali *et al*., 2022).

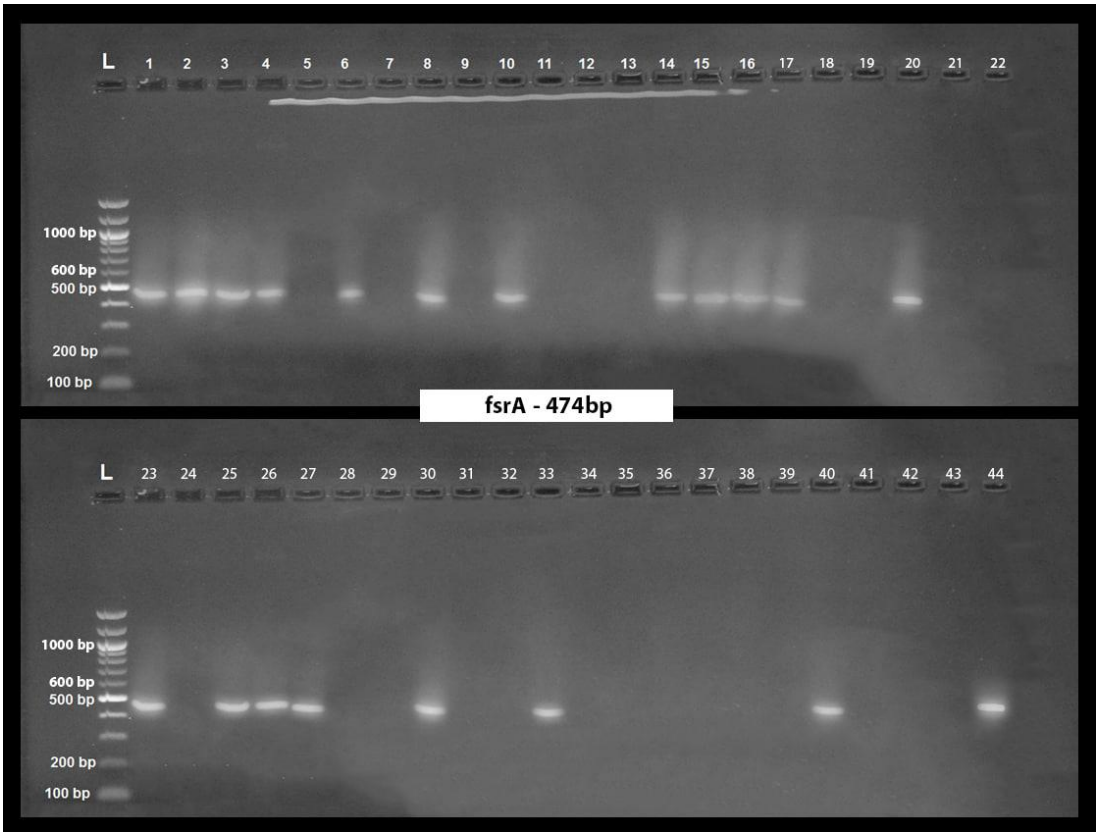

Figure (3-12): Agarose gel electrophoresis (1.5%) of PCR amplified of *fsrA* gene at (474bp) of *E. faecalis* for (55) min at 5 volt/cm2. 1x TBE buffer for 1:30 hours. L: DNA ladder (100), line (1,2,3,4,6,8,10,14,15,16,17,20,23,26,27,30,33,40,44) were showed positive results of *fsrA* gene.

In addition, the expression of gelatinase, serine protease, and enterocin O16 is regulated by the *E. faecalis* Fsr regulator system (Willett *et al.*, 2022). Human and animal models of enterococcal illness have been connected to the cytolysin and Fsr virulence factor systems (Bin-Asif & Ali, 2019). Thus, there is a strong need to learn about and manipulate these regulatory mechanisms in order to create new treatments for the prevention and treatment of enterococcal infections (Venkateswaran *et al.*, 2022).

Therapeutic drugs that decrease quorum sensing may be useful in reducing the harmful effects of *E. faecalis*. Therapeutic strategies for preventing *E. faecalis* infection are discussed, along with the control of cytolysin, the LuxS system, and the Fsr system, and their roles in *E. faecalis*-mediated infections (Desouky *et al*., 2022).

As Krishnamoorthy *et al*., (2020) report, the Fsr system is required for the expression of four genes: *gelE, sprE, ef1097*, and *ef1097b*. The FsrA protein is encoded by the *fsrA* gene and contains a DNA-binding domain of the LytTR family.

FsrA is likely a response regulator of the Fsr system since phosphorylated FsrA binds to LytTR-binding sites in the upstream region of ef1097, fsrB, and gelE. In particular, fsrA transcription is not reliant on the Fsr quorum-sensing system since it is controlled by a constitutive promoter. FsrA, a member of the AgrA family of accessory gene regulator proteins, is encoded by the *fsrA* gene (Deepika & Bramhachari, 2018).

Wu and Luo, (2021) revealed that the *E. faecalis* fsr system is the second example of a quorum-sensing system that modulates virulence gene expression during bacterial infection of both simple model organisms and mammalian hosts. Many pathogenic prokaryotes may employ quorum sensing as a key method for adjusting to novel settings. In addition to *gelE* and *sprE*, our findings suggest that, the fsr system in *E. faecalis* controls other virulence genes (McBrayer *et al*., 2020). Table (3-3) showed a total numbers of genes were detection from *E. faecalis* isolates.

3.7.8 Detection of Collagen-binding adhesion gene (*ace* gene)

Among 44 *E. faecalis* isolates tested for the presence of the *ace* gene, only 15(34%) were found to be positive. As can be seen in Figure (3-13), when the (320bp) band was compared to the allelic ladder, positive findings were obtained for the *ace* virulence gene.

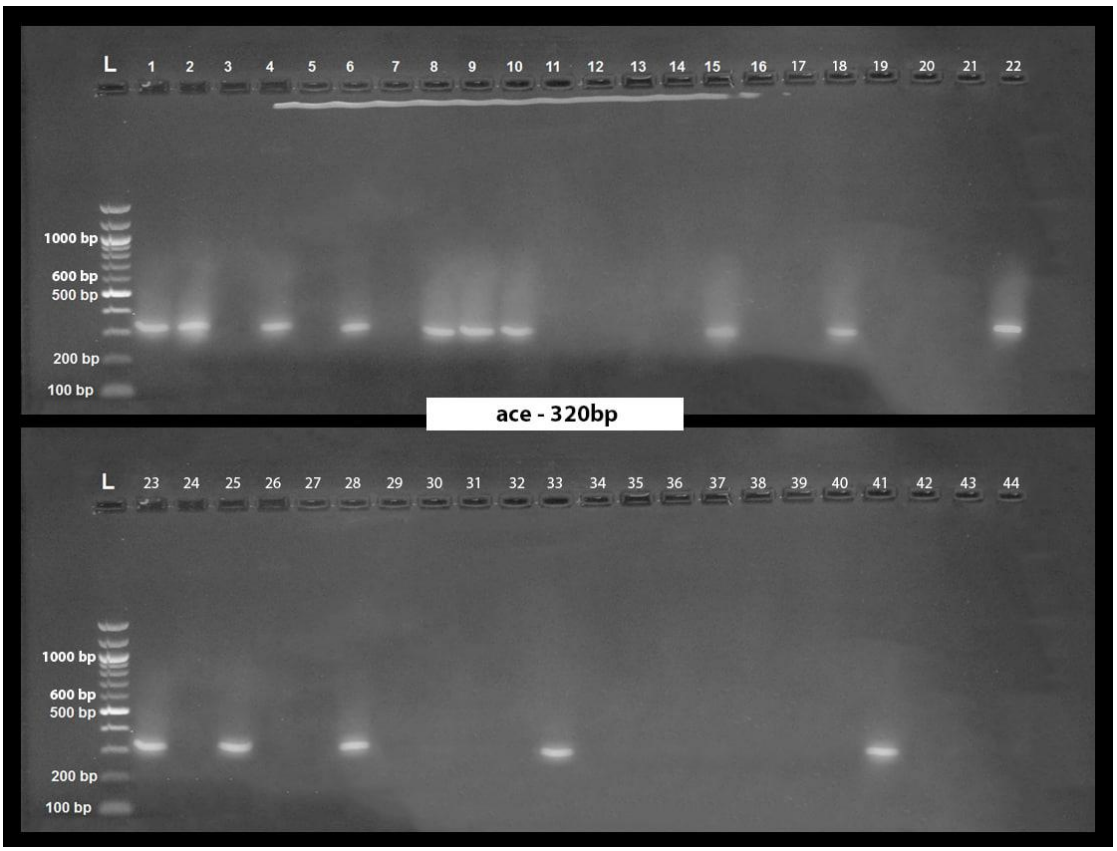

Figure (3-13): Agarose gel electrophoresis (1.5%) of PCR amplified of *ace* gene at (320bp) of *E. faecalis* for (55) min at 5 volt/cm2. 1x TBE buffer for 1:30 hours. L: DNA ladder (100), line (1,2,4,6,8,9,10,15,18,22,23,25,28,33,41) were showed positive results of *ace* gene.

E. faecalis was ability to express the *ace* gene, which encodes a member of the microbial surface component recognizing adhesive matrix molecule family, is an essential first step in establishing an infection. The discovery that some strains of *E. faecalis* do not encode *ace* suggests that, these strains do not bind collagen, as *ace* is a well-established gene encoding an adhesion that specifically binds collagens. Results obtained by Rotta *et al*., (2022), who found that only four isolates showed positive result with *ace* gene (14%), were in contrast with the results obtained by the current study.

Furthermore, the findings of this study contradicted those of Hashem *et al*., (2022), who reported that (72%) of *E. faecalis* isolates from urine samples were associated to *ace* gene.

Ace gene significantly reduced *E. faecalis* capacity to colonize host tissue, as reported by Jovanović *et al*., (2023). This suggests that *ace* mediates *E. faecalis* adherence to collagen exposed at the site of tissue injury, a critical step in the initiation of colonization. In addition, *ace* is a useful therapeutic target against human UTIs, as noted by Singh *et al*., (2019). The attachment of *Enterococcus faecalis* to extracellular matrix is thought to be an important step in the life cycle of pathogenic bacteria. This adhesion is facilitated by the *ace* gene, which produces a collagen-binding protein. Results indicated that the *E. faecalis ace* was the crucial pathogenic factor at sites with additional intestines (Francisco *et al*., 2021).

The *E. faecalis* genome database revealed a substantial match. Translation of the nucleotide sequence revealed a 74-kDa protein with a structural organization very similar to other Gram-positive bacteria.The complete sequence of the gene encoding this protein has been obtained; it

has been given the working name *Ace* (adhesion of collagen from *E. faecalis*) (Russo, 2019).

Several *E. faecalis* strains can produce *Ace* exhibited antigen-like characteristics in human hosts when infecting them. Because the *ace* gene was commonly found in *E. faecalis* strains that caused UTIs, it has been hypothesized that this protein could serve as a therapeutic target. Oftentimes, bacteria infecting a host begin by attaching to and colonizing host cells. The variables that aid *E. faecalis* in adhering to host tissues, however, remain largely unknown (Bolocan *et al*., 2019).

3.7.9 Detection of Enterococcal Surface Protein gene (*esp* gene)

Among 44 *E. faecalis* isolates tested for the presence of the *esp* gene, only 18(40.9%) were found to be positive. As can be seen in Figure (3-14), when the (933bp) band was compared to the allelic ladder, positive findings were obtained for the *esp* virulence gene.

In addition to epidemiological differences, *E. faecalis* strains isolated from various locations may contain varying frequencies of or express varying amounts of the enterococcal surface protein crucial for colonization. Yet, it would appear that they are necessary during an *E. faecalis* infection because the gene encoding the surface protein was studied in one study. Using gene-specific primers, performed polymerase chain reaction (PCR) on *E. faecalis* and found that 54% of *Enterococcus* contain the *esp* gene encoding for the *Enterococcus* surface protein (Bonyadi and Amini, 2022).

Enterococci have been shown to have a number of virulence and pathogenicity variables that aid in colonization of patient tissues, antibiotic resistance, and infection severity. Enhanced virulence, urinary

tract colonization and persistence, and biofilm formation are all linked to greater esp levels, which are notably higher in clinical isolates (Hashem *et al*., 2021).

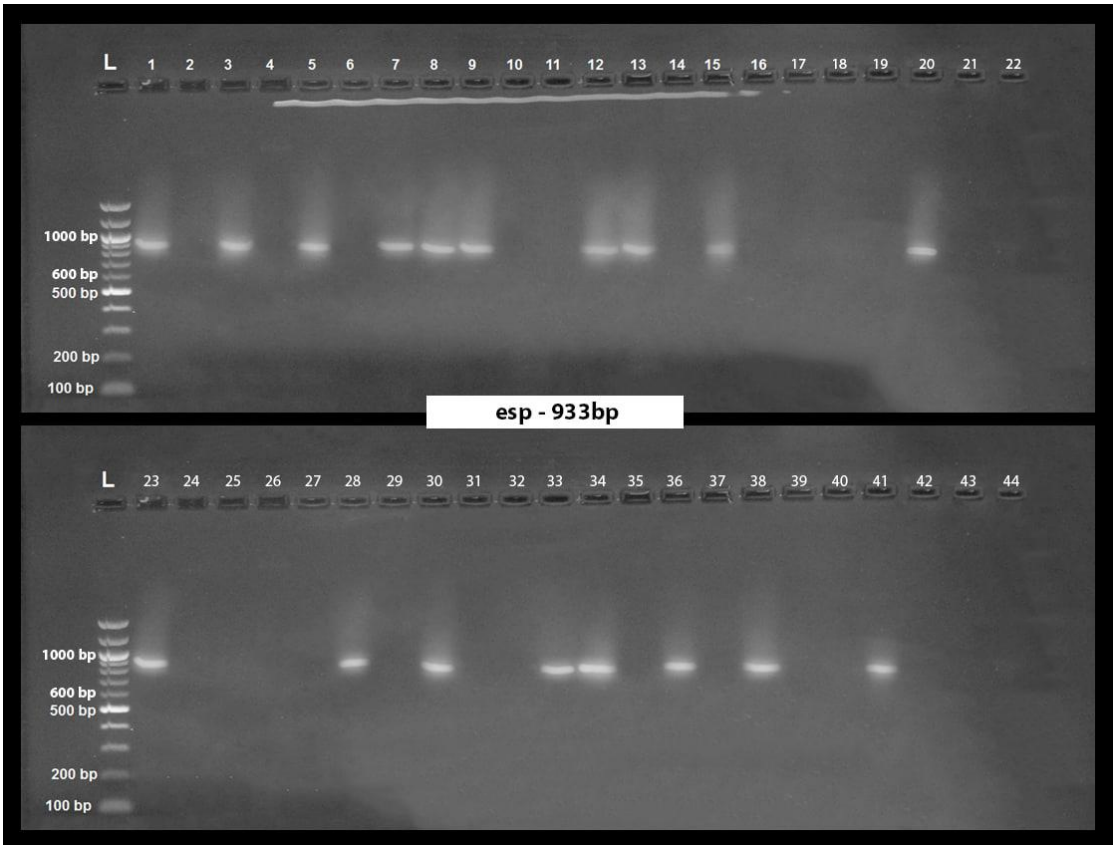

Figure (3-14): Agarose gel electrophoresis (1.5%) of PCR amplified of *esp* gene at (933bp) of *E. faecalis* for (55) min at 5 volt/cm2. 1x TBE buffer for 1:30 hours. L: DNA ladder (100), line (1,3,5,7,8,9,12,13,15,20,23,28,30,33,34,36,38,41) were showed positive results of *esp* gene.

A random sequencing study on the genome of a clinical strain of *E. faecalis* has uncovered the *esp* gene. It has been found more frequently in *E. faecalis* strains isolated from sites of infection than in strains of other, less pathogenic enterococcal species, providing strong evidence for a function of *esp* in *E. faecalis* pathogenesis (Hua *e al*., 2022).

3.7.10 Detection of endocarditis antigen (*efaA* gene)

Among 44 *E. faecalis* isolates tested for the presence of the *efaA* gene, only 15(34%) were found to be positive. As can be seen in Figure (3-15), when the (688bp) band was compared to the allelic ladder, positive findings were obtained for the *efaA* virulence gene.

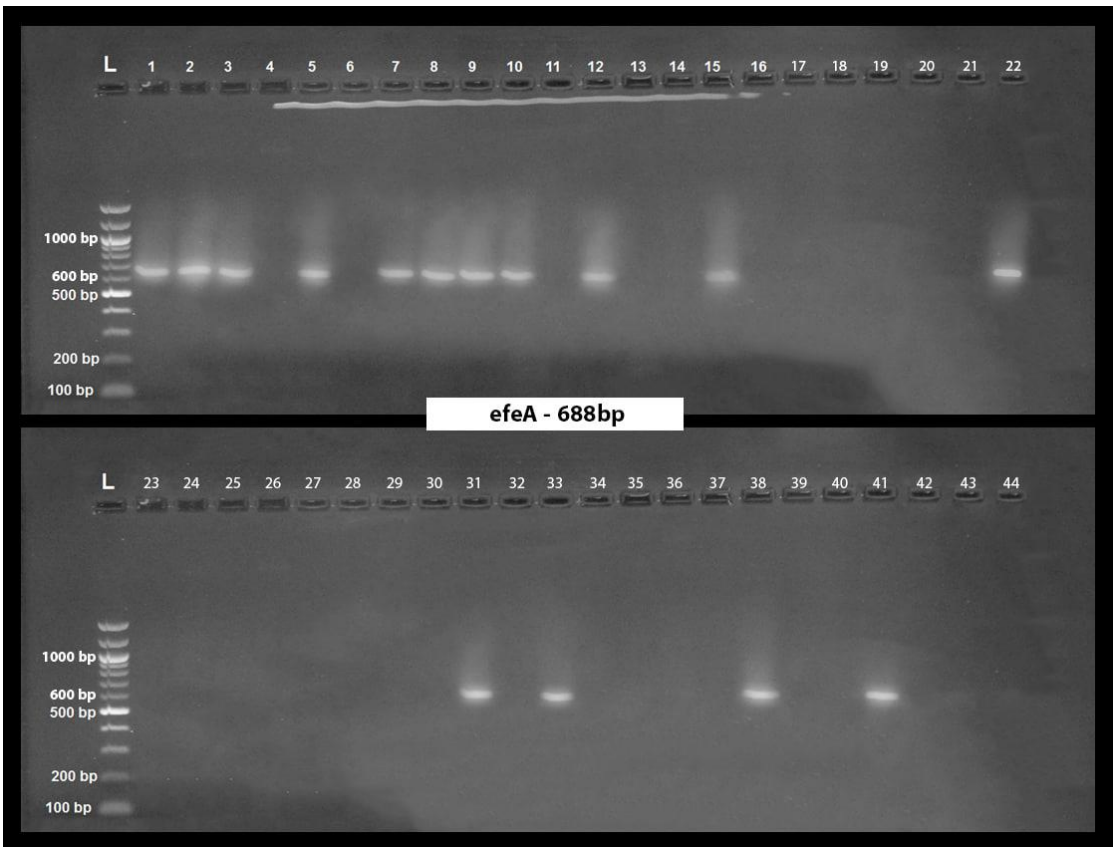

Figure (3-15): Agarose gel electrophoresis (1.5%) of PCR amplified of *efaA* gene at (688bp) of *E. faecalis* for (55) min at 5 volt/cm2. 1x TBE buffer for 1:30 hours. L: DNA ladder (100) , line (1,2,3,5,7,8,9,10,12,15,22,31,33,38,41) were showed positive results of *efaA* gene.

The result of Kayaoglu and Orstavik (2004) found Eleven out of fifteen positive *E. faecalis* samples tested positive for the presence of the *efaA* gene. Medeiros *et al*., (2014) found that, there were 10 out of

15(67%) isolates of *E. faecalis* detected positive for the *efaA* gene. A study of Golob *et al*., (2019) found that, Four out of fifteen (27%) isolates of *E. faecalis* possessed of the *efaA* gene and Leong *et al*., (2020) found one out of fifteen (6%) isolates of *E. faecalis* possessed of this gene.

Gene encoding the endocarditis antigen was the most frequently detected virulence gene in clinical *E. faecalis* isolates in the present study, which is in congruence with a study hypothesizing that *efaA* gene is important for the persistence of enterococci in UTIs (Rotta *et al*., 2022).

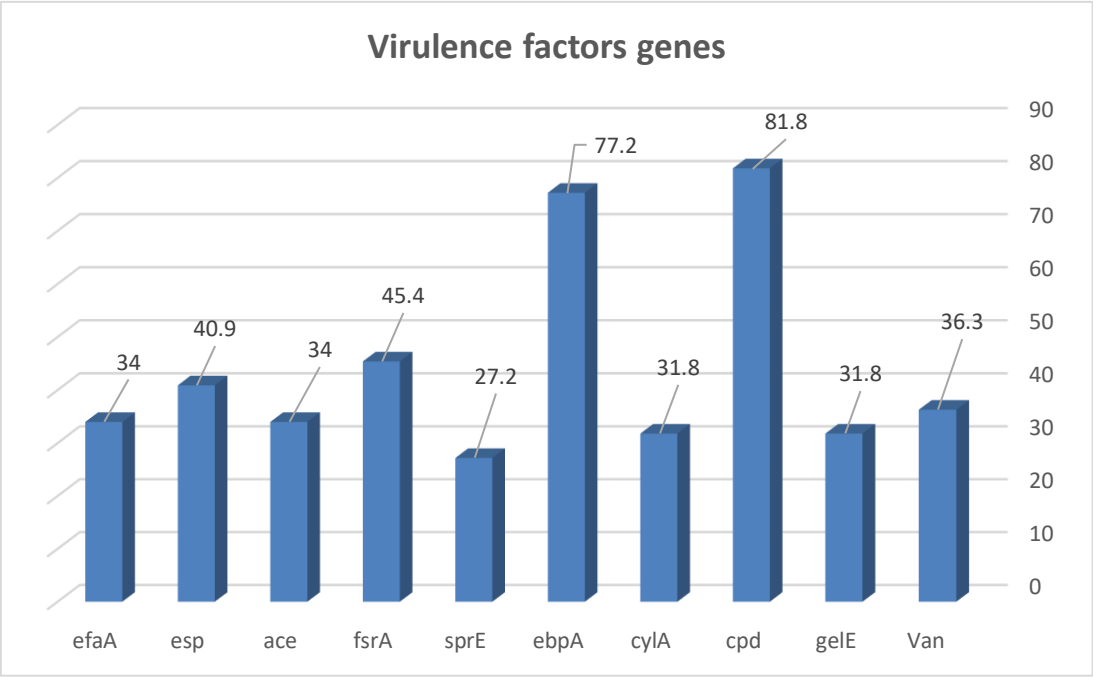

Figure (3-16): virulence factor genes determinate of *E. faecalis* isolates (Total No. =44)

3.8 DNA sequencing for some important virulence genes

After confirming the amplification of *gelE*, *sprE* and *fsrA* genes by conventional PCR, 20µl from PCR reaction with 50µl of forward primer for this genes were send to Macrogen Company to determine the DNA sequencing in these genes. Homology search was conducted using Basic

Local Alignment search Tool (BLAST) program which is available at the National Center Biotechnology Information (NCBI) online at (http://www.ncbi.nlm.nih.gov), and BioEdit program. The results were compared with data obtained from gene bank published ExPASY program which is available at the NCBI online.

3.8.1 *gelE* gene

The genes were amplified by PCR method, and send for sequencing service to Macrogen Company Korea. Thr sequencing result of *gelE* gene shows for *E. faecalis* having one transversion A/C in location (1592444 nucleotide) from the Gene Bank found part of *gelE* gene having 99% compatibility with the subject of *gelE* gene in NCBI under sequence (ID: CP0881981) as showed in Table (3-5), Figures (3-17A-D). Another part of sequencing for *gelE* gene to *E. faecalis*, the results shows compatibility of 100% in Gene Bank of *gelE* under sequence ID: CP088198.1, so no recorded change noticed from the gene in this isolate.

***Enterococcus faecalis* strain S11-6 chromosome, complete genome Sequence ID: CP088198.1Length: 2717320Number of Matches: 1**
Range 1: 1592274 to 1592460GenBankGraphicsNext MatchPrevious Match

Score	Expect	Identities	Gaps	Strand
334 bits(369)	1e-89	186/187(99%)	0/187(0%)	Plus/Minus

```
Query  1
CAATGCGTTATGGTGACACAAGTACACCAACAGGAAAAACGTATGCTTCCTCTTTAGATG  60
Sbjct  1592460
..............A.........................................  1592401
Query  61
TAGTTGGTCATGAAATGACACATGGTGTGACGGAACATACTGCCGGTTTAGAATATTTAG  120
Sbjct  1592400
.........................................................  1592341
Query  121
GACAATCAGGTGCCTTGAATGAATCTTATTCTGATTTGATGGGTTATATTATTTCGGGTG  180
Sbjct  1592340
.........................................................  1592281
Query  181      CATCTAA  187
Sbjct  1592280  .......  1592274
```

Figure (3-17A): Alignment analysis of *gelE* gene (four isolates 1, 2, 3, 4) of *E. faecalis* with Gene Bank at NCBI. Query represents of sample; subject represents a database of National Center Biotechnology Information (NCBI).

***Enterococcus faecalis* strain S11-6 chromosome, complete genome**
Sequence ID: CP088198.1Length: 2717320Number of Matches: 1
Range 1: 1592274 to 1592460GenBankGraphicsNext MatchPrevious Match

Score	Expect	Identities	Gaps	Strand
329 bits(364)	2e-88	185/187(99%)	0/187(0%)	Plus/Minus

```
Query  1
CACTGCGTTATGGTGACACAAGTACACCAACAGGAAAAACGTATGCTTCCTCTTTAGATG  60
Sbjct  1592460
..A.............A.....................................  1592401
Query  61
TAGTTGGTCATGAAATGACACATGGTGTGACGGAACATACTGCCGGTTTAGAATATTTAG  120
Sbjct  1592400
.....................................................  1592341
Query  121
GACAATCAGGTGCCTTGAATGAATCTTATTCTGATTTGATGGGTTATATTATTTCGGGTG  180
Sbjct  1592340
.....................................................  1592281
Query  181      CATCTAA  187
Sbjct  1592280  .......  1592274
```

Figure (3-17B): Alignment analysis of *gelE* gene (four isolates 1, 2, 3, 4) of *E. faecalis* with Gene Bank at NCBI. Query represents of sample; subject represents a database of National Center Biotechnology Information (NCBI).

***Enterococcus faecalis* strain S11-6 chromosome, complete genome**
Sequence ID: CP088198.1Length: 2717320Number of Matches: 1
Range 1: 1592274 to 1592460GenBankGraphicsNext MatchPrevious Match

Score	Expect	Identities	Gaps	Strand
334 bits(369)	1e-89	186/187(99%)	0/187(0%)	Plus/Minus

```
Query  1
CAATGCGTTATGGTGACACAAGTACACCAACAGGAAAAACGTATGCTTCCTCTTTAGATG  60
Sbjct  1592460
.............A.....................................  1592401
Query  61
TAGTTGGTCATGAAATGACACATGGTGTGACGGAACATACTGCCGGTTTAGAATATTTAG  120
Sbjct  1592400
.....................................................  1592341
Query  121
GACAATCAGGTGCCTTGAATGAATCTTATTCTGATTTGATGGGTTATATTATTTCGGGTG  180
Sbjct  1592340
.....................................................  1592281
Query  181      CATCTAA  187
Sbjct  1592280  .......  1592274
```

Figure (3-17C): Alignment analysis of *gelE* gene (four isolates 1, 2, 3, 4) of *E. faecalis* with Gene Bank at NCBI. Query represents of sample; subject represents a database of National Center Biotechnology Information (NCBI).

***Enterococcus faecalis* strain S11-6 chromosome, complete genome**
Sequence ID: CP088198.1Length: 2717320Number of Matches: 1
Range 1: 1592274 to 1592460GenBankGraphicsNext MatchPrevious Match

Score	Expect	Identities	Gaps	Strand
338 bits(374)	3e-91	187/187(100%)	0/187(0%)	Plus/Minus

```
Query  1
CAATGCGTTATGGTGAAACAAGTACACCAACAGGAAAAACGTATGCTTCCTCTTTAGATG  60
Sbjct  1592460
.............................................................  1592401
Query  61
TAGTTGGTCATGAAATGACACATGGTGTGACGGAACATACTGCCGGTTTAGAATATTTAG  120
Sbjct  1592400
.............................................................  1592341
Query  121
GACAATCAGGTGCCTTGAATGAATCTTATTCTGATTTGATGGGTTATATTATTTCGGGTG  180
Sbjct  1592340
.............................................................  1592281
Query  181      CATCTAA  187
Sbjct  1592280  .......  1592274
```

Figure (3-17D): Alignment analysis of *gelE* gene (four isolates 1, 2, 3, 4) of *E. faecalis* with Gene Bank at NCBI. Query represents of sample; subject represents a database of National Center Biotechnology Information (NCBI).

3.8.2 *sprE* gene

The genes were amplified by PCR method, and send for sequencing service to Macrogen Company Korea. The sequencing for *sprE* gene to *E. faecalis*, the results showed compatibility of 100% in Gene Bank of *sprE* gene under sequence ID: CP070621.1, so, no recorded change noticed from the Gene Bank in *sprE* gene as shown in Table (3-5), Figures (3-18A-D).

***Enterococcus faecalis* strain Colony534 chromosome Sequence ID: CP070621.1Length: 2996853Number of Matches: 1** Range 1: 2274299 to 2274856GenBankGraphicsNext MatchPrevious Match

Score	Expect	Identities	Gaps	Strand
1007 bits(1116)	0.0	558/558(100%)	0/558(0%)	Plus/Minus

```
Query  1
AGATCGTTACTGGACCCTGAAGACAGAAGACAAGAAGTGGCAGATACAACCGAAGCGCCT  60
Sbjct  2274856
.............................................................  2274797
Query  61
TTTGCGTCAATCGGAAGAATCATTTCCCCTGCCAGTAAACCAGGCTATATTTCTTTAGGA  120
Sbjct  2274796
.............................................................  2274737
Query  121
ACAGGCTTTGTTGTTGGAACTAATACAATTGTCACCAATAATCATGTGGCTGAAAGTTTT  180
Sbjct  2274736
.............................................................  2274677
Query  181
AAGAATGCCAAAGTATTAAATCCGAATGCCAAAGATGATGCTTGGTTTTATCCAGGTCGA  240
Sbjct  2274676
.............................................................  2274617
Query  241
GATGGCAGTGCGACACCATTTGGCAAATTCAAAGTGATTGATGTAGCTTTTTCCCCGAAT  300
Sbjct  2274616
.............................................................  2274557
Query  301
GCGGATATTGCGGTAGTGACTGTCGGCAAACAAAACGATCGTCCAGATGGCCCAGAGTTG  360
Sbjct  2274556
.............................................................  2274497
Query  361
GGAGAAATTTTAACGCCATTTGTTTTGAAAAAGTTTGAATCTTCAGATACCCATGTCACA  420
Sbjct  2274496
.............................................................  2274437
Query  421
ATATCAGGCTATCCAGGTGAGAAAAACCACACACAATGGTCTCATGAAAATGATTTGTTT  480
Sbjct  2274436
.............................................................  2274377
Query  481
ACATCTAACTTTACAGACTTAGAAAATCCATTACTATTTTATGATATCGATACAACAGGT  540
Sbjct  2274376
.............................................................  2274317
Query  541      GGTCAATCTGGTTCCCCA  558
Sbjct  2274316  ..................  2274299
```

Figure (3-18A): Alignment analysis of *sprE* gene (four isolates 1, 2, 3, 4) of *E. faecalis* with Gene Bank at NCBI. Query represents of sample; subject represents a database of National Center Biotechnology Information (NCBI).

Enterococcus faecalis **strain FDAARGOS_528 chromosome, complete genomeSequence ID: CP033787.1Length: 2963654Number of Matches: 1**Range 1: 2236578 to 2237136GenBankGraphicsNext MatchPrevious Match

Score	Expect	Identities	Gaps	Strand
1009 bits(1118)	0.0	559/559(100%)	0/559(0%)	Plus/Plus

```
Query  1
AAAAGATCGTTACTGGACCCTGAGGACAGAAAACAAGAAGTGGCAGATACAACCGAAGCG  60
Sbjct  2236578
.............................................................  2236637
Query  61
CCTTTTGCGTCAATCGGAAGAATCATTTCCCCTGCCAGTAAACCAGGCTATATTTCTTTA  120
Sbjct  2236638
.............................................................  2236697
Query  121
GGAACAGGCTTTGTTGTTGGAACTAATACAATTGTCACCAATAATCATGTGGCTGAAAGT  180
Sbjct  2236698
.............................................................  2236757
Query  181
TTTAAGAATGCCAAAGTATTAAATCCGAATGCCAAAGATGATGCTTGGTTTTATCCAGGT  240
Sbjct  2236758
.............................................................  2236817
Query  241
CGTGATGGCAGTGCGACACCATTTGGCAAATTCAAAGTGATTGATGTAGCTTTTTCCCCG  300
Sbjct  2236818
.............................................................  2236877
Query  301
AATGCGGATATTGCGGTAGTGACTGTCGGCAAACAAAACGATCGTCCAGATGGCCCAGAG  360
Sbjct  2236878
.............................................................  2236937
Query  361
TTGGGAGAAATTTTAACGCCATTTGTTTTGAAAAAGTTTGAATCTTCAGATACCCATGTC  420
Sbjct  2236938
.............................................................  2236997
Query  421
ACAATATCAGGCTATCCAGGTGAGAAAAACCACACGCAATGGTCGCATGAAAATGATTTG  480
Sbjct  2236998
.............................................................  2237057
Query  481
TTTACATCTAACTTTACAGACTTAGAAAATCCATTACTATTTTATGATATCGATACAACA  540
Sbjct  2237058
.............................................................  2237117
Query  541      GGTGGTCAATCTGGTTCCC  559
Sbjct  2237118  ...................  2237136
```

Figure (3-18B): Alignment analysis of *sprE* gene (four isolates 1, 2, 3, 4) of *E. faecalis* with Gene Bank at NCBI. Query represents of sample; subject represents a database of National Center Biotechnology Information (NCBI).

***Enterococcus faecalis* strain FDAARGOS_528 chromosome, complete genome Sequence ID: CP033787.1Length: 2963654Number of Matches: 1** Range 1: 2236578 to 2237136GenBankGraphicsNext MatchPrevious Match

Score	Expect	Identities	Gaps	Strand
1009 bits(1118)	0.0	559/559(100%)	0/559(0%)	Plus/Plus

```
Query  1
AAAAGATCGTTACTGGACCCTGAGGACAGAAAACAAGAAGTGGCAGATACAACCGAAGCG  60
Sbjct  2236578
.............................................................  2236637
Query  61
CCTTTTGCGTCAATCGGAAGAATCATTTCCCCTGCCAGTAAACCAGGCTATATTTCTTTA  120
Sbjct  2236638
.............................................................  2236697
Query  121
GGAACAGGCTTTGTTGTTGGAACTAATACAATTGTCACCAATAATCATGTGGCTGAAAGT  180
Sbjct  2236698
.............................................................  2236757
Query  181
TTTAAGAATGCCAAAGTATTAAATCCGAATGCCAAAGATGATGCTTGGTTTTATCCAGGT  240
Sbjct  2236758
.............................................................  2236817
Query  241
CGTGATGGCAGTGCGACACCATTTGGCAAATTCAAAGTGATTGATGTAGCTTTTTCCCCG  300
Sbjct  2236818
.............................................................  2236877
Query  301
AATGCGGATATTGCGGTAGTGACTGTCGGCAAACAAAACGATCGTCCAGATGGCCCAGAG  360
Sbjct  2236878
.............................................................  2236937
Query  361
TTGGGAGAAATTTTAACGCCATTTGTTTTGAAAAAGTTTGAATCTTCAGATACCCATGTC  420
Sbjct  2236938
.............................................................  2236997
Query  421
ACAATATCAGGCTATCCAGGTGAGAAAAACCACACGCAATGGTCGCATGAAAATGATTTG  480
Sbjct  2236998
.............................................................  2237057
Query  481
TTTACATCTAACTTTACAGACTTAGAAAATCCATTACTATTTTATGATATCGATACAACA  540
Sbjct  2237058
.............................................................  2237117
Query  541      GGTGGTCAATCTGGTTCCC  559
Sbjct  2237118  ...................  2237136
```

Figure (3-18C): Alignment analysis of *sprE* gene (four isolates 1, 2, 3, 4) of *E. faecalis* with Gene Bank at NCBI. Query represents of sample; subject represents a database of National Center Biotechnology Information (NCBI).

***Enterococcus faecalis* strain FDAARGOS_528 chromosome, complete genome Sequence ID: CP033787.1Length: 2963654Number of Matches: 1** Range 1: 2236578 to 2237136GenBankGraphicsNext MatchPrevious Match

Score	Expect	Identities	Gaps	Strand
1009 bits(1118)	0.0	559/559(100%)	0/559(0%)	Plus/Plus

```
Query  1
AAAAGATCGTTACTGGACCCTGAGGACAGAAAACAAGAAGTGGCAGATACAACCGAAGCG  60
Sbjct  2236578
.............................................................  2236637
Query  61
CCTTTTGCGTCAATCGGAAGAATCATTTCCCCTGCCAGTAAACCAGGCTATATTTCTTTA  120
Sbjct  2236638
.............................................................  2236697
Query  121
GGAACAGGCTTTGTTGTTGGAACTAATACAATTGTCACCAATAATCATGTGGCTGAAAGT  180
Sbjct  2236698
.............................................................  2236757
Query  181
TTTAAGAATGCCAAAGTATTAAATCCGAATGCCAAAGATGATGCTTGGTTTTATCCAGGT  240
Sbjct  2236758
.............................................................  2236817
Query  241
CGTGATGGCAGTGCGACACCATTTGGCAAATTCAAAGTGATTGATGTAGCTTTTTCCCCG  300
Sbjct  2236818
.............................................................  2236877
Query  301
AATGCGGATATTGCGGTAGTGACTGTCGGCAAACAAAACGATCGTCCAGATGGCCCAGAG  360
Sbjct  2236878
.............................................................  2236937
Query  361
TTGGGAGAAATTTTAACGCCATTTGTTTTGAAAAAGTTTGAATCTTCAGATACCCATGTC  420
Sbjct  2236938
.............................................................  2236997
Query  421
ACAATATCAGGCTATCCAGGTGAGAAAAACCACACGCAATGGTCGCATGAAAATGATTTG  480
Sbjct  2236998
.............................................................  2237057
Query  481
TTTACATCTAACTTTACAGACTTAGAAAATCCATTACTATTTTATGATATCGATACAACA  540
Sbjct  2237058
.............................................................  2237117
Query  541      GGTGGTCAATCTGGTTCCC  559
Sbjct  2237118  ...................  223713.
```

Figure (3-18D): Alignment analysis of *sprE* gene (four isolates 1, 2, 3, 4) of *E. faecalis* with Gene Bank at NCBI. Query represents of sample; subject represents a database of National Center Biotechnology Information (NCBI).

3.8.3 *FsrA* gene

The genes were amplified by PCR method, and send for sequencing service to Macrogen Company Korea. The sequencing result of *gelE* gene shows for *E. faecalis* having one transversion A/T in location (1743833) nucleotide. From the Gene Bank found part of *FsrA* gene having 99% compatibility with the subject of *FsrA* gene in NCBI under sequence (ID: CP041738.1). The sequencing for *fsrA* gene for four isolates of *E. faecalis* as seen in Table (3-3), Figures (3-19A-D).

***Enterococcus faecalis* EnGen0107 strain B594 chromosome, complete genome Sequence ID: CP041738.1Length: 3152103Number of Matches: 1** Range 1: 1743742 to 1744140GenBankGraphicsNext MatchPrevious Match

Score	Expect	Identities	Gaps	Strand
716 bits(793)	0.0	398/399(99%)	0/399(0%)	Plus/Plus

```
Query  1
TGATGATGATTGATTGATGGACTCTAATCAAATCTGGATGCAGTTGTTCAATTTTTGATA  60
Sbjct  1743742
.............................................................  1743801
Query  61
ACGTTCCATAAAATTCTCTTTTAAAATTTTTTCCAACCATTGACACACGATGATCGTATT  120
Sbjct  1743802
..............................A..............................  1743861
Query  121
CCGTTTGAAAGAAATAAATATCATTAATATCCATTTTTATAGAAGACGCTTTTAAGTTAA  180
Sbjct  1743862
.............................................................  1743921
Query  181
TGATAATATATTCACTTTTTGTCTTTATATTTTCTTTTTGTTGAATATATGCATCGACAC  240
Sbjct  1743922
.............................................................  1743981
Query  241
ATGTTTCGACCTCTTTTTGGAAATCTAGAAAATCCACGTTCTTTTGAATAAATTGAAGAG  300
Sbjct  1743982
.............................................................  1744041
Query  301
CTGAAACCATATATTTGTAGGAAGTTAAAACTAATTCTGTGTGAGTAGAAATAAATACAA  360
Sbjct  1744042
.............................................................  1744101
Query  361      TCAGACTTTCACTGTCTGTTTTTCTGATTTCTTTAGCAA  399
Sbjct  1744102  ....................................... 1744140
```

Figure (3-19A): Alignment analysis of *fsrA* gene (four isolates 1, 2, 3, 4) of *E. faecalis* with Gene Bank at NCBI. Query represents of sample; subject represents a database of National Center Biotechnology Information (NCBI).

Enterococcus faecalis **EnGen0107 strain B594 chromosome, complete genome Sequence ID: CP041738.1Length: 3152103Number of Matches: 1** Range 1: 1743742 to 1744140GenBankGraphicsNext MatchPrevious Match

Score	Expect	Identities	Gaps	Strand
716 bits(793)	0.0	398/399(99%)	0/399(0%)	Plus/Plus

```
Query  1
TGATGATGATTGATTGATGGACTCTAATCAAATCTGGATGCAGTTGTTCAATTTTTGATA  60
Sbjct  1743742
.............................................................  1743801
Query  61
ACGTTCCATAAAATTCTCTTTTAAAATTTTTTCCAACCATTGACACACGATGATCGTATT  120
Sbjct  1743802
..............................A..............................  1743861
Query  121
CCGTTTGAAAGAAATAAATATCATTAATATCCATTTTTATAGAAGACGCTTTTAAGTTAA  180
Sbjct  1743862
.............................................................  1743921
Query  181
TGATAATATATTCACTTTTTGTCTTTATATTTTCTTTTTGTTGAATATATGCATCGACAC  240
Sbjct  1743922
.............................................................  1743981
Query  241
ATGTTTCGACCTCTTTTTGGAAATCTAGAAAATCCACGTTCTTTTGAATAAATTGAAGAG  300
Sbjct  1743982
.............................................................  1744041
Query  301
CTGAAACCATATATTTGTAGGAAGTTAAAACTAATTCTGTGTGAGTAGAAATAAATACAA  360
Sbjct  1744042
.............................................................  1744101
Query  361      TCAGACTTTCACTGTCTGTTTTTCTGATTTCTTTAGCAA  399
Sbjct  1744102  ......................................  1744140
```

Figure (3-19B): Alignment analysis of *fsrA* gene (four isolates 1, 2, 3, 4) of *E. faecalis* with Gene Bank at NCBI. Query represents of sample; subject represents a database of National Center Biotechnology Information (NCBI).

Enterococcus faecalis **EnGen0107 strain B594 chromosome, complete genome Sequence ID: CP041738.1Length: 3152103Number of Matches: 1** Range 1: 1743742 to 1744140GenBankGraphicsNext MatchPrevious Match

Score	Expect	Identities	Gaps	Strand
716 bits(793)	0.0	398/399(99%)	0/399(0%)	Plus/Plus

```
Query  1
TGATGATGATTGATTGATGGACTCTAATCAAATCTGGATGCAGTTGTTCAATTTTTGATA  60
Sbjct  1743742
.............................................................  1743801
Query  61
ACGTTCCATAAAATTCTCTTTTAAAATTTTTTCCAACCATTGACACACGATGATCGTATT  120
Sbjct  1743802
..............................A..............................  1743861
Query  121
CCGTTTGAAAGAAATAAATATCATTAATATCCATTTTTATAGAAGACGCTTTTAAGTTAA  180
Sbjct  1743862
.............................................................  1743921
Query  181
TGATAATATATTCACTTTTTGTCTTTATATTTTCTTTTTGTTGAATATATGCATCGACAC  240
Sbjct  1743922
.............................................................  1743981
Query  241
ATGTTTCGACCTCTTTTTGGAAATCTAGAAAATCCACGTTCTTTTGAATAAATTGAAGAG  300
Sbjct  1743982
.............................................................  1744041
Query  301
CTGAAACCATATATTTGTAGGAAGTTAAAACTAATTCTGTGTGAGTAGAAATAAATACAA  360
Sbjct  1744042
.............................................................  1744101
Query  361      TCAGACTTTCACTGTCTGTTTTTCTGATTTCTTTAGCAA  399
Sbjct  1744102  ......................................  1744140
```

Figure (3-19C): Alignment analysis of *fsrA* gene (four isolates 1, 2, 3, 4) of *E. faecalis* with Gene Bank at NCBI. Query represents of sample; subject represents a database of National Center Biotechnology Information (NCBI).

Enterococcus faecalis **EnGen0107 strain B594 chromosome, complete genome Sequence ID: CP041738.1Length: 3152103Number of Matches: 1** Range 1: 1743742 to 1744140GenBankGraphicsNext MatchPrevious Match

Score	Expect	Identities	Gaps	Strand
716 bits(793)	0.0	398/399(99%)	0/399(0%)	Plus/Plus

```
Query  1
TGATGATGATTGATTGATGGACTCTAATCAAATCTGGATGCAGTTGTTCAATTTTTGATA  60
Sbjct  1743742
............................................................  1743801
Query  61
ACGTTCCATAAAATTCTCTTTTAAAATTTTTTCCAACCATTGACACACGATGATCGTATT  120
Sbjct  1743802
..............................A.............................  1743861
Query  121
CCGTTTGAAAGAAATAAATATCATTAATATCCATTTTTATAGAAGACGCTTTTAAGTTAA  180
Sbjct  1743862
............................................................  1743921
Query  181
TGATAATATATTCACTTTTTGTCTTTATATTTTCTTTTTGTTGAATATATGCATCGACAC  240
Sbjct  1743922
............................................................  1743981
Query  241
ATGTTTCGACCTCTTTTTGGAAATCTAGAAAATCCACGTTCTTTTGAATAAATTGAAGAG  300
Sbjct  1743982
............................................................  1744041
Query  301
CTGAAACCATATATTTGTAGGAAGTTAAAACTAATTCTGTGTGAGTAGAAATAAATACAA  360
Sbjct  1744042
............................................................  1744101
Query  361      TCAGACTTTCACTGTCTGTTTTTCTGATTTCTTTAGCAA  399
Sbjct  1744102  ......................................  1744140
```

Figure (3-19D): Alignment analysis of *fsrA* gene (four isolates 1, 2, 3, 4) of *E. faecalis* with Gene Bank at NCBI. Query represents of sample; subject represents a database of National Center Biotechnology Information (NCBI).

Table (3-3): Determinate of DNA sequencing for some important virulence genes

Source : *Enterococcus faecalis*						
No.	**Type of substitution**	**Location**	**Nucleotide**	**Sequence ID with compare**	**gene**	**Identities**
1.	**Transvertion**	1592444	A\C	**ID: CP088198.1**	***gelE***	**99%**
2.	**Transvertion**	1592458	A\C	**ID: CP088198.1**	***gelE***	**99%**
	Transvertion	1592444	A\C			
3.	**Transvertion**	1592444	A\C	**ID: CP088198.1**	***gelE***	**99%**
4.	----------	------	-----	**ID: CP088198.1**	***gelE***	**100%**
5.	----------	------	-----	**ID: CP070621.1**	***sprE***	**100%**
6.	----------	------	-----	**ID: CP033787.1**	***sprE***	**100%**
7.	----------	------	-----	**ID: CP033787.1**	***sprE***	**100%**
8.	----------	------	-----	**ID: CP033787.1**	***sprE***	**100%**
9.	**Transvertion**	1743833	A\T	**ID: CP041738.1**	***fsrA***	**99%**
10.	**Transvertion**	1743833	A\T	**ID: CP041738.1**	***fsrA***	**99%**
11.	**Transvertion**	1743833	A\T	**ID: CP041738.1**	***fsrA***	**99%**
12.	**Transvertion**	1743833	A\T	**ID: CP041738.1**	***fsrA***	**99%**

2.9 Phylogenic tree of some important genes

2.9.1 Phylogeny of *FsrA* gene

The phylogeny tree of *fsrA* gene incorporated with various strains of *E. faecalis* sequences with variable origins. The results showed the samples were distributed into closely associated positions in closely-related phylogenetic distances. It was observed that, the four samples were positioned in the immediate vicinity to several Asia and European strains of *E. faecalis*, including Chinese (GenBank acc. No. CP100596.1), Japanese (GenBank acc. No. AP025270.1), South Korea (GenBank acc. No. CP102070.1), Kazakhstan (GenBank acc. No. CP041877.1), USA (GenBank acc. No. CP041738.1), France (GenBank acc. No. CP039296.1), Switzerland (GenBank acc. No. CP091237.1), Germany (GenBank acc. No. CP091198.1), Spain (GenBank acc. No.

HF794359.1), Spain (GenBank acc. No. HG794359.1), Netherlands (GenBank acc. No. LR 962830.1), United Kingdom (GenBank acc. No. OD940420.1) and Brazil (GenBank acc. No. CPCP073085.1). these results were shown in Table (3-4), Figure (3-20).

Table (3-4): The NCBI-BLAST Homology Sequence identity (%) between local *FsrA* gene *E. faecalis* isolates and NCBI-BLAST submitted *FsrA* gene *E. faecalis* isolates in other countries

	Accession	Country	Organism/ Source	Compatibility
1.	ID: CP041738.1	USA	*E. faecalis* (*FsrA*) gene	99%
2.	ID: CP041877.1	Kazakhstan	*E. faecalis* (*FsrA*) gene	99%
3.	ID: CP039296.1	France	*E. faecalis* (*FsrA*) gene	99%
4.	ID: CP091237.1	Switzerland	*E. faecalis* (*FsrA*) gene	99%
5.	ID: CP091198.1	Germany	*E. faecalis* (*FsrA*) gene	99%
6.	ID: HG794359.1	Spain	*E. faecalis* (*FsrA*) gene	99%
7.	ID: LR962830.1	Netherlands	*E. faecalis* (*FsrA*) gene	99%
8.	ID: CP102070.1	South Korea	*E. faecalis* (*FsrA*) gene	99%
9.	ID: CP100596.1	China	*E. faecalis* (*FsrA*) gene	99%
10.	ID: AP025270.1	Japan	*E. faecalis* (*FsrA*) gene	99%
11.	ID: OD940420.1	United Kingdom	*E. faecalis* (*FsrA*) gene	99%
12.	ID: CP073085.1	Brazil	*E. faecalis* (*FsrA*) gene	99%

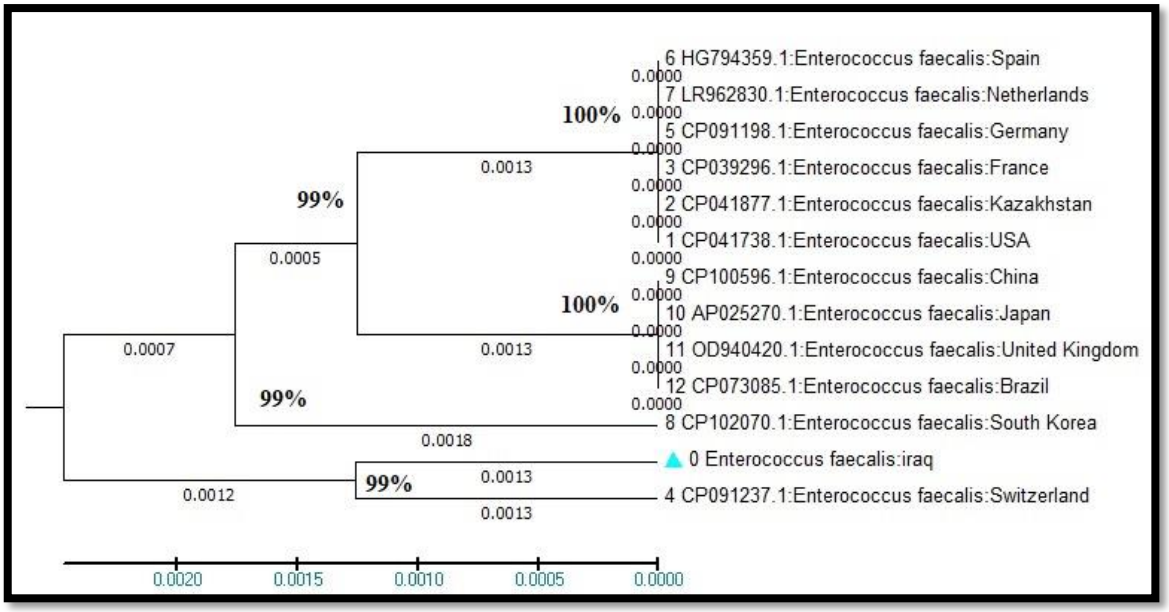

Figure (3-20): Phylogenic tree of *FsrA* gene

Furthermore, small phylogenic distances were observed among the incorporated organisms within this clade, which gives an obvious indication of the presence of high homology among these sequences. Accordingly. This high similarity between local strains and many *E. faecalis* strains isolated from Asian sources may indicate that our investigated samples has been originated from as Asian source. However, the USA and European sources of these sequences could not be ignored. This sort of four samples genetic distribution referred to the sensitivity of the investigated bacteria samples. Thus, the distinctive role of the generated phylogenetic tree in the detection of currently analyzed samples could not be excluded from the explanation. A accordingly, this notion provides a further indication of the bacterial identify and accurate genotyping of these studied bacterial samples.

By applying the UPGMA technique, we were able to deduce the evolutionary history (Sneath and Sokal, 1973). With a total branch length of 0.00921092, this ideal tree is displayed. The phylogenetic tree was inferred using evolutionary distances, and its branches are displayed to scale using those distances' units. Evolutionary distances are expressed as the average number of base changes per site and were calculated using the Maximum Composite Likelihood technique (Tamura *et al.*, 2004).

Thirteen different DNA sequences were analyzed. First, second, third, and noncoding positions of the codon table were included. Blank and empty spots have been filled in completely. The final dataset included 399 unique positions. MEGA6 was used for the evolutionary analysis (Tamura *et al*., 2013).

3.9.2 Phylogeny of *gelE* gene

A comprehensive phylogenetic tree was generated in the present study according to nucleic acid variation observed in the amplified 213bp of *gelE* gene amplicons. This phylogenetic tree was contained four samples alongside other relative nucleic acid sequences of *E. faecalis* sequences. These different genera were incorporated to assess the pattern of genera relatedness and to investigate the extent of the possible effect of the observed nucleic acid variation on the actual positioning of the investigated samples in the out-genera regions. Within this tree, investigated samples were incorporate alongside other relative sequences to constitute only one major clade of incorporated sequences within the cladogram. No other related sequences were found to be highly correlated to the incorporated sequences of *E. faecalis*.

This data indicated the highly specific ability of *gelE* gene-based amplicons to detect these bacterial particles without including any noticeable homology with other sequences of other species whether being in the same genus or other out group sequences.

The incorporated sequences within this clade showed the presence of various strains of *E. faecalis* sequences with variable origins within the major clade, the samples were distributed in to closely associated positions closely- related phylogenetic distances. It was observed that, four samples were samples were positioned in the immediate vicinity to several Asian and European strains of E. faecalis, including Chinese (GenBank acc. No. CP028835.1), Japanese (GenBank acc. No. AP018543.1), Viet Nam (GenBank acc. No. MH720045.1), Thailand (GenBank acc. No. CP070621.1), South Korea (GenBank acc. No. CP041344.1), two Switzerland (GenBank acc. No. CP088198.1 and

CP015410.2), USA (GenBank acc. No. CP046108.1), United Kingdom (GenBank acc. No. LR607378.1), Spain GenBank acc. No. CP029612.1), Germany (GenBank acc. No. CP022712.1), Czech Republic (GenBank acc. No. CP046247.1), France (GenBank acc. No. CP065784.1) and Canada (GenBank acc. No. CP050491.1). these results were shown in Table (3-5), Figure (3-21).

Table (3-5): The NCBI-BLAST Homology Sequence identity (%) between local *gelE* gene *E. faecalis* isolates and NCBI-BLAST submitted *gelE* gene *E. faecalis* isolates in other countries

No.	Accession	Country	Organism/Source	Compatibility
1.	**ID: CP088198.1**	Switzerland	*E. faecalis* (*gelE*) gene	**99%**
2.	**ID: CP046108.1**	USA	*E. faecalis* (*gelE*) gene	**99%**
3.	**ID: LR607378.1**	United Kingdom	*E. faecalis* (*gelE*) gene	**99%**
4.	**ID: CP041344.1**	South Korea	*E. faecalis* (*gelE*) gene	**99%**
5.	**ID: CP028835.1**	China	*E. faecalis* (*gelE*) gene	**99%**
6.	**ID: MH720045.1**	Viet Nam	*E. faecalis* (*gelE*) gene	**99%**
7.	**ID: CP029612.1**	Spain	*E. faecalis* (*gelE*) gene	**99%**
8.	**ID: AP018543.1**	Japan	*E. faecalis* (*gelE*) gene	**99%**
9.	**ID: CP022712.1**	Germany	*E. faecalis* (*gelE*) gene	**99%**
10.	**ID: CP015410.2**	Switzerland	*E. faecalis* (*gelE*) gene	**99%**
11.	**ID: CP046247.1**	Czech Republic	*E. faecalis* (*gelE*) gene	**99%**
12.	**ID: CP065784.1**	France	*E. faecalis* (*gelE*) gene	**99%**
13.	**ID: CP070621.1**	Thailand	*E. faecalis* (*gelE*) gene	**99%**
14.	**ID: CP050491.1**	Canada	*E. faecalis* (*gelE*) gene	**99%**

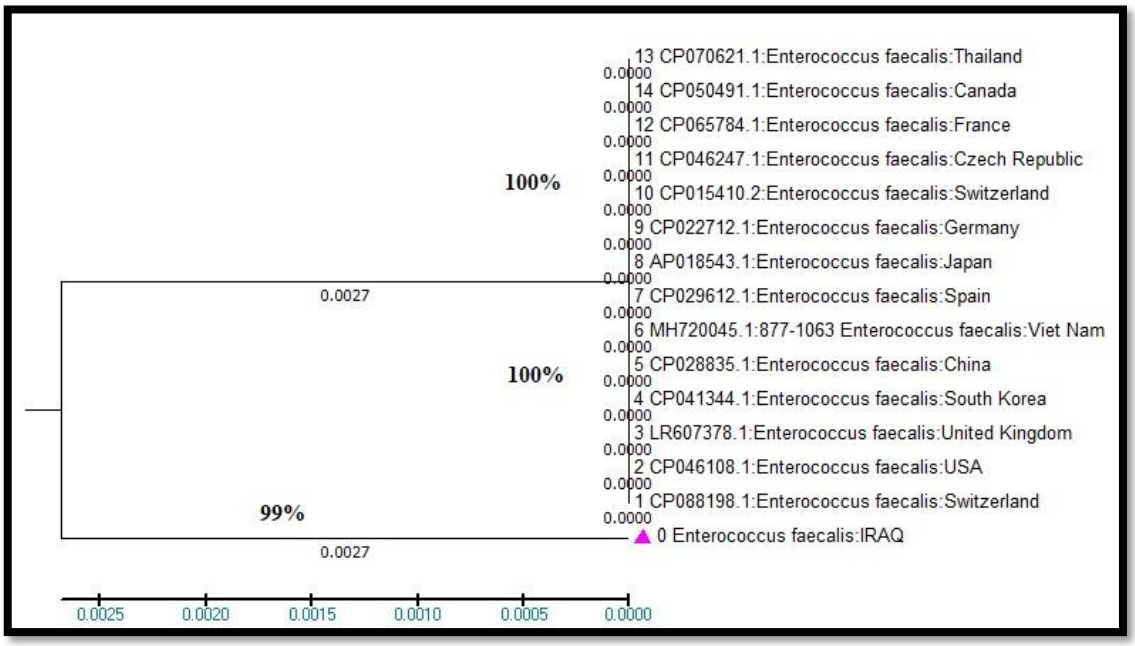

Figure (3-21): Phylogenic tree of *gelE* gene

The current observation of this tree has confirmed sequencing reactions because it explained the actual neighbour –joining- based positioning in such observed variation. Interestingly, the multinational origins of our investigated samples could not be ignored. This was due to their positioning in the vicinity to different Asian, Europeans and Americans strains belonged to the same organism. Based on the currently analyzed *gelE* nucleic acid sequences analysis is showed that, there were some variation the identity was (99%) when compared with the stranded strains, all this mutation were reduced function because they don't change the genetic code, or stop the protein translation but gave variation.

By applying the UPGMA technique, we were able to deduce the evolutionary history (Sneath and Sokal, 1973). The best tree is displayed, with a total branch length of 0.00536291. The phylogenetic tree was inferred using evolutionary distances, and its branches are displayed to scale using those distances' units. The evolutionary distances are

expressed as the average number of base substitutions per site and were calculated using the Maximum Composite Likelihood approach (Tamura *et al*., 2004).

Fifteen different nucleotide sequences were used in the study. First, second, third, and noncoding positions of the codon table were included. Blank and empty spots have been filled in completely. The final dataset included 187 distinct roles. MEGA6 was used for the evolutionary analysis (Tamura *et al*., 2013).

3.9.3 Phylogeny of *sprF* gene

The phylogenetic tree was generated which was based on the investigated sprF nucleic acid sequences in the analyzed bacterial samples. Along with other deposited DNA sequences, this phylogenetic tree contained the currently investigated samples (four samples) aligned with its highly related sequences in a neighbor- joining mode. It was observed that, the four samples were positioned in the immediate vicinity to several Asian and European strains of *E. faecalis* including Chinese (GenBank acc. No. CP086411.1), South Korea (GenBank acc. No. CP031027.1), Thailand (GenBank acc. No. CP070620.1), USA (GenBank acc. No. ID: CP033787.1), Poland (GenBank acc. No. ID: CP075604.2), France (GenBank acc. No. ID: CP065784.1), Netherlands (GenBank acc. No. ID: LR962788.1), United Kingdom (GenBank acc. No. ID: LR607371.1) and Norway (GenBank acc. No. ID: CP002491.1). These results were shown in Table (3-6), Figure (3-22).

Phylogenetic tree distance were observed among the incorporated organisms within this clade, which gives on obvious indication of the presence of high homology among these sequence of high homology among these sequences.

Table (3-6): The NCBI-BLAST Homology Sequence identity (%) between local *sprF* gene *E. faecalis* isolates and NCBI-BLAST submitted *sprF* gene *E. faecalis* isolates in other countries

No.	Accession	Country	Organism/Source	Compatibility
1.	**ID: CP033787.1**	USA	*E. faecalis* (*sprF*) gene	**100%**
2.	**ID: CP031027.1**	South Korea	*E. faecalis* (*sprF*) gene	**100%**
3.	**ID: CP086411.1**	China	*E. faecalis* (*sprF*) gene	**100%**
4.	**ID: CP075604.2**	Poland	*E. faecalis* (*sprF*) gene	**100%**
5.	**ID: CP065784.1**	France	*E. faecalis* (*sprF*) gene	**100%**
6.	**ID: LR962788.1**	Netherlands	*E. faecalis* (*sprF*) gene	**100%**
7.	**ID: LR607371.1**	United Kingdom	*E. faecalis* (*sprF*) gene	**99%**
8.	**ID: CP070620.1**	Thailand	*E. faecalis* (*sprF*) gene	**99%**
9.	**ID: CP002491.1**	Norway	*E. faecalis* (*sprF*) gene	**99%**

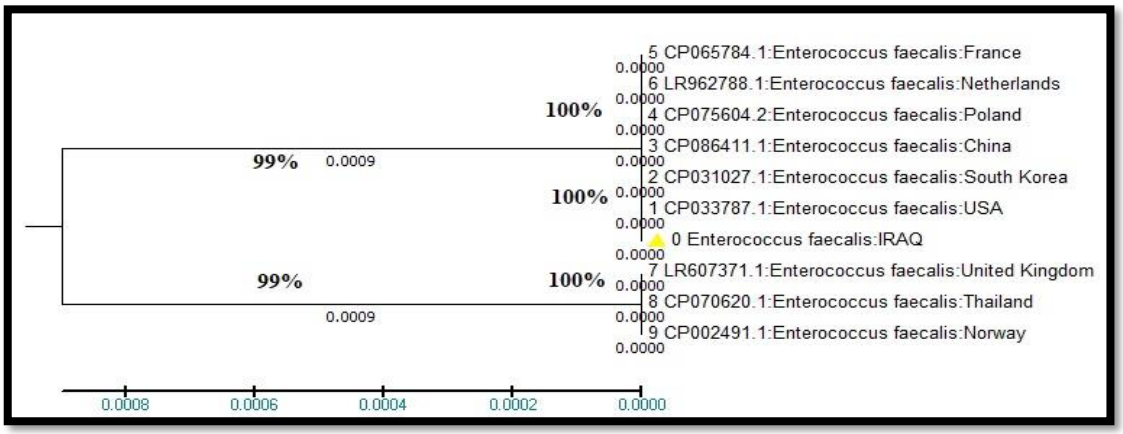

Figure (3-22): Phylogenic tree of *sprE* gene

This high similarity between local strains and many *E. faecalis* strains isolated from Asian and European source may indicate that our investigated samples has been originated from Asian and European source.

Based on currently analyzed sprF nucleic acid sequences analysis, it was showed the identity reach to (100%) when compared with standard strains.

By applying the UPGMA technique, we were able to deduce the evolutionary history (Sneath and Sokal, 1973). The best tree is displayed, with a total branch length of 0.00179518. The phylogenetic tree was inferred using evolutionary distances, and its branches are displayed to scale using those distances' units. The evolutionary distances are expressed as the average number of base substitutions per site and were calculated using the Maximum Composite Likelihood approach (Tamura *et al*., 2004).

As many as ten nucleotide sequences were analyzed. First, second, third, and noncoding positions of the codon table were included. Blank and empty spots have been filled in completely. The final dataset included 559 unique positions. MEGA6 was used for the evolutionary analysis (Tamura *et al*., 2013).

3.10 Determination of IL-23 in patients infected with *E. faecalis*

In this study, determination of IL-23 in serum patients that infected with *E. faecalis* were investigated, and the results showed that, the highest mean of IL-23 level was found among patients with UTI infected with *E. faecalis* (287.54±12.18ng/ml) comparing with the control group (188.08±2.29ng/ml) with a significant difference between the two groups ($P<0.05$), as shown in Table (3-7), Figure (3-23).

Table (3-7): Determination of IL-23 in *E. faecalis*

Parameter	sample	N	Mean ± S.E	P. value
IL23 (pg/ml)	Patients	44	287.54±12.18	0.0001
	Control	44	188.08±2.29	

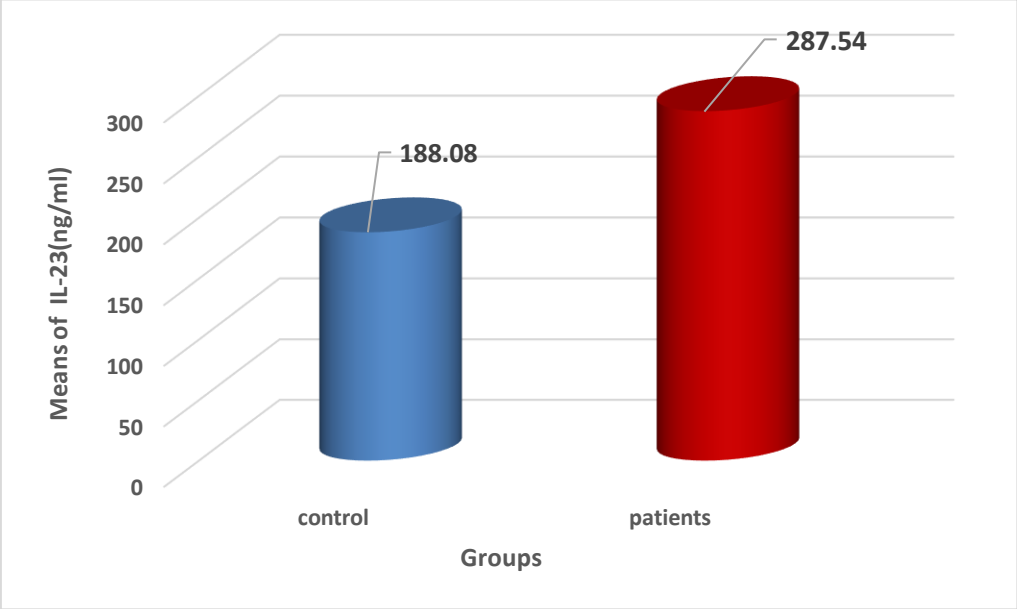

Figure (3-23): Determination of IL-23 in *E. faecalis*

Wang *et al*. (2019) discovered that, IL-23 regulated these pathways in human macrophages in a similar fashion. These findings underline the critical role for IL-23 in mediating antimicrobial actions in macrophages, which may help explain why pharmacological inhibition of the IL-23 pathways increases infection susceptibility. And they point out that even people who are protected from other immune-mediated disorders by carrying the IL-23R gene may be vulnerable to bacterial infection.

To explain the inflammatory response mechanism generated by *E. faecalis*, Bloemendaal *et al*., (2018) observed that infection with this bacteria causes the release of IL-23. In addition, *E. faecalis* was shown to activate caspase-1 and cause IL-23 production by Antushevich, (2020).

Multiple prior investigations have used immunohistochemical staining and enzyme-linked immunosorbent assay to detect elevated IL-23 levels in patients with *E. faecalis* infection (Bloemendaal *et al*., 2018). It is crucial that the host's adaptive and innate immune systems are able to

distinguish between pathogenic organisms and the commensal flora in order to maintain mucosal homeostasis (Yu *et al*., 2021).

Epithelial cells are stimulated to create antimicrobial factors by IL-23, which in turn activates the adoptive and innate immune systems to produce IL-17A, IL-17F, IL-22, and TNF. As was previously indicated, these characteristics play a significant role in host defense against a variety of infections, including *Klebsiella pneumonia*, *Candida albicans*, and *Toxoplasma gondii* (Schinocca *et al*., 2021).

Conclusions and Recommendations

Conclusions:

The present study has reached at the following conclusions:

1. Identification of *E. faecalis* by using *D-alanine ligase* gene more specific than other biochemical tests.
2. Most clinical isolates of *E. faecalis* produce many virulence factors involve hemolysin, protease, gelatinase, attached to epithelial cells, hemagglutination, lipase and hydrophobicity.
3. Most isolates of *E. faecalis* can produce biofilm by quantitative method, the biofilm formation considered an important ability to produce disease.
4. The pathogenicity of *E. faecalis* increased by presence of *vanA*, *gelE*, *cpd*, *cylA*, *ebpA*, *sprE*, *fsrA*, *ace*, *esp* and *efaA* genes.
5. The *cpd* and *ebpA* genes showed the higher prevalence among *E. faecalis* isolates.
6. *E. faecalis* were shown resistance to Vancomycin and have different behavior resistance against to one or more of antibiotics.
7. *E. faecalis* were compare with the strains deposed in NCBI.
8. Interleukin-23 is elevated in *E. faecalis* infection.

Recommendations

Depending on the finding of this study, it is recommended include:

1. Using real time for detection of *E. faecalis* depend on the copy number.
2. Direct and rapid identification of *E. faecalis* in clinical samples through using molecular technique which minimize the mixed growth.
3. The observation of this study suggest that the continuous and active surveillance programs of VRE infections are needed in all times.
4. Study the relationships between presence of *E. faecalis* in vaginitis and the cases of miscarriage among pregnant women.
5. Studding whole genome sequence of *E. faecalis*.

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Appendix

Appendix

LOCUS Seq1 201 bp DNA linear BCT 14-SEP-2022

DEFINITION DNA, partial CDS.

ACCESSION Seq1

VERSION

KEYWORDS .

SOURCE Enterococcus faecalis

ORGANISM Enterococcus faecalis

Bacteria; Firmicutes; Bacilli; Lactobacillales; Enterococcaceae;

Enterococcus.

REFERENCE 1 (bases 1 to 201)

AUTHORS Hayat,G.S. and Ilham,A.B.

TITLE Direct Submission

JOURNAL Submitted (14-SEP-2022) Department of Medical Microbiology,

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Medicine, IRAQ, Babylon, 00964, iraq

COMMENT Bankit Comment: ALT EMAIL:almutar.haydar@gmail.com

Bankit Comment: TOTAL # OF SEQS:2

##Assembly-Data-START##

Sequencing Technology :: Sanger dideoxy sequencing

##Assembly-Data-END##

FEATURES Location/Qualifiers

source 1..201

/organism="Enterococcus faecalis"

/mol_type="genomic DNA"

/strain="HaIl-1-IRAQ"

/isolate="HaIl-1-IRAQ"

/isolation_source="Patient with UTI"

/db_xref="taxon:1351"

/clone="gelE"

/country="IRAQ"

/collected_by="Hayat Ghaith Sachit; Ilham A. Bunyan"

/note="[cultured bacterial source]"

gene <1..>201

/gene="gelE gene"

CDS complement(1..>201)

/codon_start=1

/transl_table=11

/product="gelE (gelE) gene"

/product="gelE (gelE) gene"

/translation="FWDGKAMRYGDTSTPTGKTYASSLDVVGHEMTHGVTEHTAGLEY

LGQSGALNESYSDLMGYIISGAS"

BASE COUNT 64 a 49 c 30 g 58 t

ORIGIN

1 agatgcaccc gaaataatat aacccatcaa atcagaataa gattcattca aggcacctga

61 ttgtcctaaa tattctaaac cggcagtatg ttccgtcaca ccatgtgtca tttcatgacc

121 aactacatct aaagaggaag catacgtttt tcctgttggt gtacttgtgt caccataacg

181 cattgctttt ccatcccaaa a

//

Appendix

LOCUS Seq2 201 bp DNA linear BCT 14-SEP-2022

DEFINITION DNA,partial CDS.

ACCESSION Seq2

VERSION

KEYWORDS .

SOURCE Enterococcus faecalis

ORGANISM Enterococcus faecalis

Bacteria; Firmicutes; Bacilli; Lactobacillales; Enterococcaceae;

Enterococcus.

REFERENCE 1 (bases 1 to 201)

AUTHORS Hayat,G.S. and Ilham,A.B.

TITLE Direct Submission

JOURNAL Submitted (14-SEP-2022) Department of Medical Microbiology,

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Medicine, IRAQ, Babylon, 00964, iraq

COMMENT Bankit Comment: ALT EMAIL:almutar.haydar@gmail.com

Bankit Comment: TOTAL # OF SEQS:2

##Assembly-Data-START##

Sequencing Technology :: Sanger dideoxy sequencing

##Assembly-Data-END##

FEATURES Location/Qualifiers

source 1..201

/organism="Enterococcus faecalis"

/mol_type="genomic DNA"

/strain="Hall-2-IRAQ"

/isolate="Hall-2-IRAQ"

/isolation_source="female patient with vaginitis"

/db_xref="taxon:1351"

/clone="gelE"

/country="IRAQ"

/collected_by="Hayat Ghaith Sachit; Ilham A. Bunyan"

/note="[cultured bacterial source]"

gene <1..>201

/gene="gelE gene"

CDS complement(1..>201)

/codon_start=1

/transl_table=11

/product="gelE (gelE) gene"

/product="gelE (gelE) gene"

/translation="FWDGKAMRYGDTSTPTGKTYASSLDVVGHEMTHGVTEHTAGLEY

LGQSGALNESYSDLMGYIISGAS"

BASE COUNT 64 a 49 c 30 g 58 t

ORIGIN

1 agatgcaccc gaaataatat aacccatcaa atcagaataa gattcattca aggcacctga

61 ttgtcctaaa tattctaaac cggcagtatg ttccgtcaca ccatgtgtca tttcatgacc

121 aactacatct aaagaggaag catacgtttt tcctgttggt gtacttgtgt caccataacg

181 cattgctttt ccatcccaaa a

//

Appendix

LOCUS Seq1 558 bp DNA linear BCT 14-SEP-2022

DEFINITION DNA,partial CDS.

ACCESSION Seq1

VERSION

KEYWORDS .

SOURCE Enterococcus faecalis

ORGANISM Enterococcus faecalis

Bacteria; Firmicutes; Bacilli; Lactobacillales; Enterococcaceae;

Enterococcus.

REFERENCE 1 (bases 1 to 558)

AUTHORS Hayat,G.S. and Ilham,A.B.

TITLE Direct Submission

JOURNAL Submitted (14-SEP-2022) Department of Medical Microbiology,

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Medicine, IRAQ, Babylon, 00964, iraq

COMMENT Bankit Comment: ALT EMAIL:almutar.haydar@gmail.com

Bankit Comment: TOTAL # OF SEQS:2

##Assembly-Data-START##

Sequencing Technology :: Sanger dideoxy sequencing

##Assembly-Data-END##

FEATURES Location/Qualifiers

source 1..558

/organism="Enterococcus faecalis"

/mol_type="genomic DNA"

/strain="HaII-5-IRAQ"

/isolate="HaII-5-IRAQ"

/isolation_source="Patient with UTI"

/db_xref="taxon:1351"

/clone="sprF"

/country="IRAQ"

/collected_by="Hayat Ghaith Sachit; Ilham A. Bunyan"

/note="[cultured bacterial source]"

gene <1..>558

/gene="sprE gene"

CDS <1..558

/gene="sprE gene"

/codon_start=1

/transl_table=11

/product="sprE (sprE) gene"

/product="sprE (sprE) gene"

/translation="RSLLDPEDRRQEVADTTEAPFASIGRIISPASKPGYISLGTGFV

VGTNTIVTNNHVAESFKNAKVLNPNAKDDAWFYPGRDGSATPFGKFKVIDVAFSPNAD

IAVVTVGKQNDRPDGPELGEILTPFVLKKFESSDTHVTISGYPGEKNHTQWSHENDLF

TSNFTDLENPLLFYDIDTTGGQSGSP"

BASE COUNT 177 a 110 c 116 g 155 t

ORIGIN

1 agatcgttac tggaccctga agacagaaga caagaagtgg cagatacaac cgaagcgcct

61 tttgcgtcaa tcggaagaat catttcccct gccagtaaac caggctatat ttctttagga

121 acaggctttg ttgttggaac taatacaatt gtcaccaata atcatgtggc tgaaagtttt

181 aagaatgcca aagtattaaa tccgaatgcc aaagatgatg cttggtttta tccaggtcga

241 gatggcagtg cgacaccatt tggcaaattc aaagtgattg atgtagcttt ttccccgaat

301 gcggatattg cggtagtgac tgtcggcaaa caaaacgatc gtccagatgg cccagagttg

361 ggagaaattt taacgccatt tgttttgaaa aagtttgaat cttcagatac ccatgtcaca

421 atatcaggct atccaggtga gaaaaaccac acacaatggt ctcatgaaaa tgatttgttt

481 acatctaact ttacagactt agaaaatcca ttactatttt atgatatcga tacaacaggt

541 ggtcaatctg gttcccca

//

Appendix

LOCUS Seq2 558 bp DNA linear BCT 14-SEP-2022

DEFINITION DNA,partial CDS.

ACCESSION Seq2

VERSION

KEYWORDS .

SOURCE Enterococcus faecalis

ORGANISM Enterococcus faecalis

Bacteria; Firmicutes; Bacilli; Lactobacillales; Enterococcaceae;

Enterococcus.

REFERENCE 1 (bases 1 to 558)

AUTHORS Hayat,G.S. and Ilham,A.B.

TITLE Direct Submission

JOURNAL Submitted (14-SEP-2022) Department of Medical Microbiology,

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Medicine, IRAQ, Babylon, 00964, iraq

COMMENT Bankit Comment: ALT EMAIL:almutar.haydar@gmail.com

Bankit Comment: TOTAL # OF SEQS:2

##Assembly-Data-START##

Sequencing Technology :: Sanger dideoxy sequencing

##Assembly-Data-END##

FEATURES Location/Qualifiers

source 1..558

/organism="Enterococcus faecalis"

/mol_type="genomic DNA"

/strain="HaIl-6-IRAQ"

/isolate="HaIl-6-IRAQ"

/isolation_source="female patient with vaginitis"

/db_xref="taxon:1351"

/clone="sprF"

/country="IRAQ"

/collected_by="Hayat Ghaith Sachit; Ilham A. Bunyan"

/note="[cultured bacterial source]"

gene <1..>558

/gene="sprE gene"

CDS <1..558

/gene="sprE gene"

/codon_start=1

/transl_table=11

/product="sprE (sprE) gene"

/product="sprE (sprE) gene"

/translation="RSLLDPEDRRQEVADTTEAPFASIGRIISPASKPGYISLGTGFV

VGTNTIVTNNHVAESFKNAKVLNPNAKDDAWFYPGRDGSATPFGKFKVIDVAFSPNAD

IAVVTVGKQNDRPDGPELGEILTPFVLKKFESSDTHVTISGYPGEKNHTQWSHENDLF

TSNFTDLENPLLFYDIDTTGGQSGSP"

BASE COUNT 177 a 110 c 116 g 155 t

ORIGIN

1 agatcgttac tggaccctga agacagaaga caagaagtgg cagatacaac cgaagcgcct

61 tttgcgtcaa tcggaagaat catttcccct gccagtaaac caggctatat ttctttagga

121 acaggctttg ttgttggaac taatacaatt gtcaccaata atcatgtggc tgaaagtttt

181 aagaatgcca aagtattaaa tccgaatgcc aaagatgatg cttggtttta tccaggtcga

241 gatggcagtg cgacaccatt tggcaaattc aaagtgattg atgtagcttt ttccccgaat

301 gcggatattg cggtagtgac tgtcggcaaa caaaacgatc gtccagatgg cccagagttg

361 ggagaaattt taacgccatt tgttttgaaa aagtttgaat cttcagatac ccatgtcaca

421 atatcaggct atccaggtga gaaaaaccac acacaatggt ctcatgaaaa tgatttgttt

481 acatctaact ttacagactt agaaaatcca ttactatttt atgatatcga tacaacaggt

541 ggtcaatctg gttcccca

//

Appendix

LOCUS Seq1 396 bp DNA linear BCT 14-SEP-2022

DEFINITION DNA,partial CDS.

ACCESSION Seq1

VERSION

KEYWORDS .

SOURCE Enterococcus faecalis

ORGANISM Enterococcus faecalis

Bacteria; Firmicutes; Bacilli; Lactobacillales; Enterococcaceae;

Enterococcus.

REFERENCE 1 (bases 1 to 396)

AUTHORS Hayat,G.S. and Ilham,A.B.

TITLE Direct Submission

JOURNAL Submitted (14-SEP-2022) Department of Medical Microbiology,

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Medicine, IRAQ, Babylon, 00964, iraq

COMMENT Bankit Comment: ALT EMAIL:almutar.haydar@gmail.com

Bankit Comment: TOTAL # OF SEQS:2

##Assembly-Data-START##

Sequencing Technology :: Sanger dideoxy sequencing

##Assembly-Data-END##

FEATURES Location/Qualifiers

source 1..396

/organism="Enterococcus faecalis"

/mol_type="genomic DNA"

/strain="Hall-7-IRAQ"

/isolate="Hall-7-IRAQ"

/isolation_source="Patient with UTI"

/db_xref="taxon:1351"

/clone="FsrA"

/country="IRAQ"

/collected_by="Hayat Ghaith Sachit; Ilham A. Bunyan"

/note="[cultured bacterial source]"

gene <1..>396

/gene="FsrA gene"

CDS complement(1..>396)

/codon_start=1

/transl_table=11

/product="FsrA (FsrA) gene"

/product="FsrA (FsrA) gene"

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DFQKEVETCVDAYIQQKENIKTKSEYIIINLKASSIKMDINDIYFFQTEYDHRVSMVG

KNFKREFYGTLSKIEQLHPDLIRVHQSIII"

BASE COUNT 125 a 57 c 56 g 158 t

ORIGIN

1 gatgatgatt gattgatgga ctctaatcaa atctggatgc agttgttcaa tttttgataa

61 cgttccataa aattctcttt taaaattttt tccaaccatt gacacacgat gatcgtattc

121 cgtttgaaag aaataaatat cattaatatc cattttttata gaagacgctt ttaagttaat

181 gataatatat tcacttttttg tctttatatt ttctttttgt tgaatatatg catcgacaca

241 tgtttcgacc tctttttgga aatctagaaa atccacgttc ttttgaataa attgaagagc

301 tgaaaccata tatttgtagg aagttaaaac taattctgtg tgagtagaaa taaatacaat

361 cagactttca ctgtctgttt ttctgatttc tttagc

//

Appendix

LOCUS Seq2 396 bp DNA linear BCT 14-SEP-2022

DEFINITION DNA,partial CDS.

ACCESSION Seq2

VERSION

KEYWORDS .

SOURCE Enterococcus faecalis

ORGANISM Enterococcus faecalis

Bacteria; Firmicutes; Bacilli; Lactobacillales; Enterococcaceae;

Enterococcus.

REFERENCE 1 (bases 1 to 396)

AUTHORS Hayat,G.S. and Ilham,A.B.

TITLE Direct Submission

JOURNAL Submitted (14-SEP-2022) Department of Medical Microbiology,

Munsiriyah University/Dentistry college; Babylon University/College

Medicine, IRAQ, Babylon, 00964, iraq

COMMENT Bankit Comment: ALT EMAIL:almutar.haydar@gmail.com

Bankit Comment: TOTAL # OF SEQS:2

##Assembly-Data-START##

Sequencing Technology :: Sanger dideoxy sequencing

##Assembly-Data-END##

FEATURES Location/Qualifiers

source 1..396

/organism="Enterococcus faecalis"

/mol_type="genomic DNA"

/strain="HaIl-8-IRAQ"

/isolate="HaIl-8-IRAQ"

/isolation_source="female patient with vaginitis"

/db_xref="taxon:1351"

/clone="FsrA"

/country="IRAQ"

/collected_by="Hayat Ghaith Sachit; Ilham A. Bunyan"

/note="[cultured bacterial source]"

gene <1..>396

/gene="FsrA gene"

CDS complement(1..>396)

/codon_start=1

/transl_table=11

/product="FsrA (FsrA) gene"

/product="FsrA (FsrA) gene"

/translation="AKEIRKTDSESLIVFISTHTELVLTSYKYMVSALQFIQKNVDFL

DFQKEVETCVDAYIQQKENIKTKSEYIIINLKASSIKMDINDIYFFQTEYDHRVSMVG

KNFKREFYGTLSKIEQLHPDLIRVHQSIII"

BASE COUNT 125 a 57 c 56 g 158 t

ORIGIN

1 gatgatgatt gattgatgga ctctaatcaa atctggatgc agttgttcaa tttttgataa

61 cgttccataa aattctcttt taaaattttt tccaaccatt gacacacgat gatcgtattc

121 cgtttgaaag aaataaatat cattaatatc cattttttata gaagacgctt ttaagttaat

181 gataatatat tcacttttttg tctttatatt ttctttttgt tgaatatatg catcgacaca

241 tgtttcgacc tctttttgga aatctagaaa atccacgttc ttttgaataa attgaagagc

301 tgaaaccata tatttgtagg aagttaaaac taattctgtg tgagtagaaa taaatacaat

361 cagactttca ctgtctgttt ttctgatttc tttagc

//

Appendix

Enterococcus faecalis strain HaIl-1-IRAQ GelE (gelE) gene, partial cds

GenBank: OP441408.1

FASTA Graphics

Go to:

LOCUS OP441408 201 bp DNA linear BCT 14-NOV-2022

DEFINITION Enterococcus faecalis strain HaIl-1-IRAQ GelE (gelE) gene, partial cds.

ACCESSION OP441408

VERSION OP441408.1

KEYWORDS .

SOURCE Enterococcus faecalis

ORGANISM Enterococcus faecalis

Bacteria; Firmicutes; Bacilli; Lactobacillales; Enterococcaceae; Enterococcus.

REFERENCE 1 (bases 1 to 201)

AUTHORS Hayat,G.S. and Ilham,A.B.

TITLE Direct Submission

JOURNAL Submitted (14-SEP-2022) Department of Medical Microbiology, Munsiriyah University/Dentistry College; Babylon University/College Medicine, Iraq, Babylon 00964, Iraq

COMMENT ##Assembly-Data-START##

Sequencing Technology :: Sanger dideoxy sequencing

##Assembly-Data-END##

FEATURES Location/Qualifiers

source 1..201

/organism="Enterococcus faecalis"

/mol_type="genomic DNA"

/strain="Hall-1-IRAQ"

/isolation_source="Patient with UTI"

/db_xref="taxon:1351"

/country="Iraq"

/collected_by="Hayat Ghaith Sachit, Ilham A. Bunyan"

gene complement(<1..>201)

/gene="gelE"

CDS complement(<1..>201)

/gene="gelE"

/codon_start=1

/transl_table=11

/product="GelE"

/protein_id="UZH25374.1"

/translation="FWDGKAMRYGDTSTPTGKTYASSLDVVGHEMTHGVTEHTAGLEY

LGQSGALNESYSDLMGYIISGAS"

ORIGIN

1 agatgcaccc gaaataatat aacccatcaa atcagaataa gattcattca aggcacctga

61 ttgtcctaaa tattctaaac cggcagtatg ttccgtcaca ccatgtgtca tttcatgacc

121 aactacatct aaagaggaag catacgtttt tcctgttggt gtacttgtgt caccataacg

181 cattgctttt ccatcccaaa a

//

Appendix

Enterococcus faecalis strain HaIl-2-IRAQ GelE (gelE) gene, partial cds

GenBank: OP441409.1

FASTA Graphics

Go to:

LOCUS OP441409 201 bp DNA linear BCT 14-NOV-2022

DEFINITION Enterococcus faecalis strain HaIl-2-IRAQ GelE (gelE) gene, partial cds.

ACCESSION OP441409

VERSION OP441409.1

KEYWORDS .

SOURCE Enterococcus faecalis

ORGANISM Enterococcus faecalis

Bacteria; Firmicutes; Bacilli; Lactobacillales; Enterococcaceae; Enterococcus.

REFERENCE 1 (bases 1 to 201)

AUTHORS Hayat,G.S. and Ilham,A.B.

TITLE Direct Submission

JOURNAL Submitted (14-SEP-2022) Department of Medical Microbiology, Munsiriyah University/Dentistry College; Babylon University/College Medicine, Iraq, Babylon 00964, Iraq

COMMENT ##Assembly-Data-START##

Sequencing Technology :: Sanger dideoxy sequencing

##Assembly-Data-END##

FEATURES Location/Qualifiers

source 1..201

/organism="Enterococcus faecalis"

/mol_type="genomic DNA"

/strain="Hall-2-IRAQ"

/isolation_source="female patient with vaginitis"

/db_xref="taxon:1351"

/country="Iraq"

/collected_by="Hayat Ghaith Sachit, Ilham A. Bunyan"

gene complement(<1..>201)

/gene="gelE"

CDS complement(<1..>201)

/gene="gelE"

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/transl_table=11

/product="GelE"

/protein_id="UZH25375.1"

/translation="FWDGKAMRYGDTSTPTGKTYASSLDVVGHEMTHGVTEHTAGLEY

LGQSGALNESYSDLMGYIISGAS"

ORIGIN

1 agatgcaccc gaaataatat aacccatcaa atcagaataa gattcattca aggcacctga

61 ttgtcctaaa tattctaaac cggcagtatg ttccgtcaca ccatgtgtca tttcatgacc

121 aactacatct aaagaggaag catacgtttt tcctgttggt gtacttgtgt caccataacg

181 cattgctttt ccatcccaaa a

//

Appendix

Enterococcus faecalis strain HaIl-5-IRAQ SprE (sprE) gene, partial cds

GenBank: OP441412.1

FASTA Graphics

LOCUS OP441412 558 bp DNA linear BCT 14-NOV-2022

DEFINITION Enterococcus faecalis strain HaIl-5-IRAQ SprE (sprE) gene, partial cds.

ACCESSION OP441412

VERSION OP441412.1

KEYWORDS .

SOURCE Enterococcus faecalis

ORGANISM Enterococcus faecalis

Bacteria; Firmicutes; Bacilli; Lactobacillales; Enterococcaceae; Enterococcus.

REFERENCE 1 (bases 1 to 558)

AUTHORS Hayat,G.S. and Ilham,A.B.

TITLE Direct Submission

JOURNAL Submitted (14-SEP-2022) Department of Medical Microbiology, Munsiriyah University/Dentistry College; Babylon University/College Medicine, Iraq, Babylon 00964, Iraq

COMMENT ##Assembly-Data-START##

Sequencing Technology :: Sanger dideoxy sequencing

##Assembly-Data-END##

FEATURES Location/Qualifiers

source 1..558

/organism="Enterococcus faecalis"

/mol_type="genomic DNA"

/strain="HaIl-5-IRAQ"

/isolation_source="Patient with UTI"

/db_xref="taxon:1351"

/country="Iraq"

/collected_by="Hayat Ghaith Sachit, Ilham A. Bunyan"

gene <1..>558

/gene="sprE"

CDS <1..>558

/gene="sprE"

/codon_start=1

/transl_table=11

/product="SprE"

/protein_id="UZH25378.1"

/translation="RSLLDPEDRRQEVADTTEAPFASIGRIISPASKPGYISLGTGFV

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IAVVTVGKQNDRPDGPELGEILTPFVLKKFESSDTHVTISGYPGEKNHTQWSHENDLF

TSNFTDLENPLLFYDIDTTGGQSGSP"

ORIGIN

1 agatcgttac tggaccctga agacagaaga caagaagtgg cagatacaac cgaagcgcct

61 tttgcgtcaa tcggaagaat catttcccct gccagtaaac caggctatat ttctttagga

121 acaggctttg ttgttggaac taatacaatt gtcaccaata atcatgtggc tgaaagtttt

181 aagaatgcca aagtattaaa tccgaatgcc aaagatgatg cttggtttta tccaggtcga

241 gatggcagtg cgacaccatt tggcaaattc aaagtgattg atgtagcttt ttccccgaat

301 gcggatattg cggtagtgac tgtcggcaaa caaaacgatc gtccagatgg cccagagttg

361 ggagaaattt taacgccatt tgttttgaaa aagtttgaat cttcagatac ccatgtcaca

421 atatcaggct atccaggtga gaaaaaccac acacaatggt ctcatgaaaa tgatttgttt

481 acatctaact ttacagactt agaaaatcca ttactatttt atgatatcga tacaacaggt

541 ggtcaatctg gttcccca

//

Appendix

Enterococcus faecalis strain HaIl-6-IRAQ SprE (sprE) gene, partial cds

GenBank: OP441413.1

FASTA Graphics

Go to:

LOCUS OP441413 558 bp DNA linear BCT 14-NOV-2022

DEFINITION Enterococcus faecalis strain HaIl-6-IRAQ SprE (sprE) gene, partial cds.

ACCESSION OP441413

VERSION OP441413.1

KEYWORDS .

SOURCE Enterococcus faecalis

ORGANISM Enterococcus faecalis

Bacteria; Firmicutes; Bacilli; Lactobacillales; Enterococcaceae; Enterococcus.

REFERENCE 1 (bases 1 to 558)

AUTHORS Hayat,G.S. and Ilham,A.B.

TITLE Direct Submission

JOURNAL Submitted (14-SEP-2022) Department of Medical Microbiology, Munsiriyah University/Dentistry College; Babylon University/College Medicine, Iraq, Babylon 00964, Iraq

COMMENT ##Assembly-Data-START##

Sequencing Technology :: Sanger dideoxy sequencing

##Assembly-Data-END##

FEATURES Location/Qualifiers

source 1..558

/organism="Enterococcus faecalis"

/mol_type="genomic DNA"

/strain="Hall-6-IRAQ"

/isolation_source="female patient with vaginitis"

/db_xref="taxon:1351"

/country="Iraq"

/collected_by="Hayat Ghaith Sachit, Ilham A. Bunyan"

gene <1..>558

/gene="sprE"

CDS <1..>558

/gene="sprE"

/codon_start=1

/transl_table=11

/product="SprE"

/protein_id="UZH25379.1"

/translation="RSLLDPEDRRQEVADTTEAPFASIGRIISPASKPGYISLGTGFV

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IAVVTVGKQNDRPDGPELGEILTPFVLKKFESSDTHVTISGYPGEKNHTQWSHENDLF

TSNFTDLENPLLFYDIDTTGGQSGSP"

ORIGIN

1 agatcgttac tggaccctga agacagaaga caagaagtgg cagatacaac cgaagcgcct

61 tttgcgtcaa tcggaagaat catttcccct gccagtaaac caggctatat ttctttagga

121 acaggctttg ttgttggaac taatacaatt gtcaccaata atcatgtggc tgaaagtttt

181 aagaatgcca aagtattaaa tccgaatgcc aaagatgatg cttggtttta tccaggtcga

241 gatggcagtg cgacaccatt tggcaaattc aaagtgattg atgtagcttt ttccccgaat

301 gcggatattg cggtagtgac tgtcggcaaa caaaacgatc gtccagatgg cccagagttg

361 ggagaaattt taacgccatt tgttttgaaa aagtttgaat cttcagatac ccatgtcaca

421 atatcaggct atccaggtga gaaaaaccac acacaatggt ctcatgaaaa tgatttgttt

481 acatctaact ttacagactt agaaaatcca ttactatttt atgatatcga tacaacaggt

541 ggtcaatctg gttcccca

//

Appendix

Enterococcus faecalis strain HaIl-7-IRAQ FsrA (fsrA) gene, partial cds

GenBank: OP441414.1

FASTA Graphics

Go to:

LOCUS OP441414 396 bp DNA linear BCT 14-NOV-2022

DEFINITION Enterococcus faecalis strain HaIl-7-IRAQ FsrA (fsrA) gene, partial cds.

ACCESSION OP441414

VERSION OP441414.1

KEYWORDS .

SOURCE Enterococcus faecalis

ORGANISM Enterococcus faecalis

Bacteria; Firmicutes; Bacilli; Lactobacillales; Enterococcaceae; Enterococcus.

REFERENCE 1 (bases 1 to 396)

AUTHORS Hayat,G.S. and Ilham,A.B.

TITLE Direct Submission

JOURNAL Submitted (14-SEP-2022) Department of Medical Microbiology, Munsiriyah University/Dentistry College; Babylon University/College Medicine, Iraq, Babylon 00964, Iraq

COMMENT ##Assembly-Data-START##

Sequencing Technology :: Sanger dideoxy sequencing

##Assembly-Data-END##

FEATURES Location/Qualifiers

source 1..396

/organism="Enterococcus faecalis"

/mol_type="genomic DNA"

/strain="HaIl-7-IRAQ"

/isolation_source="Patient with UTI"

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                /country="Iraq"
                /collected_by="Hayat Ghaith Sachit, Ilham A. Bunyan"
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                /gene="fsrA"
     CDS        complement(<1..>396)
                /gene="fsrA"
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                /transl_table=11
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                /protein_id="UZH25380.1"
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                KNFKREFYGTLSKIEQLHPDLIRVHQSIII"
ORIGIN
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       61 cgttccataa aattctcttt taaaattttt tccaaccatt gacacacgat gatcgtattc
      121 cgtttgaaag aaataaatat cattaatatc cattttttata gaagacgctt ttaagttaat
      181 gataatatat tcacttttttg tctttatatt ttctttttgt tgaatatatg catcgacaca
      241 tgtttcgacc tctttttgga aatctagaaa atccacgttc ttttgaataa attgaagagc
      301 tgaaaccata tatttgtagg aagttaaaac taattctgtg tgagtagaaa taaatacaat
      361 cagactttca ctgtctgttt ttctgatttc tttagc
//
```

Appendix

Enterococcus faecalis strain HaIl-8-IRAQ FsrA (fsrA) gene, partial cds

GenBank: OP441415.1

FASTA Graphics

Go to:

LOCUS OP441415 396 bp DNA linear BCT 14-NOV-2022

DEFINITION Enterococcus faecalis strain HaIl-8-IRAQ FsrA (fsrA) gene, partial cds.

ACCESSION OP441415

VERSION OP441415.1

KEYWORDS .

SOURCE Enterococcus faecalis

ORGANISM Enterococcus faecalis

Bacteria; Firmicutes; Bacilli; Lactobacillales; Enterococcaceae; Enterococcus.

REFERENCE 1 (bases 1 to 396)

AUTHORS Hayat,G.S. and Ilham,A.B.

TITLE Direct Submission

JOURNAL Submitted (14-SEP-2022) Department of Medical Microbiology, Munsiriyah University/Dentistry College; Babylon University/College Medicine, Iraq, Babylon 00964, Iraq

COMMENT ##Assembly-Data-START##

Sequencing Technology :: Sanger dideoxy sequencing

##Assembly-Data-END##

FEATURES Location/Qualifiers

source 1..396

/organism="Enterococcus faecalis"

/mol_type="genomic DNA"

/strain="HaIl-8-IRAQ"

/isolation_source="female patient with vaginitis"

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                /country="Iraq"
                /collected_by="Hayat Ghaith Sachit, Ilham A. Bunyan"
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                /gene="fsrA"
     CDS        complement(<1..>396)
                /gene="fsrA"
                /codon_start=1
                /transl_table=11
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                /protein_id="UZH25381.1"
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                KNFKREFYGTLSKIEQLHPDLIRVHQSIII"
ORIGIN
        1 gatgatgatt gattgatgga ctctaatcaa atctggatgc agttgttcaa tttttgataa
       61 cgttccataa aattctcttt taaaattttt tccaaccatt gacacacgat gatcgtattc
      121 cgtttgaaag aaataaatat cattaatatc catttttata gaagacgctt ttaagttaat
      181 gataatatat tcacttttttg tctttatatt ttctttttgt tgaatatatg catcgacaca
      241 tgtttcgacc tctttttgga aatctagaaa atccacgttc ttttgaataa attgaagagc
      301 tgaaaccata tatttgtagg aagttaaaac taattctgtg tgagtagaaa taaatacaat
      361 cagactttca ctgtctgttt ttctgatttc tttagc
//
```

Table (1): Pattern phenotypic virulence factors and biofilm formation among *E. faecalis*

No. of isolates	Biofilm formation	Hemolysin production	Protease production	gelatinase production	Attached to epithelial cells	hemagglutination	Lipase production	hydrophobicity
1.	+	+	+	+	-	-	+	+
2.	-	-	-	-	+	+	-	-
3.	-	-	-	-	-	+	-	-
4.	+	-	+	+	+	-	+	-
5.	+	-	+	+	+	+	+	+
6.	+	+	-	-	-	-	+	-
7.	+	-	-	-	+	+	+	-
8.	+	-	-	+	-	-	+	-
9.	+	-	+	+	+	+	+	+
10.	+	+	-	+	-	+	+	-
11.	+	-	-	+	+	-	+	-
12.	+	-	-	+	-	+	+	-
13.	+	-	-	-	+	-	+	+
14.	+	-	+	-	-	+	+	+
15.	+	+	-	-	+	-	+	+
16.	+	-	-	-	+	-	-	+
17.	+	-	+	-	+	+	+	-
18.	-	+	-	-	-	-	-	-
19.	+	-	-	+	-	-	+	-
20.	+	-	-	-	+	+	+	-
21.	+	+	-	-	+	-	+	+
22.	+	-	+	-	-	+	-	-
23.	+	-	-	-	+	-	+	-
24.	+	-	-	-	-	-	+	+
25.	+	+	-	-	-	+	-	-
26.	+	-	-	+	+	-	+	+
27.	+	-	+	-	-	+	+	-
28.	+	-	-	-	-	-	-	-
29.	+	+	-	-	-	-	+	-

Table (1): Pattern phenotypic virulence factors and biofilm formation among *E. faecalis*

30.	-	+	-	-	+	+	-	+
31.	-	-	-	-	-	-	-	-
32.	+	-	+	+	-	+	+	-
33.	+	+	-	-	+	-	+	+
34.	+	-	-	-	-	-	+	-
35.	+	-	-	+	+	+	+	-
36.	+	-	+	-	-	-	+	+
37.	+	+	-	+	-	-	-	-
38.	+	-	-	-	-	-	+	-
39.	+	+	-	+	+	+	+	+
40.	+	+	-	-	-	-	+	-
41.	+	+	+	-	+	-	+	+
42.	+	-	-	-	+	+	+	-
43.	+	-	-	-	+	-	-	+
44.	-	-	+	-	+	-	-	-
Total	**38(86.3%)**	**14(31.8%)**	**12(27.2%)**	**14(31%)**	**22(50.0%)**	**18(40.9%)**	**32(72.7%)**	**16(36.3%)**

Table (2): Pattern of antibiotic resistance and occurrence of virulence factors genes among *E. faecalis* isolates (Total No. =44)

No. of isolates	Antibiotic resistance	*Van*	*gelE*	*cpd*	*cylA*	*ebpA*	*sprE*	*fsrA*	*ace*	*esp*	*efaA*
1.	SYN, LEV, ERT, NOR	+	+	+	+	+	+	+	+	+	+
2.	SYN, LEV, NOR, CIP	+	-	+	+	-	-	+	+	-	+
3.	SYN, LEV, ERT, CIP	-	+	+	-	+	-	+	-	+	+
4.	SYN, LEV, ERT, VAN		-	-	+	+	-	+	+	-	-
5.	SYN, ERT, CIP, VAN	+	+	+	-	+	+	-	-	+	+
6.	SYN, LEV, CIP, VAN	-	-	-	-	+	-	+	+	-	-
7.	SYN, ERT, CIP, VAN	+	+	+	+	+	+	-	-	+	+
8.	SYN, LEV, CIP, CN	-	-	+	-	+	-	+	+	+	+
9.	SYN, LEV, VAN, CN	-	-	-	-	+	-	-	+	+	+
10.	SYN, ERT, CIP, CN	-	-	+	-	-	-	+	+	-	+
11.	SYN, ERT, VAN	-	-	+	-	+	-	-	-	-	-
12.	SYN, LEV, ERT, CN	-	-	+	-	+	-	-	-	+	+
13.	SYN, ERT, CIP, CN	-	+	+	-	-	-	-	-	+	-
14.	SYN, NOR, VAN, CIP	+	+	+	+	+	+	+	-	-	-
15.	SYN, LEV, NOR, VAN	-	+	-	-	+	-	+	+	+	+
16.	SYN, ERT, NOR, VAN	-	-	+	-	+	-	+	-	-	-
17.	SYN, ERT, CIP, CN	+	+	+	+	+	-	+	-	-	-
18.	SYN, LEV, NOR, VAN	-	+	-	-	+	-	-	+	-	-
19.	SYN, ERT, CIP, CN	-	-	+	-	+	-	-	-	-	-
20.	SYN, LEV, ERT, CN	-	-	+	-	+	-	+	-	+	-
21.	SYN, NOR, VAN	+	+	+	+	+	+	-	-	-	-
22.	SYN, LEV, ERT, CN	-	-	+	-	-	-	-	+	-	+
23.	SYN, NOR, CIP, VAN	+	-	+	-	+	-	+	+	+	-
24.	SYN, LEV, ERT, VAN	-	-	+	-	-	-	-	-	-	-
25.	SYN, ERT, NOR, VAN	+	-	+	+	+	+	+	+	-	-
26.	SYN, LEV, NOR, CN	-	-	+	-	+	-	+	-	-	-

Table (2): Pattern of antibiotic resistance and occurrence of virulence factors genes among *E. faecalis* isolates (Total No. =44)

27.	SYN, ERT, CIP, VAN	-	+	+	-	+	-	+	-	-	-
28.	SYN, LEV, ERT, TEC	+	-	+	+	+	+	-	+	+	-
29.	SYN, LEV, ERT, CN	+	-	+	-	+	-	-	-	-	-
30.	SYN, LEV, CIP, VAN	-	-	+	-	+	-	+	-	+	-
31.	SYN, LEV, NOR, TEC	+	+	+	+	+	+	-	-	-	+
32.	SYN, LEV, NOR, VAN	-	-	-	-	-	-	-	-	-	-
33.	SYN, LEV, NOR, CN	-	-	+	-	+	-	+	+	+	+
34.	SYN, LEV, CIP, CN	-	-	+	+	+	-	-	-	+	-
35.	LEV, ERT, VAN	-	-	-	-	-	-	-	-	-	-
36.	SYN, LEV, CIP, TEC	+	-	+	-	+	+	-	-	+	-
37.	SYN, NOR, TEC	-	-	+	-	+	-	-	-	-	-
38.	SYN, LEV, TEC	+	+	+	+	+	+	-	-	+	+
39.	SYN, NOR, CIP	-	-	+	-	+	-	-	-	-	-
40.	SYN, NOR, TEC	+	-	+	+	+	+	+	-	-	-
41.	SYN, NOR, TEC	-	-	+	-	+	-	-	+	+	+
42.	SYN, NOR, CIP, TEC	+	-	+	-	+	+	-	-	-	-
43.	SYN, LEV, NOR	-	+	-	+	+	-	-	-	-	-
44.	LEV, NOR, CIP, TEC	-	-	-	-	-	-	+	-	-	-
Total	SYN 42(95.4%) LEV 25(56.8%) ERT 20(45.4%) NOR 20(45.4%) CIP 19(43.1%) VAN 18(40.9%) CN 13(29.5%) TEC 9(20.4%)	16(36.3%)	14(31.8%)	36(81.8%)	14(31.8%)	34(77.2%	12(27.2%)	20(45.4%)	15(34%)	18(40.9%)	15(34%)

VAN**/** Vancomycin**,** TEC**/** Teicoplanin**,** CIP/ Ciprofloxacin**,** NOR/ Norfloxacin, ERT/ Erythromycin, SYN/ Synercid, LEV/ levofloxacin, CN/ Gentamicin

الخـــــــلاصة

المكورات المعوية البرازية ، مثل الأعضاء الأخرى في جنس *Enterococcus* ، شائعة في الأفراد الأصحاء ولكنها قد تسبب عدوى مميتة، خاصة في المستشفيات بسبب مستوياتها الطبيعية العالية من مقاومة المضادات الحيوية.

هدفت هذه الدراسة إلى فحص الجينات المشفرة للعوامل المرضية المصاحبة بين عزلات *Enterococcus faecalis*.

تم جمع 200 عينة بول ومهبل من المرضى الذين يعانون من التهاب المسالك البولية والتهاب المهبل والتي تتراوح اعمارهم بين (56-4 سنة) والوافدين إلى مستشفى الكرامة والمدينة الطبية في بغداد لفترة ثلاثة أشهر من (مايس إلى تموز 2022) ، وتم جمع 44 عينة دم من الاشخاص الاصحاء كمجموعة سيطرة. تمت زرع هذه العينات لمدة (24-18) ساعة. وتم تحضينها عند (37 درجة مئوية) لمدة (24-18) ساعة على عدد من الوسائط الانتقائية المختلفة.

اعتمد التعرف على بكتيريا *E. faecalis* على شكل المستعمرة والفحوصات المجهرية والاختبارات البيوكيميائية. جميع العينات السريرية الـ 200 المزروعة كانت إيجابية، ولكن فقط 44 (22٪) من العزلات كانت *E. faecalis* ، استخدم نظام Vitek 2 الآلي بطاقات GP-ID التي تحتوي على 64 اختبارًا كيميائيًا حيويًا للتأكد من أن العزلات وقد تم تاكيدها على انها *E. faecalis*. وتم التعرف على أربعة وأربعين عزلة من بكتيريا *E. faecalis*، 32 (72.7٪) من العينات كانت من البول ، في حين أن 12(27.3٪) كانت من المهبل. تم أخذ الحمض النووي لجميع العزلات التي تم تحديدها مسبقًا واستخدامها في تفاعل البوليميراز المتسلسل التقليدي لتضخيم جين *ddl* ، أظهرت أن جميع العزلات (100٪) عند941 نقطة أساس كانت تعود ل *E. faecalis*.

نظرت الدراسة في ما إذا كانت بعض عزلات *E. faecalis* قد تنتج عوامل ضراوة أم لا. من بين 44 عزلة تم اختبارها ، وجد أن 14 عزلة (31.8٪) منتجة للهيمولايسن ، 12(27.2٪) عزلة التي كانت قادرة على تكوين بروتينيز خارج الخلية. 14(31,8٪) كانت إيجابية لإنتاج الجيلاتيناز. بالإضافة إلى ذلك، كان 22(50٪) من العزلات قادرة على الالتصاق بالخلايا الظهارية، 18(40.9٪) كانت قادرة على تكوين تراص دموي، 32(72.7٪) كانت قادرة على إنتاج انزيم اللايبيز و16(36.3٪) كانت كارهه للماء hydrophobicity .

تم تقييم القدرة على تكوين غشاء حيوي في 44 عزلة لهذه البكتريا ، 31(70.4٪) كانت منتجة قوية للأغشية الحيوية، 3(8. ٪6) منتجة متوسطة للأغشية الحيوية، 4 (9٪) منتجة ضعيفة للأغشية الحيوية بينما 6(13.6٪) كانت غيرمنتجة لهذه الاغشية.

تم تقييم مقاومة المضادات الحيوية باستخدام تقنية Kirby-Bauer لعزلات *E. faecalis*، أذ لوحظ أعلى معدل مقاومة للمضادات الحيوية المستخدمة في الدراسة الحالية كان 42(95.4٪) عزلة كانت مقاومة لـ Synercid، تليها 25(56.8٪) عزلة مقاومة ليفوفلوكساسين، 20(45.4٪) عزلة كانت مقاومة للإريثروميسين والنورفلوكساسين، 19 عزلة (56.8٪) كانت مقاومة للسيبروفلوكساسين، 18(40.9٪) عزلة كانت مقاومة لفانكومايسين، 13(29.5٪) عزلة كانت مقاومة للجنتاميسين، 9(20.4٪) عزلة كانت مقاومة لتيكوبلانين، بينما لم تحدث مقاومة

بين الكشف الجزيئي عن جينات عوامل الضراوة لبكتريا *E. faecalis* أنه من بين 44 عزلة كان 16(36.3٪) جين *vanA* عند (314 زوج أساس)، 14(31.8٪) تحتوي على جين *gelE* عند (213 زوج أساس). 36 (81.8 ٪) جين *cpd* عند (782 نقطة أساس)، 14(31.8 ٪) جين *cylA* عند (688 نقطة أساس)، 34(77.2 ٪) جين *ebpA* عند (517 نقطة أساس)، 12 (27.2 ٪) جين *sprE* عند (591 نقطة أساس)، 20(45.4٪) جين *fsrA* عند (474 نقطة أساس) ، 15 (34٪) لديهم جين الآس عند (320 نقطة أساس) ، 18 (40.9٪) لديهم جين esp عند (933 نقطة أساس) و 15(34٪) جين *efaA* عند (688 نقطة أساس).

يوضح تسلسل جين *gelE* لـ *E. faecalis* وجود تحويل واحد A / C، وتأثير Missense من بنك الجينات ، تم العثور على جزء من جين *gelE* متوافق بنسبة 99 ٪ مع جين *gelE* في NCBI تحت معرف التسلسل: CP0881981. وجزء آخر من التسلسل لجين *gelE* مع *E. faecalis* ، تظهر النتائج توافق 100٪ في *gelE* في البنك الجيني تحت معرف التسلسل: CP088198.1، لذلك لم يلاحظ أي تغيير مسجل من الجين في هذه العزلة. تسلسل جين *sprE* لـ *E. faecalis*، أظهرت النتائج توافق 100٪ في بنك الجينات لجين *sprE* تحت معرف التسلسل: CP070621.1، لذلك ، لم يلاحظ أي تغيير مسجل من بنك الجينات في جين *sprE*. تسلسل جين *fsrA* لأربع عزلات من *E. faecalis*. تم تنسيق النتائج بنسبة 99 ٪ مع وجود تحويل واحد A / T، تأثير Missense تحت معرف التسلسل: CP041738.1. علاوة على ذلك ، أشارت مسافات النشوء والتطور المجاورة في هذه الشجرة إلى تنوع بيولوجي واسع في متواليات *E. faecalis*. في هذه الدراسة، تم تحديد مستوى IL-23 في مصل الاشخاص

المصابين ببكتريا *E. faecalis*، وأظهرت النتائج أنه تم العثور على أعلى متوسط لمستوى IL-23 بين مرضى المسالك البولية المصابين (287.54 ± 12.18ng / ml) مقارنة مع مجموعة السيطرة (188.08 ± 2.29 نانوغرام / مل) مع وجود فرق معنوي بين المجموعتين (P <0.05).

استنتج أن تحديد *E. faecalis* باستخدام جين D-alanine ligase أكثر تخصصا" من الاختبارات الكيميائية الحيوية الأخرى، زادت إمراضية بكتيريا *E. faecalis* بوجود جينات *vanA*، *gelE*، *cpd*، *cylA*، *ebpA*، *sprE*، *fsrA*، *ace* ، *esp* ، efaA وان Interleukin-23 مرتفعة في اصابات *E. faecalis*.

وزارة التعليم العالي والبحث العلمي
جـــــــامعة بـــــــابل
كـــــــــلية الطب

خصائص الجينات المرتبطة بالضراوة للمكورات المعوية البرازية المعزولة من المصادر السريرية

اطروحة مقدمة إلى

مجلس كلية الطب / جامعة بابل
وهي جزء من متطلبات نيل درجة الدكتوراة فلسفة في علم الأحياء المجهرية الطبية

من قبل

حيـــــــــاة غيث ساجت الدعوم

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اشـــــــــراف

الأستاذ الدكتـــــورة

الهـــــام عباس بنيان

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