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جـــــــامعة بـــــــابل
كـــــــــلية الطب

دراسةالعلاقة الجينية بين عزلات المكورات المعوية البرازية المعزولة من عينات مختلفة في محافظة بابل

رسالة مقدمة إلى

مجلس كلية الطب / جامعة بابل
وهي جزء من متطلبات نيل درجة الماجستير في العلوم/ الاحياء المجهرية الطبية

من قبل

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اشـــــــــراف

الأستاذ الدكتـــــورة

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١٤٤٣هـ ٢٠٢٢م

Republic of Iraq
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Study the Genetic Relationship Among *Enterococcus faecalis* Isolated from Different Specimens in Babylon Province

A Thesis
Submitted to the Council of the College of Medicine,
University of Babylon, in Partial Fulfillment of
The Requirements for the Degree of Master in Science/
Medical Microbiology

By

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بِسْمِ اللَّهِ الرَّحْمَنِ الرَّحِيمِ

﴿قَالَ رَبِّ اشْرَحْ لِي صَدْرِي وَيَسِّرْ لِي أَمْرِي﴾

صدق الله العلي العظيم

سورة طه (٢٥-٢٦)

Supervisor Certification

I certify that this thesis entitled "Study the genetic relationship among *Enterococcus faecalis* isolated from different specimens in Babylon province" was prepared by Maha Abbas Najeeb Al-Hassani under my supervision at the college of Medicine, University of Babylon, as a partial requirement for the degree of Master of Science in Medical Microbiology.

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/ / 2022

In view of the available recommendation, I forward this thesis for debate by the examining committee.

Professor

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/ / 2022

Dedication

For …..

My big heart, father and mother

My husband, and My children

For…..

All who supported me in my study my sisters, brothers and friends, who shares me my hopes and supported my efforts.

I dedicate this work.

Maha/2022

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الخـــــــلاصة

من مواقع اصابات مختلفة لبكتريا المكورات المعوية البرازية جمعت١٢٠ عينة شملت (الادرار, البراز, المهبل والجروح) من المرضى بالطرق البكتريولوجية القياسية. من المرضى المراجعين لمستشفيين رئيسيين في محافضة بابل: مستشفى الحلة التعليمي الجراحي و مستشفى الهاشمية العام خلال الفترة من (اب ٢٠٢١ الى تشرين الثاني ٢٠٢١). بعد ذلك , تمت زراعة العينات على الاوساط الانتقائية و شخصت باستخدام الاختبارات البكتريولوجية و الكيموحيوية و (*ddl*) و باستخدام بادئ خاصPCR الطرق الجزيئية(تفاعل البادئ)

باستخدام المكورات المعوية البرازية من اصل ١٢٠ عينة, تم الكشف عن ٢٠ عزلة فقط من *ddl)*. وسط الكروم اجار و باستخدام جين متخصص للمكورات المعوية البرازية لجميع عزلات تمت دراسة بعض جينات عوامل الضراوة باستخدام بادئ خاص للحامض النووي من عزلة PCR, و تم اجراء *srt* كجينات الى ان ٢٠(١٠٠%) عزلة تمتلك *srt* لجين PCR اشار تضخيم المكورات المعوية البرازية هذا الجين , ست عزلات التي تضمنت ٦\ ٦ (١٠٠%) من الادرار , اربع عزلات من البراز٤\٤ (١٠٠%), ٤ عزلات من الجروح ٤\٤ (١٠٠%) , ست عزلات من المهبل ٦\٦ (١٠٠%) تمتلك هذا الجين .

, و قد لوحظ في ٩ عينة (٤٥%) عزلة *fsr*محدد للكشف عن جين PCRتم استخدام اساس من هذه البكتريا و التي تشمل عزلتين من الادرار ٢\ ٦ (٣٣.٣%) و عزلة واحدة من البراز ١\٤ (٢٥%) و عدم وجود عزلات من الجروح ٠\٤ (٠%) و ست عزلات من المهبل ٦\٦ (١٠٠%) .

كما درس جين متعدد الاشكال و متعدد السكريات *cps1* لبكتريا المكورات المعوية البرازية والذي شمل ١٨(٩٠%) من العزلات, تضمنت اربع عزلات من الادرار ٦\٤(٦٦.٦), و اربع عزلات من الخروج ٤\٤(١٠٠%) , واربع عزلات من الجروح ٤\٤(١٠٠%) و ست عزلات من المهبل ٦\٦(١٠٠%) تمتلك هذا الجين .

اوضح بان فقط ٩(٤٥%)PCR بواسطة تقنية *cps2* من الجانب الاخر التحديد الجزيئي لجين من عزلات اعطت نتائج ايجابية لهذا الجين و التي تضمنت ثلاث عزلات من الادرار ٣\٦(٥٠%) و عزلة واحدة من الخروج ١\٤(٢٥%) و اربع عزلات من الجروح ٤\٤(١٠٠%) و عزلة واحدة من المهبل ١\٦(١٦.٦%) .

اوضح بان ١٥(٧٥%) من العزلاتPCR بواسطة تقنية *cps5*بينما التحديد الجزيئي لجين اعطت نتائج موجبة لهذا الجين و التي تضمنت خمس عزلات من الادرار٥\٦ (٨٣.٣%) و عدم وجود عزلة من الخروج ٠\٤ (٠%) و اربع عزلات من الجروح ٤\٤ (١٠٠%) و ست عزلات من المهبل ٦\٦(١٠٠%) .

Atl هو عامل ضراوة في بكتريا المكورات المعوية البرازية و الذي تم الكشف عنه في ٢٠ عزلة من هذه البكتريا مختلفة المصادر اوضحت دراستنا بان من ٢٠ عزلة فقط ٩(٤٥%) عزلات اعطت نتائج ايجابية لهذا الجين و التي تضمنت عزلتين من الادرار ٢\٦(٣٣.٣%) و عزلة واحدة من الخروج ١\٤(٢٥%) و عدم وجود عزلات من الجروح ٠\٤(٠%) و ست عزلات من المهبل ٦\٦(١٠٠%).

و بالفعل اوضحت الدراسة ان الكشف عن التنوع الجيني بين العزلات البكتيرية تم اجراءه باستخدام *المكورات المعوية البرازية* و تم تاكيد وجودهم في (ERIC-PCR),(BOX-PCR)and ((GTG)5-

استطاعت تحديد مجموعات التي (GTG)5and ERIC,BOXاستخدمت الدراسة الحالية

بصمت (PCRوقد ثبت انها مفيدة في تحديد مصدر عزلات *E. faecalis* التطعيم لعزلات وثيقة الصلة وراثيا او لتقييم درجة مسافة الارتباط بين المصادر المختلفة للافراد وفقا للتسلسل المتكرر للحفظ في الجينوم البكتيري. انماطا مختلفة من تعرض *E. faecalis*ان عزلات UPGAM وجدت بيانات التسجيل من التوزيع داخل كل علامة تمت دراستها و يمكن رؤية قيم تعدد الاشكال للباندات. فيما يتعلق ب تجمعة في المصدر من نفس المجموعة *E. faecalis* ان النتائجتم عرض , UPGMA مجموعات مختلفة من الكتلة و تداخلت مع مصدر عزل اخر. الى جانب المؤشرات التمييزية التي باستخدام مؤشر سيمبونز للتنوع الذي كان٠,٠٤١٩٩ ,BOX, ERIC5(GTG تم حسابها ل ٠,٣٠٧٨ \٠,٢٣٥٤ و هي قيمة ثقة مقبولة لتغيير مستوى التمييز .

هي الطريقة 5(GTG) بناء على تحليل كتلة النتائج و تحليل الوضيفة التمايزية , وجد ان

5(GTG) .تتمتع.BOXو ERIC متبوعا ب *E. faecalis*المناسبة للطعن في التصنيف الجزيئي ل

, و بالتالي يمكن ان تكون اداة مفيدة لتحليل *E. faecalis* بقدرة تمييزية على التمايز بين

E. faecalis .

جميع المكورات المعوية البرازية التي تم تحديدها و المعزولة من مصادر مختلفة في المختبر

لاختبار الحساسية للمضادات الحيوية عن طريق نشر قرص كيربي باور . وجد ان عزلات

الكانامايسين هي ٢٠(١٠٠%) عزلة مقاومة لكل من الكليندامايسين و *E. faecalis*

, ١٨(٩٠%) عزلة كانت مقاومة للامبينيم, ٢٠(١٠٠%) عزلة مقاومة للسيبروفلوكساسين

, ١٧(٨٥%) عزلة كانت مقاومة لكل من الاريثرومايسين و الفانكومايسين , ٢(١٠%)

عزلة مقاومة لليفوفلوكساسين , ١(٥%) عزلة كانت مقاومة لكل من البيبراسلين و التيكوبلانين .

Summary

A total of 120 clinical specimens ,were collected from patients suffering from urinary tract infection, vaginitis, diarrhea, bacteremia and wound infection who admitted to two main hospitals of Babylon Governorate: Al-Hilla Surgical Teaching Hospital and Al-Hashimiyah General Hospital during a period extending from (August 2021 to November 2021). Then, the specimens were cultured in selective media (chrom agar media) and identified by using bacteriological, biochemical tests and molecular method (PCR) using specific gene (*ddl*).

Out of 120 specimens, only 20 isolates of *E. faecalis* were detected by culture, biochemical test and specific gene six isolates obtained from urine specimen, four isolates from wound, six isolates from women vagina , four isolates obtain from stool specimen and there was no isolate obtained from blood specimens.

Some virulence factors genes of all *E. faecalis* isolates were studied as sortase peptide(*srt*) gene, PCR was performed using specific primer on genomic DNA from *E. faecalis* isolate. PCR amplification of *srt* gene indicated that all isolates 20(100%) were carried this gene, which include six from urine isolates6/6(100%), four isolates from stool 4/4(100%), four isolates from wound 4/4(100%) and six isolates from vagina 6/6(100%).

Specific PCR primer was used for detection of *fsr*(faecal Streptococci regulator) gene, it was observed in 9(45%) of these 20 isolates, which include two isolates from urine 2/6(33.3%), one isolate from stool 1/4(25%), no isolates from wound 0/4(0%)and six isolates from vagina 6/6(100%) .

Capsule polysaccharide(*cps1*) gene polymorphism was also studied and it was found that most isolates were carried *cps1* marker, observed in 18(90%) isolates out of 20 *E. faecalis*, , which include four isolates from urine 4/6(66.6%),six isolates from vagina 6/6(100%),four isolates from stool 4/4(100%),four isolates from wound 4/4(100%).

On the other hand, molecular detection of *cps2* gene by PCR technique showed that only 9(45%) isolates gave positive results for this marker which include three isolates from urine 3/6(50%), one isolate from stool 1/4(25%), four isolates from wound 4/4(100%)and one isolate from vagina 1/6(16.6%) .

While molecular detection of *cps5* gene by PCR technique showed that 15(75%) isolates gave positive results of this marker which include five isolates from urine 5/6(83.3%), no isolate from stool 0/4(0%), four isolates from wound 4/4(100%)and six isolates from vagina 6/6(100%) .

Autolycin is a virulence factor in *E. faecalis* and it was investigated in 20 *E. faecalis* isolated from different sources. This study showed that only 9(45%) isolates gave positive results to this gene, which include two isolates from urine 2/6(33.3%), one isolates from stool 1/4(25%), no isolates from wound 0/4(0%) and six isolates from vagina 6/6(100%) .

Indeed, the study implicated the detection of genetic diversity among bacterial isolates through using (ERIC-PCR), (BOX-PCR) and ((GTG)$_5$-PCR) and there were confirmed present in *E. faecalis*. The study employed the ERIC, BOX and (GTG)$_5$ fingerprint it was able to determining the source groups of *E. faecalis* isolates and may prove to be useful for determintion the source of genetically closely related *E. faecalis* isolates or to evaluate the degree of relatedness distance between different source of specimens according to conservation repetitive sequences in bacterial genome.

The scoring data from UPGMA found the *E. faecalis* isolates display different patterns of distribution within each marker studied can be seen with the polymorphism values of bands. So regarding to UPGMA, the result was shown the *E. faecalis* isolated from the same source were clustered into different groups of cluster, and had cross with other isolates source. Besides, discriminatory indexes (DI) were calculated for (GTG)$_5$, BOX and ERIC- PCR by using Simpson's index of diversity which were 0.4199, 0.3578 and 0.2353 respectively which is acceptable confidence value for interpreting the level of discrimination.

Summary

Based on result of cluster analysis and discriminant function analysis, $(GTG)_5$ – PCR was found to be the most suitable method for molecular typing of *E. faecalis* followed by BOX and ERIC. The $(GTG)_5$ has a discriminatory power for the differentiation of *E. faecalis* and therefore can be useful tool for the analysis of *E. faecalis*.

All the identified *E. faecalis* isolated from different sources were subjected in *vitro* to antibiotic susceptibility test by modified Kirby-Bauer disc diffusion method. It has been found that *E. faecalis* isolates were 20(100%) isolates were resistant to clindamycin, ciprofloxacin and kanamycin for each, 18(90%) isolates were resistant to imipenem, 17(85%) isolates were resistant to erythromycin and vancomycin for each, 2(10%) isolates were resistant to levofloxacin, 1(5%) isolates were resistant to teicoplanin and piperacillin.

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Abbreviation

Abbreviation	***Meaning***
°C	Celsius degree
Ace	Collagen binding protein
Agg	Aggregation substance
AgrB	Accessory gene regulator protein
Asa1	Aggregation substance
AtlA	Acetylglucosaminidase
BHI	Brain heart infusion
bps	Biofilm and pilus -associated sortase
Can	Collagen-binding proteins
CLSI	Clinical Laboratory Standard Institute guidelines
Cpd	Sex pheromone-responsive plasmids
CIP	Ciprofloxacin
CWSS	Cell wall sorting signal
coNS	Coagulase negative Staphyllococci
ddl	D-Alanine D-Alanine Ligase
DI	Discrimination index
DNA	Deoxyribonucleic acid
DW	Distilled water
E. faecalis	*Enterococcus faecalis*
Ebp	Endocarditis- and biofilm-associated pili
ECM	Extracellular matrix

eDNA	Extracellular DNA
EfaA	Endocarditis antigen A gene
Epa	Enterococcal polysaccharide antigen
ERIC	Enterobacterial Repetitive Intergenic Consensus
Esp	Enterococcal surface protein
erm	Erythromycin gene
GBAP	Gelatinase biosynthesis-activating pheromone
GelE	Gelatinase gene
GI	Gastroenteritis
GTF	Glycosyl transferases
gm	Gram
fsr	Faecal streptococci regulator
HA	Hyaluronic acid
HAI	Hospital-acquired infections
HGT	Horizontal gene transfer
ICU	Intensive Care Units
IBD	Inflammatory bowel disease
hyl	Hyaluronidase Enzyme
kD	Kilo Dalton
LAB	Lactic acid bacteria
Luxs	luminescence
LP*X*TG	Endopeptidase enzyme
LPXTGX	Leu-Pro-any-Thr-Gly
MDR	Multidrug resistant
MIIEs	Miniature Inverted Transposable Elements
ml	Milliliter
msr	Specifies resistance to macrolide
mes	Macrolide efflux
mef	Efflux pump resistance to macrolide
PADI	Pheromone-responsive plasmids
PID	Pelvic inflammatory disease
PBP	Penicillin Binding Protein
PCR	Polymerase Chain Reaction
PFGE	Pulcid field gel electrophoresis
PMNs	Polymorphnuclear leuckocyte
SprE	Serine protease
srt	Sortase peptide gene
tet	Tetracycline gene

UPGMA	Unweight Group Pair Method with Arithmetic Averages
UTIs	Urinary tract infections
VRE	Vancomycin resistant enterococci
µl	Microliter

Chapter One

Introduction and Literatures Review

1.1 Introduction:

Enterococcus faecalis formerly classified as part of the group D Streptococcus system is a Gram-positive, commensal bacterium inhabiting the gastrointestinal tract of human (Khan *et al*., 2018). It is a type of bacteria that inhabit gastrointestinal tract, there are at least 18 different species of these bacteria. It is one of the most common species. These bacteria also live in the root chanal for teeth and vagina (Javed and Manzoor, 2020).

E. faecalis are an opportunistic pathogen facultative anaerobic, Gram-positive that is spherical or oval in shape bacteria and has emerged as a major cause of nosocomial infections worldwide. The bacterium is non spore-forming, esculin-positive, catalase and oxidase negative, non-motile (Sreeja, 2010).

Like other species in the genus *Enterococcus*, *E. faecalis* is found in healthy humans, but can cause life-threatening infections, especially in the nosocomial (hospital) environment, where the naturally high levels of antibiotic resistance found in *E. faecalis* contribute to its pathogenicity (Chayon, 2018). *E. faecalis* is found in most healthy individuals, but can cause endocarditis and sepsis, urinary tract infections (UTIs), meningitis, and other infections in humans (Poolman and Wacker, 2016). However, Enterococci have proven very competent in causing opportunistic infections in hospitalized patients, particularly in debilitated hosts (Selleck *et al*. 2019).

Urinary tract infections (UTIs) are common in women and are one of the most frequent human bacterial infections . UTIs are responsible for significant worldwide morbidity and loss of workplace productivity . Although most UTIs (80%–90%) are caused by extra-intestinal *Escherichia coli* strains, *Enterococcus faecalis* strains have more frequently been isolated

and have been reported to be causative agents in up to 20% of all cases (Abat *et al*.,2016).

While *E. faecalis* colonization is normally asymptomatic, certain populations are at risk for severe disease, including urinary tract infections, wound infections, pelvic inflammatory disease (PID), infective endocarditis, and adverse birth effects during pregnancy (Schindlbeck *et al.*,2014).

Generally, the pathogenesis of Enterococcal infections follows a common sequence of events and different virulence factors of *E. faecalis* can play a role in each of these steps. First, Enterococci possessing various virulence traits asymptomatically colonize the gastrointestinal tract. Factors that enhance the virulence of Enterococci through this way are found more frequently among isolates from hospitalized patients compared to isolates from the community. Finally, Enterococci can cause symptomatic disease at the level of tissue destruction or toxicity (Okwen, 2016).

To confirm the identity of *E. faecalis*, we resorted to a PCR assay detecting internal fragments of the gene encoding D-alanine-D-alanine ligase, *ddl*, as this gene was described to be diagnostic and to differentiate the two major clinically important species *E. faecalis* and *E. faecium(*Hashem *et al*.,2021).

Different virulence factors has been found in *E. faecalis*, such as faecal streptococci regulator(*fsr*), capsule polysaccharide (*cps*), sortase peptide(*srt*), and autolysine(*Atl*). Knowledge of the virulence characteristics of circulating Enterococcus strains may help to understand the complex pathogenic process of these opportunistic microorganisms (Comerlato *et al*., 2013).

A major quorum sensing system in *E. faecalis*, the *fsr* regulator locus, is encoded by *fsrA, fsrB* and *fsrC* genes, which regulate the expression of both gelatinase and serine protease. The *fsr* quorum-sensing system controls biofilm

development through regulating the production of gelatinase (Hashem *et al*.,2021).

Sortase (*srt*) is a transmembrane protein responsible for covalently anchoring several virulence factors and adhesins to the cell wall of Gram-positive organisms, including *E. faecalis* (Mitra,2018).

Autolysin, is assist in pathogenesis due to its ability to break down the wall or lyse a portion of the invading Enterococci and release potentially lethal toxins into the cell(Smith *et al*.,2000).

Some strains of *E. faecalis* produce capsule, which contributes to pathogenesis through evasion of host defenses, and its production is dependent on the capsule (*cps*) operon polymorphism(Pinheiro *et al*.,2012).

E faecalis, particularly vancomycin-resistant strains are an important cause of nosocomial infections such as bacteremia, sepsis, endocarditis, urinary tract infection (UTI) and wound infection (Heidari *et al*.,2017). The combination of a cell wall active agent (penicillin, or vancomycin) and an aminoglycoside, has been used frequently for treatment of serious enterococcal infections. However, treatment of enterococcal infections could be difficult due to increasing resistance of enterococci to antimicrobial agents such as Beta-lactams, high-level resistance to aminoglycosides and more recently to glycopeptides (Miller *et al*.,2016).

The repetitive element sequence-based PCR (rep-PCR) was proven to be a useful tool for identification of enterococci on species (Jurkovič *et al*., 2007). (GTG)5-PCR and ERIC-PCR were applied for evaluation of genetic relatedness using total genomic DNA. Malathum *et al*., (1998) used the BOX- PCR and REP-PCR for typing of *E. faecalis* strains.

Aim of study:

Study the genetic relationship among *E. faecalis* Isolated from different specimens in Babylon province.

Objectives:

1-Isolation and identification of *E. faecalis* from different clinical samples (urine, stool, vagina swab, blood, wound swab) .

2- Molecular diagnosis of *E. faecalis* by using specific primer.

3- Study antibiotic susceptibility test on bacteria.

4- Detection of some virulence genes of *E. faecalis* by PCR technique

5- Study of genotyping in *E. faecalis* isolated from different clinical specimens. by using BOX -PCR , ERIC-PCR and (GTG)5.

1.2 Literatures review:

1.2.1 Characteristics of *Enterococcus faecalis*:

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

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

E. faecalis is a non-motile microbe; it ferments glucose without gas production, catalase and oxidase negative, capsulated bacteria (Morandi *et al*., 2012). It shows consistent growth throughout nutrient broth which is consistent with being a facultative anaerobe. It catabolizes a variety of energy sources, including glycerol, lactate, malate, citrate, arginine, and many keto acids. Enterococci survive in very harsh environments, including extremely alkaline pH (9.6) and salt concentrations. (John *et al*., 2015).

Enterococcus is a large genus of lactic acid bacteria that have the ability to grow under various aggressive conditions. Enterococci are Gram-positive cocci that often occur in pairs (diplococci) or short chains, and are difficult to distinguish from streptococci on physical characteristics alone (Bloushy *et al*., 2018). Enterococcus species are facultative anaerobic organisms that can survive at 60°C for short times and can grow in high salt concentrations. However the genus *Enterococcus* is composed of thirty-eight species, *E. faecalis* (*E. faecalis*) and *E. faecium* (*E. faecium*) are considered the most

common commensal two species normally inhabitant in the intestine of both humans and animals.(John and Carvalho, 2011)

Enterococcus, previously known as group D Streptococcus, is a gram-positive commensal organism that is universally found in the human gastrointestinal tract. It is an organism of low virulence but has long been recognized as a cause of endocarditis (third following staphylococci and) in the community and has proven to be an important cause of streptococci hospital-associated infections, especially infections related to the gastrointestinal tract, the urinary tract, and catheter-associated infections. Endocarditis, in particular, is associated with high mortality rates(Magill *et al*.,2014; Arias *et al*.,2015).

E. faecalis can resistance oxidative stress, sanitizers, hefty metals, ethanol, and sodium azide for quite a long time or even months (Tan *et al*., 2019). Moreover, it can endures warming for 30 minutes at 60°C or 10 minutes at 65°C. Another distinctive component is that it develops well at 6.5 % NaCl focuses and at pH 4.0 to 9.6 in unequivocally acidic or soluble conditions. Within the sight of 40% bile salts, *E. faecalis* can likewise hydrolyze leucin-pyrrolidonyl naphthylamide and esculin (Saito *et al*., 2018).

The enterococci are a group of non-spore, disposed individually, in pairs, chains of various lengths or in groups. They are facultative anaerobes and chemo-organ trophic organisms. Enterococci have an optimal growth between 35 ºC to 37 ºC and a wide range of growth temperature, from 10 to > 45 ºC They have been considered as part of lactic acid bacteria being classified as homofermentative. They produce lactic acid as the main and predominant product of carbohydrate fermentation, without gas production(Manimala,2019).

Enterococcus can express alpha, gamma, or beta hemolysis on blood agar. Haemolysin producing strains of enterococci have been shown to be virulent in a human infections, and to be associated with increased severity of infection. Trypticase soy agar or Columbia agar with 5% (v/v) defibrinated sheep blood may be used to assess the haemolysis produced by enterococci. If

human or horse blood is used, hemolysis is based on cytolysis activity and may cause a β-haemolytic reaction, Bacterial colonies appear gray on blood agar and turquoise color on chromogenic agar medium(Atlas and Snyder, 2015).

Enterococci are often recovered from cultures of intra- abdominal, pelvic, and soft tissue infections. They are almost always isolated as only one component of mixed microbial flora and rarely cause monomicrobial infection at these sites. The importance of enterococci in wounds and pus has been debated at length. However, with enterococcal bacteremia commonly associated with pelvic and intra-abdominal abscess and wounds (Gilmore *et al*., 2014).

1.2.2 Classification of *Enterococcus faecalis*:

Human microbiome research has shown that the body is inhabited by approximately 5000 species of microorganisms belonging to 2000 genera and 25 phyla, which possess a total of 316 million genes (Thomas and Segata ,2019). It is estimated that there are 9 million different genes of bacterial origin related to the human digestive system alone .The gastrointestinal (GI) tract is mainly colonized by species belonging to the phylum Firmicutes, which accounts for up to 65% of all bacteria(Yang *et al*.,2009).Enterococci belong to the phylum Firmicutes in the family Enterococcaceae, which includes a great variety of species. Enterococci are a natural component of the human microbiota. They colonize the lower GI tract, the oral cavity, and the genital tract .There are approximately 106 to 107 *Enterococcus* in the human intestine (Qin *et al*., 2010).

Kilpper and Scheifer.,(1984) were used nucleic acid hybridization and RNA sequencing to separate the intestinal bacteria from the genus Streptococci once and for all and put them in to a new genus based on those studies in addition to serological and biochemical tests ,bringing the number of species to 28(Doming *et al* .,2003 Shanks *et al* .,2006).

E. faecalis is classified in to:

Kingdom : Eubacteria

Phylum : Firmicutes

Class : Bacilli

Order : Lactobacillales

Family : Enterococceae

Genus : *Enterococcus*

Species*: E. faecalis*

E. faecalis formerly classified as part of the group D *Streptococcus* system is a Gram-positive, commensal bacterium inhabiting the gastrointestinal tracts of humans and other mammals. Enterococci are becoming one of the most common causes of infection in elderly population. *E. faecalis* is the most common among enterococci responsible for 63-81% of cases ,and malignancy is the most common comorbidity (Kajihara *et al*.,2015).

Principle component analysis loading line plots highlight the inter-genus and inter-species differences at various wavelengths, which are mostly assigned to cell-wall compounds such as polysaccharides. The best artificial neural network identification models give 98.8% and 86.3% classification rates at the genus and species level, respectively(Treguier *et al*.,2019).

1.2.3 Pathogenesis of *Enterococcus faecalis* :

Enterococci are considered as commensals of the human gut flora. They don't normally cause infections in healthy peoples, with the exception of occasional urinary tract infection. However, enterococci have proven very competent in causing opportunistic infections in hospitalized patients, particularly in debilitated hosts (Dorsch *et al*., 2019). It can cause endocarditis ,sepsis, urinary tract infections (UTIs), meningitis, and other infections in humans (Kline & Lewis, 2017).

The majority of enterococcal infections are caused by *E. faecalis*. It is accounts for the majority (65.80%) of nosocomial infections caused by enterococci. That the majority of enterococcal infections are caused by the species *E. faecalis* may reflect either a greater level of bacterial virulence, or the increased prevalence of *E. faecalis* in intimate association with the host, or both (Zou & Shankar, 2016).

E. faecalis possesses the ability to survive for up to 72 hours in peritoneal macrophages. They also showed that enterococci possess the ability to translocate from the intestinal lumen into the mesenteric lymph nodes, liver, and spleen (Barnes *et al*., 2017)

Bacteria including enterococci can be introduced directly into the urinary tract, or can colonize surgical wounds and the bloodstream by colonizing sutures, catheters, or other indwelling devices. *E. faecalis* bloodstream isolates from catheterized patients exhibited a stronger propensity to form biofilm when compared to isolates from no catheterized patients or isolates of unclear clinical significance (Goh *et al*., 2017).

In order for the enterococci to cause disease, they must first adhere to host tissues. Toward this end, they possess surface adhesion proteins, such as aggregation substance, that allow them to bind to the cells of the human intestine. When “sex pheromones” are produced and secreted by enterococci, certain strains of enterococci respond by producing the aggregation substance (Sava *et al*., 2010) .

The aggregation substance is a hair-like protein that is embedded in the cytoplasmic membrane and it enables cell-to-cell contact between donor and recipient strains for conjugation (Fisher and Phillips, 2009).

For enterococci to cause disease several barriers must first be overcome. An initial barrier is the ability to overcome colonization resistance provided by competing microbes, and host defenses such as gastric acid and bile, and colonize the intestinal tract. From this reservoir the bacteria can amplify in

number and spread to sites vulnerable to infection(Fiore *et al*., 2019). A basic prediction from such a model is that the probability of infection should be a function of the intestinal burden of bacteria in the gut reservoir – the more bacteria, the greater the probability of contamination of a potential infection site in numbers large enough to overcome host defenses. Indeed, colonization of the GI tract has been shown to be directly associated with risk of infection (Taur *et al*.,2012). Infection occurs when enterococci overwhelm host defenses, replicate at rates that exceed clearance, and when pathologic changes result through direct toxin activity, or indirectly by bystander damage from the inflammatory response(Garsin *et al*.,2014).

Colonization and proliferation of hospital-adapted lineages of enterococci is usually associated with antibiotic-induced disruption of the community structure . Therefore, effectively managing the human microbiome in health and disease represents a theoretically promising strategy for preventing hospital infection(Ubeda *et al*.,2010).

In order to produce infection, enterococci must be able to colonize host tissues, resist the host's non-specific and immune defense mechanisms and produce pathological changes. With regard to colonization of host tissues, adherence assays have shown that enterococci can attach to intestinal and urinary tract epithelial cells and heart cells by means of adhesins expressed on the bacterial surface. The expression of these adhesins by enterococci has further been shown to be affected by bacterial growth conditions. In addition, the adherence of *E. faecalis* to renal tubular cells in vitro is enhanced if the organisms produce aggregation substance, a proteinaceous surface material that aggregates donor and recipient bacteria to facilitate plasmid transfer. Bacterial growth conditions also affect the interaction of enterococci with polymorphonuclear leucocytes (PMNLs), with serum-grown organisms showing less association with PMNLs than organisms grown in broth. Efficient killing of enterococci by PMNLs in vitro requires the presence of serum

complement proteins and is enhanced by anti-enterococcal antibodies. Enterococci produce a number of factors that may be associated with pathological changes in the host(Guzman *et al*.,1989).

Both sex pheromones and plasmid-encoded pheromone inhibitors produced by *E. faecalis* are chemotactic for PMNLs in *vitro*, and may mediate, at least in part, the inflammatory response often associated with enterococcal infection. *E. faecalis* may also produce a plasmid-encoded haemolysin, which is associated with increased severity of infection. In addition, enterococci are capable of inducing platelet aggregation and tissue factor-dependent fibrin production, which may be relevant to the pathogenesis of enterococcal endocarditis. Although questions concerning the pathogenicity of enterococci remain unanswered, it is clear that we are now beginning to understand the mechanisms by which this important group of microorganisms produce disease(Alan,1994).

The immune response to the bacteria can cause septic shock and sepsis which has a relatively high mortality rate. Bacteria can enter the bloodstream as a severe complication of infections (like pneumonia or meningitis), during surgery (especially when involving mucous membranes such as the gastrointestinal tract), or due to catheters and other foreign bodies entering the arteries or veins (including intravenous drug abuse) (Chen and Zervos ,2009).

1.2.4 Virulence factors of *Enterococcus faecalis*:

Enterococci have a variety of virulence factors that allow them to bind to host cells and extracellular matrix, invade tissues, modulate the immune system, and cause toxin-mediated damage (Dickey *et al*., 2017).

Virulence factors are traits or molecules that are produced by pathogens that help these pathogens with colonization, immunoevasion, and immunosuppression of their hosts. Consequently, these virulence factors are often responsible for causing disease. Though the enterococci do not produce potent toxins like some other bacteria, they do possess virulence factors in the

form of aggregation substance, enterococcal surface protein (*Esp*), cytolysin, gelatinase, and antibiotic resistance genes (Sava *et al*., 2010) . In addition, they can release superoxide to the extracellular environment (Wang and Huycke, 2007) . Many of the genes for the enterococcal virulence factors are found on conjugative plasmids or encoded within transposons and are easily transferable (Palmer *et al*., 2010).

These opportunistic bacteria possess various virulence factors including enterococcal surface protein and aggregation substance which could enhance the colonization process in the host and binding to the host epithelium, respectively. Others such as cytolysin, enterolysinA, capsule, gelatinase, hyaluronidase, and Zinc.metalloendopeptidase, enhanced expression of pheromone and adhesion-associated protein (*E. faecalis* endocarditis antigen A) have been reported to be among the most important virulence factors (Madsen *et al*., 2017). On the other hand, *E. faecalis* produces cell walls anchored proteins that aid in binding host cells including adhesion to collagen of *E. faecalis* which also plays a role in virulence (Kenia *et al.,* 2019).

Many factors determine the virulence *of Enterococcus* species, for example, the ability to colonize the gastrointestinal tract or to adhere to a range of extracellular matrix proteins or to the epithelial cells . Several enterococcal virulence genes that may be involved in the onset of a disease in humans or exacerbation of the disease symptoms(Anderson *et al* .,2016).

1.2.4.1Capsular Polysaccharid polymorphism(*cps*)genes:

Capsular polysaccharides are major contributors to the virulence of many microorganisms. The presence of capsule allows these microbes to escape detection and clearance by the host immune system (Graveline *et al*.,2007). Several attempts have been made to establish a serotyping system for *E. faecalis* capsular polysaccharides. These serotyping schemes include differences in capsular polysaccharide antigens but are also based on

differences in surface antigens, including lipoteichoic acid (Theilacker *et al*.,2006). Two loci that have been reported to contain putative genes for capsule production are the enterococcal polysaccharide antigen(*epa)* and *cps* operons (Hancock *et al*.,2002). The polysaccharide produced by the *epa* locus is thought to be the cell wall rhamnopolymer , but it cannot be detected on the surface of the bacterium . Although rhamnopolymer production is reported to be abrogated by mutation. The full nature of rhamnopolymer production is to be determined for many *E. faecalis* strains. (Hufnagel *et al*.,2005).

E. faecalis has several types of capsules; one of them is based on the type of antigen in the polysaccharide capsule .Polysaccharide capsules can also be used to identify *E. faecalis* genotypes by considering the type of capsule. *E. faecalis* polysaccharide capsules have 11 different variations, namely, *cpsA–cpsK*. Only seven groups of *E. faecalis* capsules have been identified, i.e. *cpsC, cpsD, cpsE, cpsG, cpsI, cpsJ,* and *cpsK* (Pinheiro *et al* .,2012).

The *cps* operon encodes the polysaccharide capsule and has 11 open reading frames (from *cpsA* to *cpsK)* and 3 polymorphisms: *cps1*, comprising *cpsA* and *cpsB*; *cps2*, comprising all 11 genes in the operon; and *cps5*, comprising all genes, except *cpsF*. Only *cps2* and *5* encode the capsular polysaccharide whereas *cps1* do not (Pinheiro *et al*.,2012).

Genetically, the synthesis of polysaccharide capsule operon coding and a polymorphism locus was found in the clinical isolates of *E. faecalis. cps2* is the major bacterial strain that has been implicated in root canal persistent intraradicular infection relative to *cps1* and *cps5* strains. *E. faecalis cps2* is comparatively common in Indonesians who require root canal treatment (Bachtiar *et al* .,2015).

Polysaccharide capsules act as important virulence factors expressed by bacteria. This is due to the polysaccharide capsule being able to protect bacteria from the host's immune system, which can make the infection persist longer.

Bacterial strains that produce capsules are more resistant than bacteria that do not have capsules (Bachtiar *et al*.,2015).

1.2.4. Faecal Streptococci Regulator(*fsr*) gene :

The important virulence Factors in *Enterococcus* spp. are regulated by quourum sensing, including the *fsr* (faecal streptococci regulator) locus. Changes in the activity of particular genes lead to maturation of the biofilm and the appearance of appropriate phenotypic traits, depending on the conditions (Mohamed & Huang,2007).

The *fsr* locus of *E. faecalis* encodes a two-component regulatory system that senses the cell density and regulates virulence . The *fsr* locus is 2.8 kb in size and comprises four genes: *fsrA, fsrB, fsrD,* and *fsrC* . The *fsrA* gene encodes the *fsrA* protein, which belongs to the LytTR family of DNA-binding domains (Del Papa *et al.*,2011). The binding of phosphorylated *fsrA* to LytTR-binding sites in the upstream region of ef1097, *fsrB*, and *gelE* suggested that *fsrA* is a response regulator of the *fsr* system (Cook *et al*.,2011). Notably, *fsrA* transcription is under the control of a constitutive promoter; therefore, it is independent of the *fsr* quorum-sensing system. The *fsrB* gene encodes a transmembrane protein, *fsrB*, which belongs to the accessory gene regulator protein B (*AgrB*) family. *fsrB* processes a propeptide, *fsrD* (encoded by *fsrD*), to generate GBAP (Gelatinase biosynthesis-activating pheromone) (a lactone ring containing a short cyclic peptide of 11 amino acid residues), which is further exported out of the cell . The fourth gene, *fsrC,* encodes the transmembrane histidine protein kinase *fsrC,* the sensor-transmitter of the *fsr* operon (Cook *et al*.,2014).

In *E. faecalis*, the *fsr* system up regulates expression of gelatinase (*gelE*) and serine protease (*sprE*) virulence factors in a cell density-dependent manner and in this regard resembles the much-studied regulation of toxin synthesis by the *Staphylococcus aureus Agr* quorum-sensing system (Wang *et al*.,2017).Indeed, the *fsr* system is the main activator of *gelE* expression , but

has also been implicated in virulence independently of *gelE* (Bourgogne *et al*., 2006).The peptide mediator of *fsr* quorum sensing is a peptide lactone encoded by *fsrD* located just downstream of, and in frame with *fsrB* (Nakayama *et al*.,2006).

In *E. faecalis*, the *fsr* quorum sensing system, is an important regulator with both positive and negative effects, regulating *gelE* and *sprE* expression that are important for biofilm formation, along with genes implicated in several metabolic pathways. The earliest reports of *E. faecalis* in associated with infection-related biofilm were probably from the studies that identified strains in infected vascular ports from patients and in a urinary stone. Moreover, isolates from endocarditis and intravascular catheter associated bloodstream infections display particularly robust formation of biofilms(Roilides *et al* .,2015).

Quorum sensing (QS) is a cell-to-cell communication process that regulates major pathogenic attributes in bacteria including biofilm formation, secretion of virulence factors, and antimicrobial resistance. The two-component *fsr*-QS system of the nosocomial pathogen *E. faecalis* controls the production of extracellular gelatinase that contributes to biofilm development by enhancing the release of nucleic acids into the biofilm matrix. However, the contribution of this system to the deposition of other biofilm matrix components such as polysaccharides and proteins remains unknown. Using wild type and mutant strains, the biofilm formation was attenuated by inactivation of the *fsr* system or its downstream gelatinase production. Inactivation of the *fsr* system caused a modest, the significant reduction in biofilm metabolic activity without affecting cell counts. Inactivation of the QS-signal sensor *fsrC* and response regulator *fsrA* resulted in decreased extracellular polysaccharides and proteins in biofilms in a temporal manner. Irrespective of biofilm age, eDNA levels were reduced in the gelatinase mutant strain (Barnes *et al*.,2012; Asfahl *et al*.,2017; Ali *et al*., 2021).

Moreover, further studies are required to understand the exact mechanisms of *fsr*'s functions. It is speculated that there might be additional systems that play different roles in *E. faecalis* virulence, either directly or indirectly. Understanding *fsr* quorum-sensing would help to develop new and effective anti virulence drugs against *E. faecalis* pathogenesis(Dale *et al*.,2015).

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

E. faecalis molecular detection of virulence genes also showed significant correlations between the presence of *gelE* and *sprE* genes and the strength of biofilm formed, and between *fsrB* and gelatinase activity, but confirmed prior findings that the presence of *gelE* is not sufficient to predict gelatinase activity, whereas the quorum sensing *fsr* locus was an important predictor(Hashem *et al* .,2021)

1.2.4. 3 Sortase Peptide (*Srt*)gene:

Sortases (*srtA* and *srtC*) are enzymes spatially localized at the septal region in majority of gram-positive bacteria during the cell cycle, which in-turn plays an important role in proper assembling of adhesive surface proteins and pilus on cell membrane. the both *srtA* and *srtC* were focally localized in *E. faecalis* and essential for efficient bacterial colonization and biofilm formation on the host tissue surfaces. The sortase mutants produce defective pili and found to be less virulent than the wild type strain (Spirig *et al* ., 2011).

The peptides targeting sortase family proteins were identified as potential therapeutics to kill multidrug resistant bacterial strains, which is an emerging field in the drug discovery process(Culp & Wright.,2017).

Sortases are membrane-bound transpeptidases that cleave the sorting signal of the secreted protein to form an isopeptide bond between the secreted protein and peptidoglycan. They are either responsible for covalently anchoring specific surface proteins or polymerizing pilin sub-units to form a proteinaceous structure termed pili (Hendrickx *et al*., 2011). Sortase-displayed surface structures play a pivotal role in displaying virulence and pathogenesis properties without affecting the growth and viability of cells. They are responsible for cell attachment, heme transport, nutrient uptake, sporulation and aerial hyphae formation (Weiss *et al*., 2004; Cheng *et al*., 2009).

Class A sortase is well characterized and found mostly in low GC content Gram-positive bacteria. They play a housekeeping role in anchoring a variety of functionally distinct surface proteins with an LPXTG((Leu-Pro-any-Thr-Gly) recognition sequence. Class C sortases are responsible for constructing complex pili polymers by recognizing the LPXTG (Endopeptidase enzyme) motif (Girolamo *et al*., 2019).

SrtA possesses a single transmembrane helix and a positively charged cytoplasmic tail which hypothesize to play a role in the focal localization of the protein to the septum. Indeed, the positively-charged C-terminal tail of *srtC*, the pilin polymerizing sortase in *E. faecalis*, is necessary for its localization and function (Mitra.,2018).

Sortases are membrane-bound transpeptidases that cleave the sorting signal of the secreted protein to form an isopeptide bond between the secreted protein and peptidoglycan. They are either responsible for covalently anchoring specific surface proteins or polymerizing pilin sub-units to form a proteinaceous structure termed pili (Susmitha *et al*., 2021).

In most gram-positive bacteria, membrane-anchored transpeptidase enzymes known as sortases are responsible for covalently anchoring cell surface proteins bearing an LPXTG motif to the cell wall .Thus far, only class A and class C sortases have been implicated in biofilm formation and the virulence of *E. faecalis*. Deletion of *srtC*, also known as bps(biofilm and pilus-associated sortase), which encodes sortase C (*srtC*), resulted in a significant reduction in biofilm production and attenuation of virulence in a mouse model of urinary tract infection, unlike deletion of *srtA*, which had minor effects under similar conditions (Kemp *et al*.,2007).

SrtC co-localizes with *srtA* and the general secretion machinery at distinct foci on the cell membrane and mislocalization of *srtC* results in an overall decrease in piliated *E. faecalis* cells .This decrease in piliated cells suggest that proper localization of *srtC* is necessary for pilus biogenesis(Mitra *et al*.,2018).

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

1.2.4.4Autolysin(*atl*)gene:

An endogenous lytic enzymes that break down the peptidoglycan components of biological cells which enables the separation of daughter cells following cell division. They are involved in cell growth, cell wall metabolism, cell division and separation, as well as peptidoglycan turnover and have similar functions to lysozymes. Autolysin is formed from the precursor gene, *atl*. Amidases, gametolysin, and glucosaminidase are considered as types of autolysins(Clarke,2018).

Autolysins exist in all bacteria containing peptidoglycan and are potentially considered as lethal enzymes when uncontrolled. They target the glycosidic bonds as well as the cross-linked peptides of the peptidoglycan matrix(Atilano *et al.*,2014) .The peptidoglycan matrix functions for cell wall stability to protect from turgor changes and carries out function for immunological defense (Zhang *et al.*,2019; Pazos *et al*., 2019).

These enzymes break down the peptidoglycan matrix in small sections to allow for peptidoglycan biosynthesis (Clarke,2018). Autolysins breaks down old peptidoglycan which allows for the formation of newer peptidoglycan for cell growth and elongation. This is called cell wall turnover. Autolysins do this by hydrolyzing the β-(1,4) glycosidic bond of the peptidoglycan cell wall and the linkage between N-acetylmuramoyl residues and L-amino acid residues of certain cell-wall glycopeptides (Jump *et al*, 2018).

Autolysins are naturally produced by peptidoglycan containing bacteria, but excessive amounts will degrade the peptidoglycan matrix and cause the cell to burst due to osmotic pressure. Previous studies have found that the byproducts of autolysin during cell wall breakdown are highly immunogenic(Smith *et al*,2000).

Atl, an autolysin involved in peptidoglycan hydrolysis plays an important role in the separation of daughter cells following replication. In *E. faecalis*, an *atlA* deletion mutant presents a long chaining phenotype under light microscopy ,with strings of cells attached end to end due to incomplete septum cleavage. The impact of *atlA* on septal cleavage is further demonstrated by the addition of *atlA* protein to an *atlA* deletion mutant, resulting in short chaining cells(Mesnage *et al*,2008).

Autolysin activities in *E. faecalis* have been reported, including one which could lyse heat-killed Micrococcus lysodeikticus cells and another which could lyse heat-killed *E. faecalis* cells; the proteins with autolytic activities were

shown to have molecular masses and substrate specificities similar to those of *E. hirae* (Be´liveau *et al.*,1991).

This autolysin has a predicted size of 74 kDa. However, the physiological functions of the autolysin of *E. faecalis* are still unknown(Xu *et al*.,1997).

The enzymes are referred to as autolysins as they contribute to cell lysis in response to various stresses, including exposure to antibiotics(Leclerc and Asselin,1989).

Sequence comparisons with known autolysins revealed that the *E. faecalis* genome encodes 20 putative peptidoglycan hydrolases, including 6 glucosaminidases, 7 muramidases, 2 amidases, 2 endopeptidases, and 3 proteins related to lytic transglycosylases. Among these putative enzymes, *atlA* was previously shown to be an N-acetylglucosaminidase (Eckert *et al*.,2006).

E. faecalis produces several autolysins, which were identified and characterized . The major *E. faecalis* autolysin, *atn* (also known as *atlA*), is an N-acetylglucosaminidase important for daughter cell separation during cellular division (Eckert *et al*.,2006). Disruption of *atn* in *E. faecalis* resulted in increased chaining, a defect in primary attachment, and decreased biofilm production (Thomas *et al*.,2009). Thomas *et al*.,(2009) provided evidence that inactivation of this autolysin results in a decrease in DNA release similar to that of gelatinase-deficient mutants. Furthermore, they showed that *gelE* and *sprE* can differentially cleave *atn* in *vitro*, and this processing may underlie the mechanism of cell death and DNA release in *E. faecalis* during biofilm formation.

1.2.5 Phylogenetic analysis of *Enterococcus faecalis* isolates:

ERIC-PCR is one of the most widely adopted PCR typing methods and is chosen for analyses of genetic diversity (Chen *et al*., 2014; Xie *et al*., 2015). This method provide discriminatory value and is a rapid method for *E. faecalis* typing. ERIC-PCR is a relatively simple and cost-effective method, which has

been successfully used for genotyping of different bacterial pathogens and for tracking the different bacterial source (Martin-Platero *et al*., 2009).

The rep-PCR with (GTG)5 primer has also been presented as a reliable method for species identification of all enterococci strains which grouped clearly into well-separated clusters and representing single species (Švec *et al*., 2005).

This techniques based on primers complementary to repetitive sequences dispersed in bacterial genomes (Lupski and Weinstock, 1992) which have been widely applied in bacterial taxonomy for identification of different bacterial Taxa.

The BOX-PCR genotypes are assumed to reflect phylogenetic relatedness. BOX-PCR typing targets sequences located between interspersed repetitive DNA elements, resulting in amplification products of different sizes that generate a genomic fingerprint of individual bacterial isolates. Most of the Box sequences were encounter in close proximity to gene, suggesting their potential role as a regulatory element controlling coordinate virulence or competence related gene. Probably, Box elements are key elements in adaptive bacterial evolution.

Variation in genome sizes, as well as the location of BOX elements among different strains of a particular *Enterococcus* species, leads to generation of multiple strain-specific fingerprint patterns, which allows BOX-PCR typing to discriminate among different strains of the same species (Nayak *et al*.,2011) .

The repetitive-sequence-based PCR system (rep-PCR) is based on non-coding repetitive sequence elements within the genome of organisms to create primers and amplify regions between these sequences(Russello *et al*.,2011).

1.2.6Antibiotics Profile in *Enterococcus faecalis* :

An antibiotic is a type of antimicrobial substance active against bacteria and is the most important kind of antibacterial agent for fighting bacterial infections. Antibiotic medications are widely used in the treatment and prevention of such infections. They may either kill or inhibit the growth of bacteria (Combarros-Fuertes *et al.,* 2020).

Most antibiotics fall into their individual antibiotic classes. An antibiotic class is a grouping of different drugs that have similar chemical and pharmacologic properties. Their chemical structures may look comparable, and antibiotics within the same class may kill the same or related bacteria (Meade *et al*., 2020).

Several trends have been identified in the epidemiology of enterococcal infections: an increasing incidence of enterococcal infections particularly among the severely ill hospitalized patients, an increasing proportion of nosocomial enterococcal infections caused by *Enterococcus*, and an increasing level of resistance to piperacillin, aminoglycosides, and glycopeptides (Alotaibi & Bukhari, 2017).

Aminoglycosides are another class of clinically important antibiotics for treating various bacterial pathogens. The increasing resistance of clinical isolates against aminoglycosides, however, has compromised the effectiveness of this class of antibiotics. A major mechanism of aminoglycoside resistance is the production of aminoglycoside-modifying enzymes(Shi *et al*.,2013). Two enzymes with aminoglycoside-modifying activities aminoglycoside kinase and Aminoglycoside6-N-acetyltransferase is another clinically important enzyme prevalent in a wide variety of Gram-negative pathogens(Ramirez *et al*.,2013) .

The relative importance of *Enterococcus* as a pathogen has increased with the occurrence of high-level resistance to multiple antimicrobial drugs, such as imipenem ,aminoglycosides and vancomycin (Sparo *et al*., 2018).

The emergence of vancomycin resistance enterococci (VRE) has alarmed the global infectious diseases community due to few option left for disease management. Besides drug resistant enterococci colonizing the gastro- intestinal tract of hospitalized patients are the major source of infection as well as nosocomial spread (Haghi *et al*., 2019).

Enterococcus species have a broad range of resistance genes, and are able to exchange the resistance genes. Enterococci are the third common cause of nosocomial infections (Nis) and health-care-associated blood stream infections (BSIs) and have been estimated to be responsible for 25–50% of the mortality rate among hospitalized patients. Also, simultaneous multiple resistance mechanisms lead to the emergence of multi resistant or pan-resistant Enterococci that do not respond to the common first-line antibiotics, leading to amplified morbidity and mortality rates and eventually more financial burdens on hospitals and patients(Shiadeh *et al*.,2019).

Furthermore, the hospitalization of patients and progress in medical technology and treatment, as well as increase in antibiotic usage, have contributed to a increase in infections due to multidrug resistant (MDR) enterococci and vancomycin resistant enterococci (VRE) (Yim *et al*., 2017).

VRE are of very worried in that vancomycin is a powerful antibiotic used to treat gram positive bacterial infections, both intrinsic and acquired forms of resistance do occur in enterococci. VRE have become an important cause of serious invasive infections globally to such an extent that clinical microbiology laboratories are encouraged to speciate enterococcal isolates from hospitals and screen them for vancomycin resistance (Nellore *et al*., 2019).

A high mortality rate of enterococcal infections is due to increasing resistance of the organism to β-lactam antibiotics, aminoglycosides, and glycopeptides(eg:vancomycin and teicoplanin) and inadequate response to the treatment. Pandemic spread of vancomycin-resistant enterococci (VRE) and acquisition of resistance to newer antimicrobials warrant continued surveillance

and early detection of VRE along with Minimum Inhibitory Concentrations (MIC). Beta-lactam antibiotics, such as penicillin, piperacillin and imipenim are uniformly effective against most strains of *Enterococcu*s (Combarros-Fuertes *et al*.,2020).Ciprofloxacin(1-cyclopropyl-6-fluoro-1,4-dihydro-4-oxo-7 piperazinylquinolone-3- carboxylic acid) (CIP) is a large spectrum fluoroquinolone antibiotic that is widely used for the treatment of numerous bacterial infections in joints, bones, skin, tooth, gastrointestinal, and urinary and respiratory tracts (Herizchi *et al*.,2016).

Enterococci with high level aminoglycoside resistance and glycopeptide resistance have emerged posing a therapeutic challenge to physicians due to the ease of acquiring and transferring antimicrobial drug resistance (Guo *et al*., 2020).

Aminoglycoside antibiotics(eg:kanamycin) play a crucial role in providing high-fidelity translation of genetic material rendering the ribosome unavailable for translation and thereby resulting in cell death (Hobson *et al*., 2021).

However, the aminoglycosides are seldom drugs of first choice for monotherapy of infections, except for some cases of uncomplicated urinary tract infections. Because of their synergism with cell wall synthesis inhibitors, they are recommended as part of an empirical combination therapy for severe infections such as septicemia, nosocomial respiratory tract infections and enterococcal endocarditis (Vestergaard *et al*., 2019).

Antimicrobial-resistant *Enterococcus* spp. have the potential to cause zoonotic diseases, being possessed of intrinsic resistance to various antimicrobial agents including aminoglycosides and cephalosporin's, and able to acquire resistance genes from other bacteria by conjugation via plasmids or transposons and bacteriophages. This phenomenon has led to an increase in the prevalence rate of multidrug resistant (MDR) (Jahan *et al*.,2015).

The main problem associated with enterococcal infections is antimicrobial resistance. On one hand enterococci are intrinsically resistant to

some antimicrobials; on the other hand they are very able to acquire and transfer resistant genes from other bacteria via plasmids and/or transposons (Selleck *et al*.,2019).The selective pressure linked to widespread use of antimicrobials drives an accumulation process of resistance genes in these bacteria and the selection of multidrug-resistant strains. As consequence, antimicrobial therapy of *Enterococcus* infection in humans need to be constantly modulated and changed. One of the main raised problems was the resistance to vancomycin, considered as last line therapy (Miller *et al*.,2020).

Enterococci have become resistant to antimicrobials through a number of mechanisms: efflux pumps − Tetracycline (tet(K)), tet(L), efflux pump resistance to macrolide (mef(A/E)), specifies resistance to macrolide (msr(A/B)); modification of target molecule: modification of the ribosomal target −Erythromycine erm(A), erm(B), erm(T), alteration in penicillin-binding protein (PBP) antibiotic inactivation:- aminoglycoside modifying enzymes According to the established criteria, the highest resistance in *E. faecalis* isolates was noted for erythromycin (51.3%) alteration in penicillin-binding protein (PBP) antibiotic inactivation: aminoglycoside modifying enzymes(Arias& Murray,2015).

Chapter Two

Materials and Methods

2.1 Materials:

2.1.1 Laboratory instruments and equipment:

Table (2-1): Laboratory instruments and equipment in used study

No.	Instruments	Company	Country
1.	Autoclave	Stermite	Japan
2.	Bensen burner	Satorins	Germany
3.	Centrifuge	Hettich	Germany
4.	Cooling box	Ningbo	China
5.	Digital camera	Samsung	Japan
6.	Distillator	GFL	Germany
7.	DNA extraction tubes.	Eppendorf	Germany
8.	Gel electrophoresis	Clever	USA
9.	Gel documentation system (imager)	Biometra	Germany
10.	Hood	Labogene	Danemark
11.	Incubator	Memmert	Germany
12.	Light microscope	Olympus	Japan
13.	Micropipettes 5-50 µl, 100-1000 µl, 0.5 – 10 µl, 2-20 µl.	Eppendorf	Germany
14.	Millipore filter (0.45mm)	Satorins membrane Filter Gm, BH, W.	Germany
15.	Nano drop	Memmert	Germany
16.	Oven	Memmert	Germany
17.	PCR system	Clever	USA
18.	PCR thermocyling	Invitrogen	USA
19.	PCR tubes	Eppendorf	Germany
20.	Plastic Test tubes 10ml.	AFCO	Jordan
21.	Platinum wire loop	Himedia	India
22.	Refrigerator	Concord	Italy.
23.	Screw capped bottles 30 ml	Hirschmann	Germany
24.	Sensitive electron balance	A & D	Japan
25.	Sterile swab for streaking	Lab. Service	S.P.A.
26.	UV-transilluminator	Clever	USA
27.	Vortex	Germmy	Twain
28.	Water bath	Memmert	Germany
29.	Wooden sticks	Supreme	China

2.1.2 Chemical and Biological Materials:

2.1.2.1 Chemical Materials:

Table (2-2) Chemical Materials

N0.	Chemicals	Company/country
1.	Absolute ethanol	Fluka/ Germany
2.	Carbohydrates (glucose, maltose, lactose)	Fluka chemika/Switzerland
3.	Catalase reagent	Schuchariot/ Germany
4.	Ethidium bromide, Loading dye (bromophenole blue), Agarose, Master mix	(Promega, USA)
5.	Glycerol	Fluka /England
6.	Gram Stain kit	Crescent/KSA
7.	NaCl , NaOH	Merk Darmstadt/ Germany
8.	Nuclease free water (1.25) ml	Promega/(USA)
9.	Oxidase reagent	Himedia / India
10.	Tris EDTA (TE)	Bio basic Canada
11.	Tris-Borate-EDTA (TBE10x) buffer	Bio Basic/ Canada

2.1.2.2 Biological Materials

Table (2-3) Culture Media

No.	Culture Media	Company/country
1	Agar agar	Oxoid /UK
2	Bile esculin Agar	Himedia /India
3	Brain Heart Infusion Agar	BBL /France
4	Brain Heart Infusion Broth	Conda/Spain
5	Chromogenic Agar	Conda/Spain
6	MacConky Agar, Blood Agar , Pepton water	Himedia /India
7	Mueller-Hinton Agar	Mast /UK
8	Nutrient Agar, Nutrient Broth	Himedia /India

2.1.2.3 Antibiotics Disks:

Table (2-4) Antibiotics Disks (Origin / Turkey)

No.	Antibiotics	Assembly	Potency (µg per disk)
1.	Piperacillin	PRL	100
2.	Clindamycin	DA	15
3.	Vancomycin	VAN	30
4.	Levofloxacin	LEV	5
5.	Ciprofloxacin	CIP	10
6.	Erythromycin	E	15
7.	Imipenem	IMI	10
8.	Kanamycine	K	30
9.	Teicopanin	TEC	30

2.1.3 Commercial kits:

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

No.	Type of kits	Company/country
1.	DNA extraction kit	Geneaid / UK
2.	DNA ladder 100bp-1500pb	Promega-USA
3.	DNA ladder 100bp-3000pb	Promega-USA
4.	Green master mix 2X Kit	Promega-USA
5.	Primers of *srt*,*fsr*,*ddl*,*atl*,*cps1*,*cps2*/*cps5*,Box1A,ERIC, and(GTG)5	ALPHA DNA/Canada

Table (2-6) DNA extraction kit (Geneaid/ UK).

DNA extraction kit
Materials: GT Buffer 30 ml GB Buffer 40ml W1 Buffer 45 mlWash Buffer 25 ml+100 ml Ethanol Elution Buffer 30ml
DNA ladder
Materials: 1. Ladder consist of 11 double-stranded DNA with size 100-1500 bp. 2. Ladder consist of 11 double-stranded DNA with size 100-10000 bp 3. Loading Dye which has a composition of (15% Ficoll, 0.03% bromophenol blue, 0.03% xylene cyanol, 0.4% orange G, 10mM Tris-HCl (pH 7.5) and 50mM EDTA)

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

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

2.2 Methods:

2.2.1 Preparation of Reagents and Solutions:

2.2.1.1 Reagents:

2.2.1.1.1 Oxidase Reagent:

This reagent was prepared according to the method recommended by the manufacturing company .

2.2.1.1.2 Catalase Reagent:

Hydrogen peroxide (3%) was prepared from stock solution in a dark bottle and it has been used for detection of the ability of the isolates to produce catalase enzyme (Forbes *et al*., 2007).

2.2.1.2 Solutions:

2.2.1.2.1 Normal Saline Solution:

It was prepared by dissolving 8.5 gm of NaCl in a small volume of distilled water, then completed to 1000 ml, pH fixed at 7.2 and sterilized in autoclave at 121^{o}C for 15 minutes, then kept at 4 ºC (MacFaddin, 2000).

2.2.1.2.2 Agarose Gel:

Agarose gel was prepared according to the method of Sambrook and Rusell (2001) by adding 1gm 10 100ml of 1x TBE Buffer. The solution was heated to boiling (using water bath) until all the gel particles dissolved. The solution was allowed to cool down within 50-60 ºC, and mixed with 0.5 mg/ml ethidium bromid.

2.2.1.2.3 Sugar Solutions:

The solution was prepared by dissolving 1gm of (glucose, lactose and maltose) in 100 ml distilled water, and sterilized by filtration (Gadeberg *et al*., 1983).

2.2.2 Preparation of Culture Media:

A group of culture media were prepared according to the instructions of the company and serialized by autoclaving at 121^{O}C for 15 minutes.

2.2.2.1 Chromogenic agar medium:

Chromogenic agar medium was prepared according to the manufacturing company (72 gm /L) after autoclaved and cool, add (0.24 naldic acid dissolve in 5 ml D.W) with 5 drop of NaOH (MacFaddin, 2000).

2.2.2.2 Bile-Esculin Agar Medium:

Bile-esculinagar medium was prepared according to the manufacturing company (56.25 gm/1L) . It is a selective medium used for isolating and identifying of *Enterococcus faecalis* (MacFaddin, 2000).

2.2.2.3 MacConkey Agar Medium:

MacConkey agar medium was prepared according to the method recommended by the manufacturing company (52 gm /L) and it was used for the primary isolation of most enteric bacteria and differentiation of lactose fermentative from the non-lactose fermentative, and used for isolation and differentiation of enteric microorganisms and permitting growth of Enterococci (Winn *et al*., 2006).

2.2.2.4 Nutrient Agar Medium:

Nutrient agar medium was prepared according to the manufacturing company (28 gm/1L). It was used for general experiments, cultivation and activation of bacterial isolates when it is necessary (MacFaddin, 2000).

2.2.2.5 Blood agar medium:

Blood agar medium has been prepared according to MacFaddin, (2000) by dissolving 40gm blood agar base in 1000 ml D.W. and autoclaved at 121ºC for 15 min, then cold to 50ºC and 5% of human blood was added. This medium was used to cultivate bacterial strains and to determine their ability to blood cell hemolysis (MacFaddin, 2000:82).

2.2.2.6 Muller-Hinton Agar Medium:

Muller-Hinton agar was prepared according to the manufacturing company (38mg/IL). It was used in anti-bacterial susceptibility testing (MacFaddin, 2000).

2.2.2.7 Motility Medium (semi-solid medium):

This medium was prepared by adding 40gm of agar to 100ml of brain-heart infusion broth and completed with 1000ml distilled water. It was then sterilized by autoclave at 121^{o}C for 15minutes. It was distributed in tubes. This medium was used to detect bacterial motility (MacFaddin, 2000).

2.2.2.8 Brain Heart Infusion Broth:

This medium using to activate, grow and as stock culture for isolates *Enterococcus* spp.; it is prepared by dissolving (37 gm.) of medium in (1L.) of distilled water, and adding 20 % glycerin, then pouring to sterile test tubes and sterilizing by autoclave (MacFaddin, 2000).

2.2.2.9 Maintenance medium:

Maintenance media for bacterial isolates; the bacterial isolates have been preserved on brain heart infusion agar slant at 4 ºC. The isolates have been maintained monthly during the study by culturing on new culture media. For long preservation, brain heart infusion broth supplemented with 20% glycerol has been used and the isolates have been preserved frozen (-20 ºC) for long term (several months) (Collee *et al*., 1996).

2.2.2.10 Sugar fermentation medium:

This medium consists of medium base: 0.0082 gm of α-phenol red as indicator was added to 100 ml of Brain heart infusion broth, the pH was adjusted to 7.4, and then this media had been autoclaved.

Sugar solution: 1 gm of each of the following sugars (glucose, lactose and maltose), were added to the broth separately and sterilized by filtration by Millipore filter, later poured into sterile plain tubes (Forbes., 2007).

2.2.2.11 Brain heart infusion agar

Brain-heart infusion agar was prepared according to the manufacturing company (52 gm/L) (MacFaddin, 2000).

2.3 Subjects of the Study:

A total of (120) clinical specimens were collected from patients suffering from urinary tract infections, vaginitis, diarrhea, wound infection and bacteremia , Who admitted two hospitals of Babylon Governorate :Al-Hashimiyah General Hospital and Al-Hillah Surgical Teaching Hospital, during a period of two months (from August 2021 to November 2021).

2.3.1 Exclusion criteria:

More than 19 cases excluded from the study when routine clinical assessments identified such as diabetes, chronic urinary tract infection, immunocompromised and patient use antibiotic were excluded after taken the history of patients.

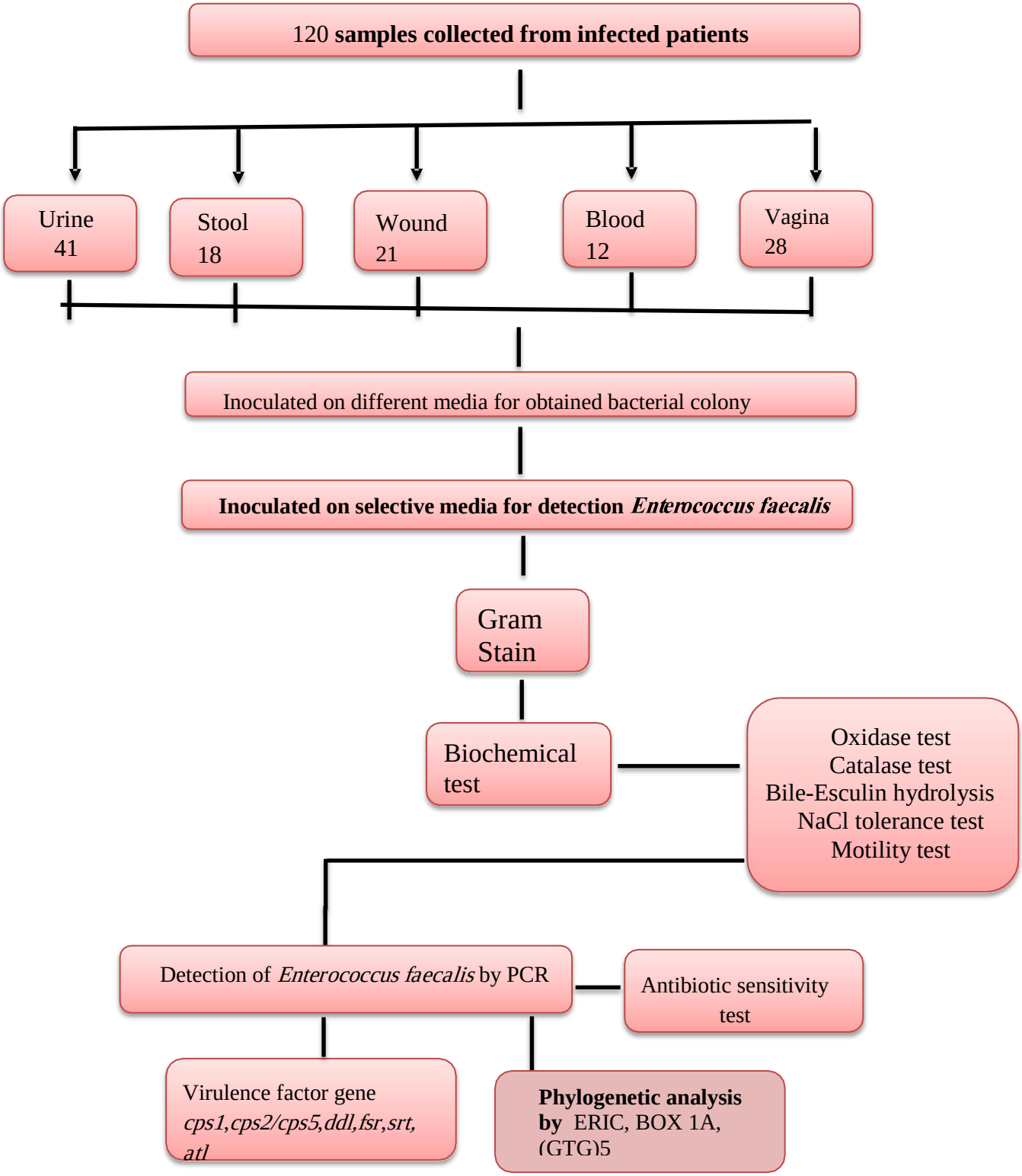


Figure (2-1) Scheme of study design

2.3.2 Ethical approval:

The study was conducted in accordance with the ethical principles that have their origin in the Declaration of Helsinki. Verbal consent was taken from each patient parents before sampling. Investigative standards were rigidly preserved, primarily concerning confidentiality. Moreover, this study was undisclosed, participation of patients was optional, and verbal consent was received before data uptake process was started. The study protocol and the subject information and consent form were reviewed and approved by a local ethics committee (at College of Medicine University of Babylon).

2.3.3 Isolation and identification of *Enterococcus faecalis*:

2.3.3.1 Clinical specimens:

The proper specimens collected for bacteriological analysis are described below. Those specimens were collected in proper ways to avoid any possible contamination (Collee *et al*., 1996).

2.3.3.1.1 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 culture media and incubated aerobically at 37°C for 24h (Vandepitte *et al*, 1991).

2.3.3.1.2 Vagina 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 culture media and incubated aerobically at 37°C for 24h.

2.3.3.1.3 Stool samples:

The specimens were collected from patients suffering from diarrhea, by disposable sterile clean, leak-proof container proper way to avoid any possible contamination. These are taken and closed it then transported to laboratory the college of medicine, fecal specimen were diluted serially 10-fold steps in pepton water and cultivated on selective media (Chromogenic agar) and inoculated aerobically at 37°C for 24h , the lowest level of detection was 400 cfu/g of feces .

2.3.3.1.4 Blood samples:

Blood sample was collected from patients, (5) ml of fresh venous blood samples were collected from suspected patients by sterile syringes which delivered into special screw cupped of culture bottle containing (100) ml of brain heart infusion broth and incubated at (37°C) for at least (3) days placed in bact/alert 3D apparatus for a week. If positive sample, each specimen was inoculated using direct method of inoculation on culture of selective media.

2.3.3.1.5 Wound swabs:

The samples were generally collected by twice rolating a sterile cotton 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 culture media and incubated aerobically at 37°C for 24h.

All samples were transferred by means of a cooled box to the Faculty of Medicine Laboratory / Babylon University for the purpose of identifying the bacteria and performing laboratory analyzes.

2.4 Laboratory Diagnosis of *Enterococcus faecalis*:

2.4.1 Colonial Morphology and Microscopic Examination:

A single colony was taken from each primary positive culture and its identification depended on the morphological properties (Colony size, shape, color, translucency, edge, elevation and texture). Bacterial smear stained with Gram stain was used to check the morphological properties of bacterial cells, including gram reaction, shape and arrangement of bacteria.

2.4.2 Biochemical Tests:

2.4.2.1 Catalase Test:

Catalase is an enzyme that catalyses the release of oxygen from hydrogen peroxide. Nutrient agar medium was streaked with the selected bacterial colonies and incubated at 37ºC for 24 hrs, then the growth was transferred by the wooden stick and it was put on the surface of a clean slide, a drop of (3% H_2O_2) prepared at (2.2.1.1.2) was added. Formation of gas bubbles indicates a positive result (Collee *et al.*, 1996; Forbes *et al*., 2007).

2.4.2.2 Oxidase Test:

The test depends on the presence of certain bacterial oxidases that would catalyze the transport of electrons between electron donors in the bacteria and a redoxdye (tetramethyl-ρ-phenylene-diamine dihydrochloride), the dye was reduced to a deep purple color.

A strip of filter paper was soaked with a little freshly made reagent, and the colony to be tested was picked up with a sterile wooden stick and smeared over the filter paper. A positive result was indicated by an intense deep purple color which appeared within 5-10sec. (Forbes *et al*., 2007).

2.4.2.3 Bile-Esculine Hydrolysis Test:

It is used for rapid detection of esculin hydrolysis in presence of 40% bile for differentiation of group D streptococci from non-group D streptococci, as the positive result can detected by the darkening of the medium (MacFaddin, 2000).

2.4.2.4 NaCl Tolerance Test:

To check the ability of enterococci to tolerate 6.5% NaCl, BHI broth supplemented with NaCl was prepared by dissolving 6.5 g NaCl in 100 ml. Inoculated tube containing BHI broth without NaCl was used as a positive control to compare the turbidity (Collee *et al*., 1996; Koneman *et al*., 1997).

2.4.2.5 Motility Test:

This test was done by incubating the tube that contained semisolid media with tested bacteria by stabbing method and was incubated at 25-30^{o}C for 24-48 hrs. The dissemination of growth out of the stability line was an indication for positive result (MacFaddin, 2000).

2.4.2.6 Sugar Fermentation:

This test was done to detect the ability of bacteria to ferment different types of carbohydrate including (glucose, lactose and maltose). This test was performed as follows: Sugar fermentation medium was inoculated by the suspected bacterial colonies and the tubes were incubated at 35-37ºC for 18-24hrs, incubation as long as 30 hrs may be needed to confirm a negative result. Looking for the results next day:

1. Change in the color of broth from red to yellow indicates positive result (acid formation i.e. sugar fermenter).
2. No change in color indicates negative result (no sugar fermentation) (Collee *et al.*, 1996; MacFaddin, 2000).

2.4.2.7 Growth at 10ºC and 45ºC:

Two tubes of BHI broth were inoculated by bacterial isolates, one tube is incubated at 10 ºC and the other at 45ºC for 18-24 hrs then the turbidity was checked and compared with negative control (uninoculated tube) and positive control (inoculated tube which incubated at 37ºC) (MacFaddin, 2000).

2.4.2.8 Growth at alkaline pH (9.6):

Brain-heart infusion broth was prepared and then its pH was adjusted to 9.6 by the addition of 0.1 M NaOH (Collee., 1996).

2.5 Hemolysin production:

Hemolysin production was carried out by inoculating bacterial isolate on blood agar medium at (37˚C) for (24-48) hours, 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 Antimicrobial Susceptibility Test: Disk Diffusion Test (DDT):

Antibiotic diffusion test (the Kirby-Bauer susceptibility test).

1. It was performed by using a pure culture of previously identified bacterial organism.
2. The inoculum to be used in this test was prepared by adding growth from 5 isolated colonies grown on brain heart infusion plates to 5 ml of broth; this culture was then incubated for 2 hours to produce a bacterial suspension of moderate turbidity.
3. A sterile swab was used to obtain an inoculum from the standardized culture, this inoculum was then swapped on Mueller – Hinton plate and left to dry.

4. The antibiotic discs were placed on the surface of the medium at evenly spared intervals with flamed forceps or a disc applicator, incubation was usually for an overnight at 37^{O}C.
5. Antibiotics inhibition zones were measured using a caliper, zone size was compared to standard zones to determine the susceptibility or resistance of organism to each antibiotics (CLSI, 2019).

2.7 Genotyping Assays:

2.7.1 DNA Extraction:

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

a. Cultured bacterial cells were transferred to 1.5 ml microcentrifuge tube, centrifuged for 1 minutes at 14-16,000xg and the supernatant was discarded.

b. A volume of 200 ml of Gram Buffer was added to 1.5ml microcentrifuge tube then 200ml of lysozyme buffer was added to the Gram Buffer then vortex to completely dissolve the Lysozyme.

c. A volume of 200 ml of Gram Buffer in the 1.5 microcentrifuge tube, incubated at 37^{O}C for 30 minutes. During incubation the tube was inverted every 10 minutes.

d. A volume of 20 ml of proteinase K was added then mixed by vortex, incubated at 60^{O}C for at least 10 minutes. During incubation the tube was inverted every 3 minutes.

e. A volume of 200 ml of GB Buffer was added to the sample and mix by vortex for 10 minutes.

f. The sample lysate was incubated at 70^{O}C for at least 10 minutes. During incubation, the tube was inverted every 3 minutes. At this time, the required Elution Buffer (200 ml per sample) was pre-heated to 70^{O}C (for step 5 DNA Elution).

g. Following 70^{O}C incubation, 5ml of RNase A (10mg ml) was added to the clear lysate and mixed by shaking vigorously.

h. The lysate was incubated at room temperature for 5 minutes.

i. A volume of 200 ml of absolute ethanol was added to the clear lysate and immediately mixed by shaking vigorously, the precipitate was broken up by pipetting.

j. A GD Column was placed in a 2ml collection tube.

k. All of the mixture was transferred (including any precipitate) to the GD column, centrifuged at 14000-16000xg for 2 minutes.

l. The 2ml collection tube was discarded containing the flow-through and the GD column was placed in a new 2 ml collection tube.

m. A volume of 400 ml of W1 buffer was added to the GD Column, Centrifuged at 14000-16000 g for 30 second.

n. The flow-through was discarded and placed the GD column back in the 2ml collection tube.

o. A volume of 600 ml of wash buffer (ethanol added) was added to the GD column, centrifuged at 14000-16000xg for 30 seconds.

p. The Flow-through was discarded and placed the GD column back in the 2ml collection tube, Centrifuged again for 3 minutes at 14000-16000xg to dry the column matrix.

q. The dried GD column was transferred to a clean 1.5 ml centrifuge tube .

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

2.7.2 Estimation of DNA Concentration

The extracted genomic DNA is checked by using Nanodrop spectrophotometer which measures DNA concentration (ng/µl) and checks the DNA purity by reading the absorbance at (260 /280 nm).

2.7.3 The mixture of PCR reaction:

Amplification of DNA was carried out in final volume of 25 µl containing the following as mentioned in Table (2-8):

Table (2-8) Contents of the Reaction Mixture

No.	Contents of reaction mixture	Volume
1.	Green master mix	12.5 µl
2.	Upstream primer	2.5µl
3.	Downstream primer	2.5 µl
4	DNA template	5 µl
5.	Nuclease free water	2.5 µl
Total volume		25 µl

2.7.4 Primer Sequences:

Molecular assay in this study includes 9 gene, each one has specific nucleotide and product size. The primer sequences and PCR conditions that used are listed in Table (2-9), (2-10),(2-11).

2.7.5 Agarose gel documentation:

The gel documentation system was used to detected electrophoresis results. The positive results were distinguished when DNA band base

pairs of sample equal to the target product size. The biometra gel documentation system eas used to photograph the gel.

2.7.6 Detection of some of *Enterococcus faecalis* virulence genes:

DNA (extract from bacterial cells) was used as a template in specific PCRs for the detection of some of *Enterococcus 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 (2-10).

Table (2-9): Primers sequences and PCR condition of *ddl* gene for identification of *E. faecalis*

Gen	Primer sequence (5′-3′)	Size of produc	PCR condition	Reference
E. faecalis (*ddl*	F: TCAAGTACAGTTAGTCTTTATTAG R:ACGATTCAAAGCTAACTGAATCAGT	941	94 ºC-60 s 1x 94 ºC-40s 54 ºC- 60s 30 x 72 ºC 7min 1x	(Saffari *et al.*, 2017)

Table(2-10):-Virulence factor primer sequences with their amplicon size base pair and their condition

Genes	Primer sequence (5′-3′)	Size of prod	PCR condition	Reference
Fsr	F:CAAGGCACTATTTCTTACTTAGG R: AGCGCATAAATCAACCAAG	1016	94 ºC-5 min 1x 94 ºC-60s 55 ºC-60s 35x 72 ºC-60s 72 ºC10 min 1x	(Song*et al.*,2019)
Srt	F:GTATCCTTTTGTTAGCGATGC R:TGTCCTCGAACTAATAACCGA	612	94 ºC-5 min, 1x 94 ºC-1min 56 ºC-1min 35 72 ºC-1min 72 ºC-10min 1x	(Hashem *etal.*,2017)
Cps1	F:CCAGGACATGGTGGTATTTTAGATC R:CGCCAATAAACAATCTTTACCAGAGC	950	95 ºC-5 min, 1x 94 ºC-1min 52ºC-1min 35 x 72 ºC -1min 72 ºC -10min 1x	Pinheiro *et et al.*,(2012)

Cps2/ cps5	F:GAACCTACAACAATTAAAAAAGC R:GCATAGTATGTTAAGATTGATCCA	1098/199	94 ºC-5 min, 1x 94 ºC-1min 52ºC-1min 35x 72 ºC-1min 72 ºC-10min 1x	Pinheiro *et t al*.,(2012)
Atl	F:CTGCTCCAGCTGTTACACCA R:ACCCCAACCAGATTCAACAA	206	94 ºC-5 min, 1x 95 ºC-1min 60ºC-1min 35x 72ºC-1min 72 ºC-10min 1x	(Design this study)

Table (2-11):-Oligonucleiotides primers *E. faecalis* isolates using genotyping analysis

ERIC- PCR	F:5'CAGCCATGAACAACTGGTGGCG-3' R:5'TGCTTTGCGCAGGGAAGATTCC-3'	95 ºC-7 min, 1x 90 ºC-30s 52ºC-1min 35 x 68 ºC-8min 65 ºC-16min 1x	(Versalovi *et al*.,1991)
BOXAIR- PCR	5′-CTACGGCAAGGCGACGCTGACG-3	95 ºC-3 min, 1x 95 ºC-45s 55ºC-1min 40 x 72 ºC-2min 72 ºC-5min 1x	Colombo *et al*.,2009
(GTG)5	5′GTGGTGGTGGTGGTG-3′	95 ºC-7 min, 1x 90 ºC-30s 40ºC-1min 30 x 65 ºC-8min 65 ºC-16min 1x	(Versalovic *et al*.,1994)

2.8 Detection of Amplified products by Agrose Gel Electrophoresis

The PCR amplification of products were analysed by agarose gel electrophoresis using 1.5% agarose gel prepared by dissolving 1.5 g of agarose (Paiao *et al*., 2012) mixed with 100 ml of 10 x Tris - Borate EDTA (TBE) buffer (10ml TBE+90ml sterile distilled water) heated to boil on hot plate. The agarose gel was cooled down to 45°C where 5µl of Ethidium bromide stain. The comb was fixed at one end of the tray for

making wells used for loading DNA sample. The agarose was powered gently into the tray, and allowed to solidify at room temperature for 30 min. The comb was then removed gently from the tray. The tray was fixed in an electrophoresis chamber which was filled with TBE buffer covering the surface of the gel, 5µl of DNA sample was transferred into the signed wells in agarose gel, and in one well we put the 5µl DNA ladder mixed with 1µl of loading buffer. The electric current was allowed at 75 volt for 60 min. UV transilluminater was used for the observation of DNA bands, and gel was photographed using a digital camera.

2.9 Gel analysis

Analysis of fingerprinting gel images was done by BIONUMERICS v8.0 and to build phylogenetic tree using UPGMA (unweighted pair group method with arithmetic mean) method

2.9.Simpsons (Discriminatory) Index Determination:

In order to calculate the average probability in which the molecular typing methods will assign a different type from two unrelated strains randomly sampled from the Salmonella isolates, a discriminatory index (D) was calculated at different levels of similarity index according to the formula :

$$D=1-1/N\,(N-1)\sum xj(xj-1)$$

where D= index of discriminatory power, N= number of unrelated isolates tested, $\sum$ = number of different types, and xj = number of strains belonging to jth type. D value in a range of 0 (identical type) to 1.0 indicates that the typing method of interest is capable of distinguishing each member of a population from all other members of that population(Hunter and Gaston,1988).

Chapter Three

Results and Discussion

3. Results and discussion

3.1 Isolation of *E. faecalis*:

A total of 120 clinical specimens were collected during this study which obtained from patients suffering from different infection such as UTI, vaginitis, wound infection, gastroenteritis and bacterimia who admitted to two main hospitals of Al-Hilla City: Al-Hilla Surgical Teaching Hospital and Al-Hashimiyah General Hospital during a period extending from (August 2021 to November 2021). All specimens were subjected to aerobic culturing on different media and it was found that out of the total (120) specimens, 87 (72.5%) specimens showed positive bacterial culture. No growth was seen in other 33(27.5%) specimens which indicated the presence of microorganisms that may be cultured with difficulty such as virus, fungi and other agent or may be due to difference in the size and nature of the samples. Among (87) positive culture, further culturing on chromogenic agar medium was (selective media), of them 20(22.98%) isolates were identified as *E. faecalis* as shown in the Table (3-1).

Identification of *E. faecalis* depends mainly on the cultural, biochemical characteristics and also microscopic patterns. The results were shown in table(3-3).

These isolates then subjected to molecular detection method using specific primer based on D-alanine D-alanine ligase gene as a genetic marker for confirmed identification of *E. faecalis* by PCR, the results revealed that 20(100%) were positive for *ddl* as shown in Table (3-1).

Table (3-1): isolation rate of *E. faecalis* among 120 clinical specimen

No. of samples	Negative bacteria	Positive culture of other bacteria	Positive culture of *E. faecalis*
120	33 (27.5%	87 (72.5%)	20 (22.98%

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

The result of this study was related that 20 isolates 0f related to *E. faecalis*, collected from the following site, 6 isolates (30.0%) obtained from urine samples, 4 isolates (20.0%) from stool, and wound for each, 6 isolates (30.0 %) from vagina. While no bacteria 0(0.0%) were isolated from blood samples as shown in Table (3-2).

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

Site of infection	**No. of specimens**	***E. faecalis***	**%**
Urine	41	6	30 .0%
Wound	21	4	20.0%
Blood	12	0	0.0%
Vagina	28	6	30.0%
Stool	18	4	20.0%
Total	**120**	**20**	**100%**

This table indicated that *E. faecalis* were frequently isolated from urine and vagina samples at percentage (30.0%) were frequently for each followed by stool specimens and wound at percentage (20.0%) for each.

Seenaa and Lamees.,(2020) in Babil/Iraq observed that (31.33%) isolates were recorded related to *E. faecalis*, collected from the following site, 11 isolates (23.40%) obtained from urine specimens, 10 isolates (21.28%) from stool, 10 isolates (21.28%) from wound, 6 isolates (12.76 %) from vagina, 10 isolates (21.28%) from pus. While no bacteria 0(0.0%) were isolated from blood samples

AL-Khafaji,(2021) observed that the isolates were recorded related to *E. faecalis*, it was collected from the following site, 14(51.85%) positive culture for *E. faecalis* were isolated from urine, 10(37.03%) positive culture for this bacteria isolated from stool.

AL-saadi, (2013) found that (40.62%) *E. faecalis* isolates that isolated included 18 isolates (46%) obtained from stool samples, 14 isolates (36%) from urine samples, and 7 isolates (18%) from vaginal swabs.

Kandela, (2012) in Baghdad/ Iraq was found that the isolates belonged to *Enterococcus faecalis* of which, 23 isolates (46%) from stool, 27(54%) isolates from clinical cases distributed between 16 (32%) from urine, 6 (12%) from wounds and 5(10%) from vaginal.

Golob *et al*., (2019) who found that, *E. faecalis* were isolated from clinical specimens have the ability to grow on selective *Enterococcus* agar, the highest numbers of isolates were distributed among urine specimens at rate (29.6%). Kadhem and Flayyih, (2014) in Baghdad/Iraq who found that, twenty isolates of the genus *E. faecalis* were isolated from clinical specimens have the ability to grow on selective *Enterococcus* agar, they found that the

highest numbers of isolates were distributed among urine specimens and the lowest one was observed among wound infection specimens.

On the other hand, Santos *et al*., (2017) found that, prevalence of *E. faecalis* infection was (55.6%), the commonest sites of infections were urinary tract followed by stool in a rate (40%). Yilema *et al*., (2017) found *E. faecalis* infections among patients with UTIs, wound infections were higher than the other infections.

Khazim *et al*., (2018) found that, *E. faecalis* isolated from stool (18.6%), (12.3%) urine, and (6.1%) vagina.

E. faecalis strains are frequently isolated from urine and are major causative agents of chronic UTIs(Folliero *et al*., 2020).

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

Kafil *et al*.,(2015) found from clinical isolates of urinary tract infections of Enterococci, and by biochemical differentiation and PCR for specific genes, 56.7% as *E. faecalis*.

Urinary tract infections (UTIs) are common in women and are one of the most frequent human bacterial infections . UTIs are responsible for significant worldwide morbidity and loss of workplace productivity. Although most UTIs (80%–90%) are caused by extra-intestinal *Escherichia coli* strains, *E. faecalis* isolates have more frequently been isolated and have been reported to be causative agents in up to 20% of all cases (Abat *et al*.,2016).

E. faecalis is one of the most frequently isolated bacterial species across all types of wounds, including diabetic foot ulcers, burns, and surgical sites. In surgical site infections, *E. faecalis* is the third most commonly isolated organism (Chong *et al*.,2017).

Rajkumari *et al*.,(2014) found that (41%) from patients with respect to the number of wound and tissue infections by *E. faecalis*.

Javed *et al*.,(2020)showed prevalence of *E. faecalis* (7% vs. 14%) in non-pregnant and pregnant females' respectively when take specimens from vaginal swab.

E. faecalis infections is one of bacteria which is isolated from vaginal swabs., it is associated with preterm birth, very low birth weight delivery and puerperal sepsis which causes substantial morbidity and mortality. It had been reported that *E . faecalis* is the most common etiological agent of aerobic. Vaginitis at a rate of 32.26% followed by *E. coli* 8-25% (Sangeetha *et al* .,2015)

Although *E. faecalis* can live peacefully in the GIT of the host , if it grows unchecked in the gut or gains access to extra-intestinal sites, especially in susceptible hosts, it can transform into an opportunistic pathogen. *E. faecalis* overgrowth in the GIT is often associated with antibiotic treatment and host inflammation, which can lead to subsequent translocation to other sites . In individuals with inflammatory bowel diseases (IBD), which include ulcerative colitis(Kao *et al*.,2019).

Changes in vaginal microflora that show a critical role in promising vaginal colonization (Aiyegoro *et al*., 2007), and the hypothesis is that the reason intestinal bacteria are associated with urinary tract and vaginal infections is due to the close proximity of the anal opening to the vagina and urethra, so contamination from the anus can lead to bacteria being found in the vagina area, though this is a much less common occurrence (Inabo and Obanibi, 2006).

Recent year have witnessed increased interest in enterococci not only because of their ability to cause serious infections but also because of their increasing resistance to many antimicrobial agents (Reinseth *et al*., 2021). Urinary tract infections are the most common cause of infectious disease produced by enterococci both inside and outside hospital settings.

3.3 Identification of *Enterococcus faecalis*:

Identification of *E. faecalis* depends mainly on the cultural, and biochemical characteristics and also microscopic patterns. The results were shown in Table (3-3).

The organisms are grams-positive small cocci, point colony, convex with an entire margin non-spore forming ,non-motile , oxidase negative, and catalase negative. The sodium chloride concentrations (6.5% NaCl)and the organisms are able to grow in a wide range of temperatures (10 and 45ºC with an optimum of 30°C to 37°C) . On blood agar medium colonies appears alpha, beta and gamma hemolysis, this considered as a good enrichment medium, to supply the bacteria with the needed nutritional factors. On Chromogenic agar medium, the colonies trend to Small and blue-green color.

specimens were culture on different media such as (MacConkey agar, blood agar, and bile esculin agar), on the blood agar colony appear as white to gray color, while in MacConkey agar colony appear as small size, smooth and circular shape with a pink color due to its ability to ferment lactose. While on selective media (bile esculin agar) gave round shape colony with smooth edges, white or creamy color and convert media color into black (Al-Halaby *et al*., 2017).

The *E. faecalis* colonies on chromogenic agar were small (0.5 mm in diameter), convex, circular, entire margin, turquoise in shade and having a slightly deeper peripheral thin rim in some colonies. On prolonged incubation, the grew larger; at the end of 48 hr and 72 hr the colony diameters were 1.5-2 mm which reached around 2.5 by 96 hours (Dunny *et al* 2014).

Table (3-3): Diagnostic Features of *E. faecalis*

Tests	Results
Colonies morphology	Small cocci, point colony, convex with an entire margin
Gram Stain	gram positive
Catalase	Negative
Oxidase	Negative
Growth at 10 and 45ºC	Positive
Growth at pH 9.6	Positive
Growth at 6.5% NaCl	Positive
Esculin hydrolysis	Positive
Sugar fermentation	Positive
Motility	Non motile
Chromogenic agar medium	Positive Small (blue-green
Type of hemolysis on blood agar medium	Beta, alpha, gamma hemolysis

3.4 Molecular Confirmation of *E faecalis* by D-alanine D-alanine ligase(ddl) gene by PCR technique:

To confirm the diagnosis of *E. faecalis* DNA was extracted from all suspected isolates that previously identified *E. faecalis* by selective media (Chromogenic agar medium) and biochemical tests, so the conventional PCR was carried out using these DNA samples for the amplification of specific *ddI* primer. D-alanine D-alanine ligase gene (*ddI)* is present in *E. faecalis* and this gene is specific for *E. faecalis*, So it can facilitated downstream analyses such as molecular detection.

The results recorded that all isolates 20 were produced the specific 941bp DNA fragment when compared with allelic ladder, as shown in Figure (3-1),table (3-4).

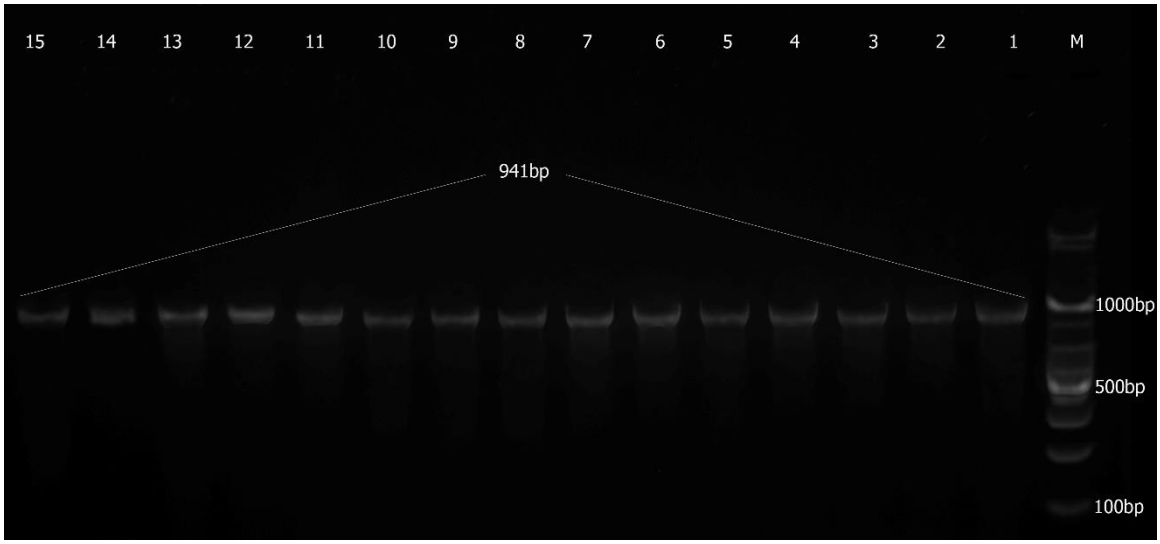


Fig. (3-1):1.5% Agarose gel electrophoresis image at 75V for 1 hour that showed PCR products analysis of D-alanine D- alanine ligase gene in *Enterococcus faecalis* isolated from clinical specimens .Where M: (100-1500bp)and Lane (1-15) showed (1,2,3,4,5,6 vagina , 7,8,9,10 wound , 11,12,13,14 stool ,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

Table(3-4) isolation rate of *E. faecalis* among 120 clinical specimen

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

To confirm the identity of *E. faecalis*, PCR assay detecting internal fragments of the gene encoding D-alanine-D-alanine ligase *ddl*, as this gene was described to be diagnostic and to differentiate the two major clinically important species *E. faecalis* and *E. faecium* (Hashem *et al* .,2021).

Sahib and Lamees (2020) found the results that all isolates taken from different clinical specimens were produced the specific 941bp DNA fragment when compared with allelic ladder.

Al-Halaby *et al*.,(2017)found that all isolates taken from urine sample gave positive result for *ddl* gene which was specific for diagnosis of *E.faecalis*

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

The result obtained by Kafil and Asgharzadeh, (2014) found that from (100) clinical isolates only 34(34%) isolated *E. faecalis* using specific primer. In this study, specific target was obtained and utilized in conventional PCR, which was proven more rapid, convenient and accurate

for identification of *E. faecalis,* than previous methods. The results of PCR approach demonstrated that comparative genomic methodology was successfully identifying specific target.

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

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

In the present study phenotypic methods are not highly sufficient, it is recommended to use polymerase chain reaction technique with primers for *ddI E. faecalis* hat provides a rapid, accurate , more sensitive, and less time-consuming detection of these bacteria.

3.5 Detection of Some Virulence Factors Genes:

E. faecalis secrete virulence factors that contribute to the severity of their infection. Cytolysin, a secreted toxin expressed in response to pheromones, contributes to the pathogenicity of *E. faecalis* by causing blood hemolysis. (Mohamed *et al*.,2007).

Numerous factors are associated with a greater hazard of acquiring enterococcal infections. These factors including antimicrobial impedance

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 hospital pathogens (Soheili *et al.,* 2014).

These opportunistic bacteria possess various virulence factors including enterococcal surface protein and aggregation substance which could enhance the colonization process in the host and binding to the host epithelium, respectively. Others such as autolysin, enterolysin A, gelatinase, hyaluronidase, Zinc-metalloendopeptidase, enhanced expression of pheromone and adhesion-associated protein (*E. faecalis* endocarditis antigen A) have been reported to be among the most important virulence factors (Madsen *et al*., 2017). On the other hand, *E. faecalis* produces cell walls anchored proteins that aid in binding host cells including adhesion to collagen of *E. faecalis* which also plays a role in virulence (Kenia, 2019).

Conventional PCR assay, in the present study is used to detect the presence of virulence factor genes, the virulence gene in this study were *srt*, *fsr , atl , cps1*, *cps2/cps5*.

3.5 Detection of Capsule Polysaccharide gene(*cps*) Polymorphism

Cps1 is non capsule polysaccharide factor was investigated in *E faecalis.* It was found that *cps1* marker was observed in 18(90%) isolates of *E. faecalis* out of 20 isolates of this bacteria .The positive result were detected by 950 bp bands when compared with allelic ladder as shown in figure (3-2)and table (3-5)and (3-6), while the *cps2* in 9(45%)isolates and *cps5*was present in 15(75%)isolates. The positive results were detected by1098/ 199bp

when compared with allelic ladder as shown in figure (3-3) and table (3-5), (3-6).

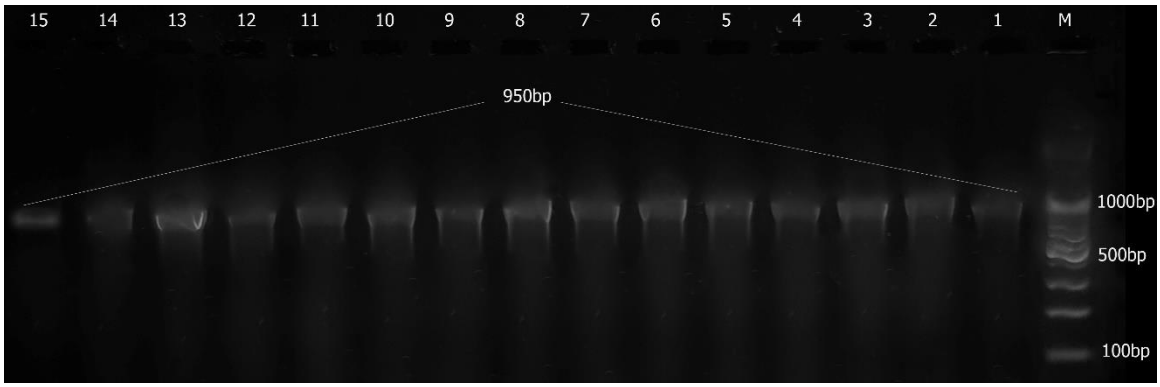


Fig. (3-2):1.5% Agarose gel electrophoresis image at 75V for 1 hour that showed PCR products analysis of *cps1* gene in *Enterococcus faecalis* isolated from clinical specimens. Where M: (100-1500bp)and Lane (1-15) showed (1,2,3,4,5,6 vagina , 7,8,9,10 wound , 11,12,13,14 stool , 15 urine) at (950bp)

.

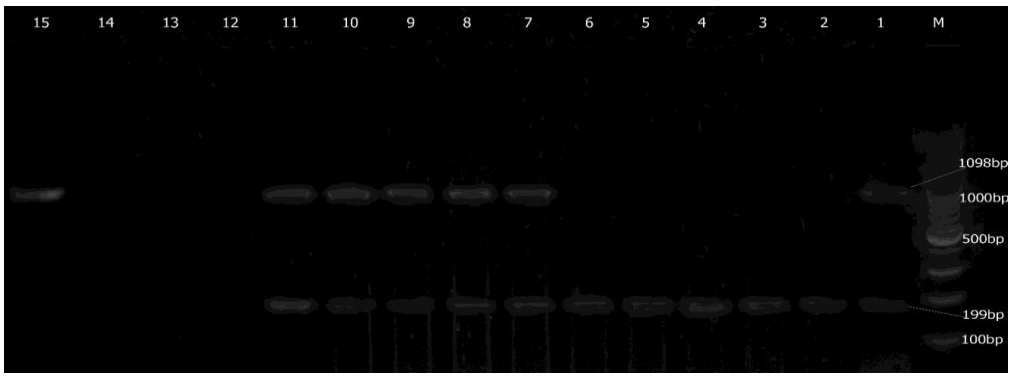


Fig. (3-3): 1.5% Agarose gel electrophoresis image at 75V(20mA) for 1 hour that showed PCR products analysis of *cps2*-*cps5*-specific primer. Where lanes 1-15 represent the identified *cps2*/*cps5* gene products in *E. faecalis* with 1,098/199bp, Lane M represent 100bp DNA ladder. Lane (1-15) showed (1,2,3,4,5,6 vagina,7,8,9,10 wound,11,12,13,14 stool,15urine).

Table (3-5): Identification of *cps* gene of *E. faecalis* in all studied specimens

Results	*cps1* N (%)	*cps2* N (%)	*cps5* N (%)	P value
Positive	18 (90)	9 (45)	15 (75)	<0.0001*
Negative	2 (10)	11 (55)	5 (25)	
Total	20	20	20	

* represent a significant difference at p<0.05.

Table (3-6): Identification of *cps* gene of *E. faecalis* in patients with different specimens sources.

specimens Sources	*cps1* N (%)	*cps2* N (%)	*cps5* N (%)
Vagina	6 (100)	1 (16.6)	6 (100)
wound	4 (100)	4 (100)	4 (100)
Stool	4 (100)	1 (25)	1(25%)
Urine	4(66.6)	3 (50)	4(66.6%)
Total	18/20	9/20	15/20
P value	1.000	<0.0001*	<0.0001*

- represent a significant difference at p<0.05.

The results were shown that the most common type among clinical isolates *cps1*(90%) indicating that these isolates do not have in their chromosome the essential gene in the *cps* operon for capsule production. The *cps2* and *cps5* which are associated with capsule producing isolates were found in prevalence (45%) and (75%) respectively.

Saffari *et al*.,(2018)showed that amplification of the *cps* operon revealed that *cps1* was the most common polymorphism, expressed by(63%) of the isolates, while only two isolates (9%) expressed *cps2*.

Pinheiro *et al* .,(2012) showed *cpsA* and *cpsB* were the only detected genes within the *cps* operon in 62.5% of *E. faecalis* strains (14/22), indicative of genotype *cps1*, which lacks capsule expression.

The present study show that the essential genes in the *cps* operon for capsule production were detected in the all strains. A limited primer set, optimized to detect the differences between these 3 capsule operon polymorphisms, was then used to determine which of the three *cps* types were present in the remaining isolates of the collection. All isolates tested yielded one of the three *cps* polymorphisms, based on characteristic PCR products. The occurrence of *cps1,2* and5 polymorphisms among the strains studied, as identified by this approach.

In the present study most isolates expressed the *cps* type 1 polymorphism, thus lacking essential genes of the *cps* operon that encode the polysaccharide capsule. The difference between the *cps* in *E. faecalis* isolates in this study and other studies may be due to the influence geographic condition, movement the virulence gene by transposon or in integron addition to plasmids are a major mechanism for the spread of virulence gene in bacterial population by conjugation and, or host genetic factor.

Sensitivity tests for *E. faecalis cps2* bacteria were performed because these bacteria have capsules that possess higher resistance to antibiotics compared to bacteria that do not have capsules (Bachtiar *et al*.,2015).

Wherever the collection contained multiple isolates within a sequence type, *cps* type was invariant among those strains. The most common *cps* type among

the diversity of lineages was type 1. When strains were examined by decade of isolation, *cps* type 1 remained most common(McBride *et al*.,2007).

The expression of the *cps* locus is regulated in order to modulate the presence of polysaccharide antigens during colonization or infection (Vebø *et al*., 2009; Corcionivoschi *et al*., 2009). Absence of expression of the *cps* locus could allow optimal adherence to host cells.

cps2-isolates are associated with an enrichment of virulence traits suggesting that *cps2*- isolates may be more prone to survive and/or colonize hospitalized patients (McBride *et al*., 2007).

capsule-producing *E. faecalis* isolates are more resistant to complement-mediated opsonophagocytosis than non capsulated isolates . Moreover, the presence of the capsule has been associated with the pathogenic lineages of *E. faecalis* isolated from hospitalized patients (McBride *et al*.,2007).

Presence of *cps* genes associated with virulence, is not sufficient to predict the corresponding phenotype. Although this fact has been previously established on other genes ,the *cps* locus highlight the need to improve and deepen the knowledge on enterococci serotypes, as *cps2* type may not necessarily be expressed in a *cps* type 2 genotype. It would be interesting to determine if silencing of *cps* type 2 genes also applies in clinical isolates(Gaspar *et al*.,2012).

E. faecalis has several types of capsules; one of them is based on the type of antigen in the polysaccharide capsule .Polysaccharide capsules can also be used to identify *E. faecalis* genotypes by considering the type of capsule. Genetically, the synthesis of polysaccharide capsule operon coding and a polymorphism locus was found in the clinical isolates of *E. faecalis* . *E.faecalis cps2* is the major bacterial isolate that has been implicated in root canal

persistent intraradicular infection relative to *cps1* and *cps5* strains (Bachtiar *et al*.,2015).

3.5.2 Detection of Feacal Streptococci Regulator gene(*fsr*) :

Feacal Streptococci regulator gene (*fsr*)was also detected in *E. faecalis* isolates and found that from 20 bacterial isolates, only 9(45%) isolates gave positive result to this gene The amplicon was detected in gel electrophoresis with molecular length (1016bp) when compared with allelic ladder as shown in figure (3-4)and table(3-7),(3-8).

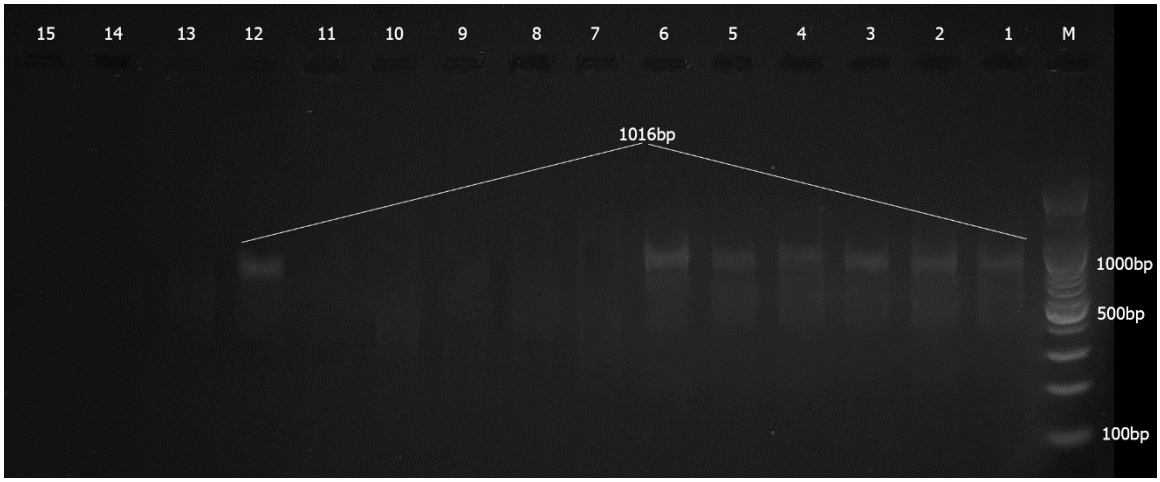


Fig. (3-4):1.5% Agarose gel electrophoresis image at 75V for 1 hour that showed PCR products analysis of *fsr* gene in *E. faecalis* isolated from clinical specimens. Where M: (100-1500bp)and Lane (1-15) showed (1,2,3,4,5,6 vagina , 7,8,9,10 wound , 11,12,13,14 stool , 15 urine) at (1016bp) .

Table (3-7): prevalence of *fsr* gene in *E. faecalis* isolated from different specimens

Results	N	Percentage	P value
Positive	9	(45)	<0.0001*
Negative	11	(55)	
Total	20	100%	

* represent a significant difference at p<0.05.

Table (3-8): Distribution of *fsr* gene of *E. faecalis* in patients with different specimens sources.

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

* represent a significant difference at p<0.05.

Song *et al.,(*2019) found the presence of virulence gene *fsr* in *E. faecalis* (64.0%) when take the bacteria from clinical specimens. While Roberts *et al*.,(2004) found *fsr* gene at rate (71%) in *E. faecalis* isolates. The presence of the *fsr* locus production affects the course of *E. faecalis* infections in humans or the outcome of the treatment.

The *fsr* system has an important role in the regulation of surface proteins and several metabolic pathways in *E. faecalis* . The presence of *fsr* locus in 100% of endocarditis isolates and over (50%) of fecal isolates strongly suggests that this system is a promising target to develop non-toxic inhibitors, thus attenuating the pathogenicity of *E. faecalis* (Bourgogne *et al*.,2008).

In this study the high prevalence of *fsr* gene in *E. faecales* isolated from vagina indicate an association of the prevalence of *fsr* gene and emergence of vaginitis. On the other hand, the absence of this gene in almost isolates may be the *fsr* system may be deleted due to horizontal transfer and recombination or rearrangement. The studies suggested that *E. faecalis* isolates isolated from different site in addition to the epidemiological differences may contain

different frequencies of *fsr* gene. However, the presence of the *fsr* gene in some isolates implies that, they would appear to be required during *E.faecalis* infection. However, the low frequency of *fsr*, might be due to regional differences and collection data of the isolates. The *fsr* virulence factor system is linked to enterococcal disease that affect the health of human. However, the *fsr* regulatory system in *E. faecalis* regulate much of their pathogenicity.

The greater variability of genes regulated by *fsr* suggests that this system is not only involved in virulence, but also alterations to metabolic activities, and biofilm-related components could play an important role(Teixeira *et al*.,2013).

The *fsr* quorum-sensing system and glycosyl transferases(GTF) promote biofilm formation . GTFs might be involved in the synthesis and processing of cell wall polysaccharides, which sequester the antibiotics present in the vicinity of cell walls, thus prevent absorption of the antibiotics (Dale *et al*.,2015).

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

fsr regulator locus responsible for bacterial quorum sensing are the most important virulence factors for biofilm development and pathogenicity, and severity of subsequent infections(Goh *et al*.,2017; Bin-Asif & Ali, 2019).

3.5.3 Detection of Sortase peptide gene (*srt*):

Sortase peptide is a virulence factor in *E. faecalis*, Present study showed that all 20 isolates, gave positive results to this gene, which gave molecular length 612bp as shown in figure(3-5) , table(3-9) and(3-10).

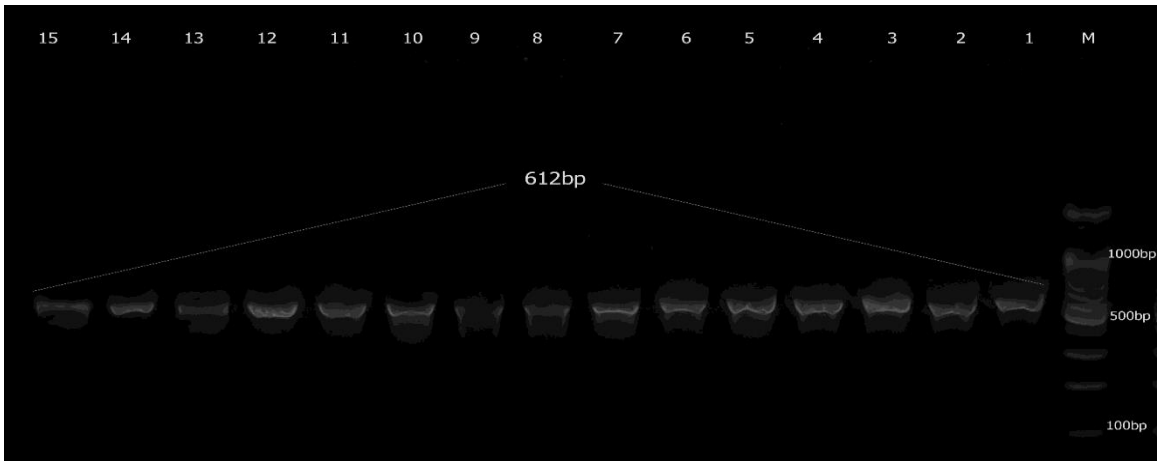


Fig. (3-5):1.5% Agarose gel electrophoresis image at 75V for 1 hour that showed PCR products analysis of *srt* gene in *Enterococcus faecalis* isolated from clinical specimens .Where M: (100-1500bp)and Lane (1-15) showed (1,2,3,4,5,6 vagina , 7,8,9,10 wound , 11,12,13,14 stool , 15 urine) at (612bp) .

Table (3-9): distribution of *srt* gene of *E. faecalis* in all studied specimens.

Results	N	Percentage	P value
Positive	20	(100)	<0.0001*
Negative	0	(0)	
Total	20	100%	

* represent a significant difference at p<0.05

Table (3-10): Distribution of *srt* gene gene of *E. faecalis* in patients with different specimen sources.

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

- represent a significant difference at $p<0.05$.

Stępień *et al*.,(2019) found that the *srt gene* was present in *E. faecalis* (96.3% isolates). While Hashem *et al*.,(2017) found *srt* gene in *E. faecalis* isolated from clinical specimens in (94%) of the isolates. On the other hand, Talebi *et al*.,(2015) showed that The presence of *srt* gene was confirmed in all patient of the isolates when take *E. faecalis* isolated from clinical specimens.

However ,in the present study the prevalence rates are different from those reported from other countries. Prevalence of the gene may vary according to the clinical status of the host and the genetic makeup of the isolates causing UTI, vaginitis, diarrhea, and wound infections.

Sortase-displayed surface structures play a pivotal role in displaying virulence and pathogenesis properties without affecting the growth and viability of cells. They are responsible for cell attachment, heme transport, nutrient uptake, sporulation and aerial hyphae formation . The surface proteins recognized by the sortase enzyme contain a C-terminal pentaglycine recognition motif followed by a stretch of hydrophobic amino acids and a positively

charged tail (Susmitha *et al*., 2021). Sortase proteins play an important role in initial attachment of planktonic bacterial cells, and subsequent biofilm formation . In *E. faecalis*, the cell wall anchoring of virulence factors such as aggregation substance and pili were facilitated by sortase enzymes. Therefore, sortase family protein was considered as the docking receptor and the antibiofilm active peptides were used as ligands. (Kurcinski *et al*.,2019).

The sortase enzyme accepts the nucleophiles which might vary in different Gram-positive bacteria, as the composition of peptidoglycan layers in the cell envelope vary from strain to strain(Comfort and Clubb, 2004).

However, given the ubiquitous nature of sortases and the limited knowledge of the activity and substrates of the sole *srt* characterized in *E. faecalis*, it is plausible that this enzyme may play an important role in *E. faecalis* physiology and/or pathogenesis under different conditions(Kristich *et al* .,2005).

The studies have proved that the both *srtA* and *srtC* were focally localized in *E. faecalis* and essential for efficient bacterial colonization and biofilm formation on the host tissue surfaces and was identified as an attractive drug target(Natarajan *et al*.,2017).

Were performed protein-peptide flexible docking to identify potential biofilm active peptides that can bind to sortase family protein thereby inhibiting its function. Also the identified peptide binding *Efsrt* residues can be considered as potential target sites for the development of potential peptide based therapeutics against biofilm associated infections(Natarajan *et al*.,2017).

3.5.4 Detection of Autolycin gene(*atl*) :

Molecular study of autolycin gene (*atl*) in *E. faecalis* were done for all 20 *E. faecalis* isolates by using specific PCR marker. It was found that (*atl*) marker was observed in 9(45%) isolates of these bacteria as shown in table(3-

11)and(3-12) . It was amplified products produced a band at the level of (206bp) when compared with the allelic ladder .

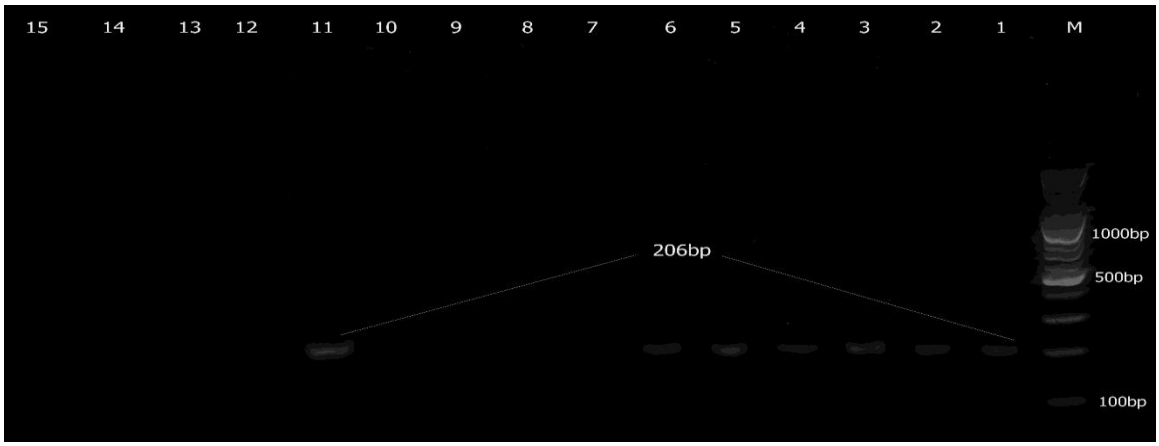


Fig. (3-6):1.5% Agarose gel electrophoresis image at 75V for 1 hour that showed PCR products analysis of *atl* gene in *E. faecalis* isolated from clinical samples. Where M: (100-1500bp)and Lane (1-15) showed (1,2,3,4,5,6 vagina , 7,8,9,10 wound , 11,12,13,14 stool , 15 urine) at (206bp) .

Table (3-11): distribution of *atl* gene of *E. faecalis* in all studied specimens.

Results	N	Percentage	P value
Positive	9	(45)	<0.0001*
Negative	11	(55)	
Total	20	100%	

* represent a significant difference at p<0.05.

Table (3-12): distribution of *atl* gene of *E. faecalis* in patients with different specimen sources.

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

- represent a significant difference at p<0.05.

In the current study the results obtained by genotypic characterization revealed a lower percentage of isolates that produce *atl* gene. This may be due to the presence of silent and undetected genes, or the fact that it was detected a single gene inside an operon. The prevalence of autolysin gene was varied based on the strain variation of *E. faecalis* . Expression of the gene may also influenced by some of the suitable environmental condition.

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

The potential implications that post-translation modification of *atlA* could have on *E. faecalis* virulence by regulating colonization and dispersion of infection(Palma *et al*.,2014).

when *gelE* cleavage of *atlA* has not occurred and long cell chains are observed. As *gelE* reaches maximum activity levels at later growth stages, after colonization is established, *atl* would be cleaved and short cell chains would result. These short chains could potentially aid in bacterial dissemination or help *E. faecalis* evade host immune defense. Thus, *gelE* could aid in bacterial cell dissemination by cleaving *atl* at a later growth stage, resulting in short chains. Further suggestion that the *gelE* processing of *atl* may impact *E. faecalis* virulence(Salamaga *et al*.,2017).

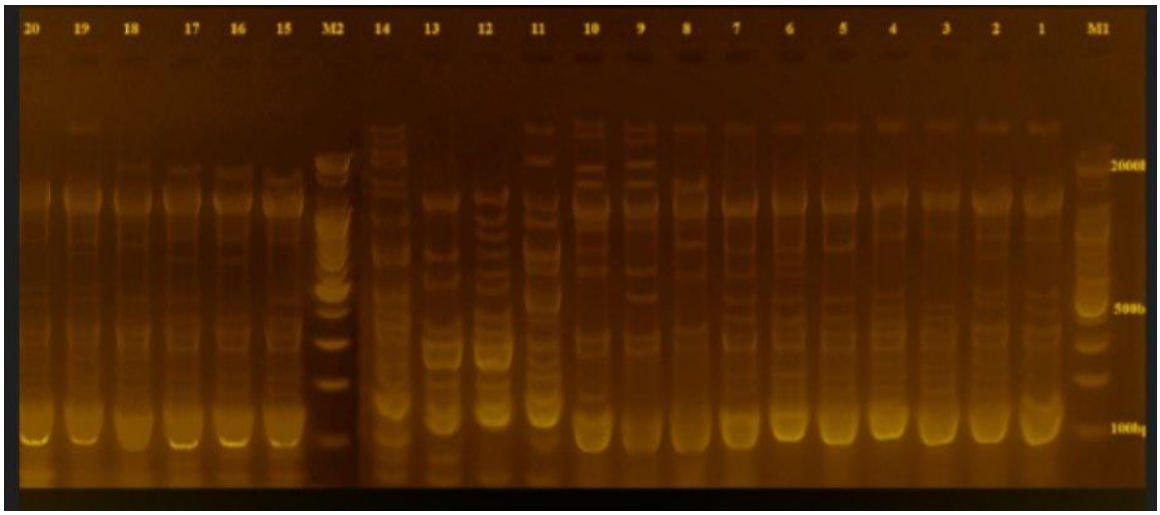
E. faecalis produces several autolysins, which were recently identified and characterized . The major *E. faecalis* autolysin, *atn* (also known as *atlA*), is an N-acetylglucosaminidase important for daughter cell separation during cellular division . Disruption of *atn* in *E. faecalis* resulted in increased chaining, a defect in primary attachment, and decreased biofilm production. Thomas *et al*.,(2009) provided evidence that inactivation of this autolysin results in a decrease in DNA release similar to that of gelatinase-deficient mutants. Furthermore, they showed that *gelE* and *sprE* can differentially cleave *atn* in *vitro*, and this processing may underlie the mechanism of cell death and DNA release in *E. faecalis* during biofilm formation(Thomas *et al*.,2009).

Atn and *srtA* were important factors in the DNA-independent initial attachment step. Additionally, *atn* played a role during the accumulative stage and in DNA release, which were demonstrated to be crucial for the growth, maturation, and structural stability of *E. faecalis* biofilms. Previous reports have implicated *atn* in primary attachment and biofilm production (Kristich *et al*.,2008). Unlike autolysins described for other gram-positive bacteria, *atn* contributed to initial adhesion in a DNA-independent fashion. Since *atn* is important for cellular growth as well as cell lysis(Qin *et al*.,2007).

3.6 Phylogenetic analysis of *Enterococcus faecalis* isolates:

3.6.1 Detection of Enterobacterial Repetitive Intergenic Consensus (ERIC)-elements fingerprint in *E. faecalis*:

ERIC are palindromes sequence include conserved intervening repeat that occur in multiple copies in the genomes of enteric bacteria. The study investigated the distribution of these elements in the genome sequence of *E. faecalis* by using ERIC – PCR. There was considerable variation among isolates with respect the presence of an element in any particular intergenic region. Since some copies appear to have been conserved among bacterial genome and this variation give rise for divergence of bacteria isolates. A total of (20) *E. faecalis* isolated from different clinical specimen were analyzed in this study. The size of ERIC product ranged from 90 to 3000 bp. DNA fingerprint of ERIC in this study obtained (10-19) band as shown in Figure (3-7).



Figure(3-7) 1.5% Agarose gel electrophoresis image at 75V for 1 hour that showed DNA fingerprinting patterns of 20 isolates of *E. faecalis* with (90-3000bp)by using ERIC primer and Lane (1-20) showed (1,2,3,4,5,6 vagina , 7,8,9,10 wound , 11,12,13,14 stool , 15,16,17,18,19,20 urine) .

ERIC typing of 20 *E. faecalis* isolates were found genetically diverse and heterogeneous within test species and their recovery source, all generated DNA patterns are relatively complex. The isolates in each clusters with heterogeneous specimens. However, the result of ERIC analysis is revealed that the amplified products of most isolates vary in molecular size patterns even with equal total fragment.

The total 20 isolates subjected to ERIC PCR, 14 patterns were observed from the binary scores of the fingerprints a dendrogram was constructed for ERIC fingerprints bands. The dendrogram constructed from ERIC by using UPGMA revealed two major cluster as shown in fig(3-8).The first cluster (A) contained three isolates (15%)which include 8EF, 9EFand 10EF) among which the isolates no. (9EFand 10EF) show similarity(64%).

Moreover the second cluster (B)contained 17 isolates(85%) this cluster divided 17 isolates in to two sub cluster, the first sub cluster contained three isolates (1-EF, 2-EF, and 14 EF) among which isolates (1EFand 2EF) revealed similarity(80%). Whereas the second sub cluster divided in to two branches, the first branch divided more to two sub branches and contained(6) isolates, among which isolates (4EFand 7EF), (5EFand 6EF) show similarity of rate(78% and 77%) respectively. While the second branch divided more to two sub branches and contained(8) isolates among of which isolates (16EFand 17EF),(19EFand 20EF) with similarity at rate (93% and 92%) respectively.

However, the cluster (A) the isolates collected from wound. These data suggest that the influence of epidemiological relatedness on the clustering of *E. faecalis* circulating strains in Babylon province, as cluster B with high relatedness were recovered from the same period of isolation and location.

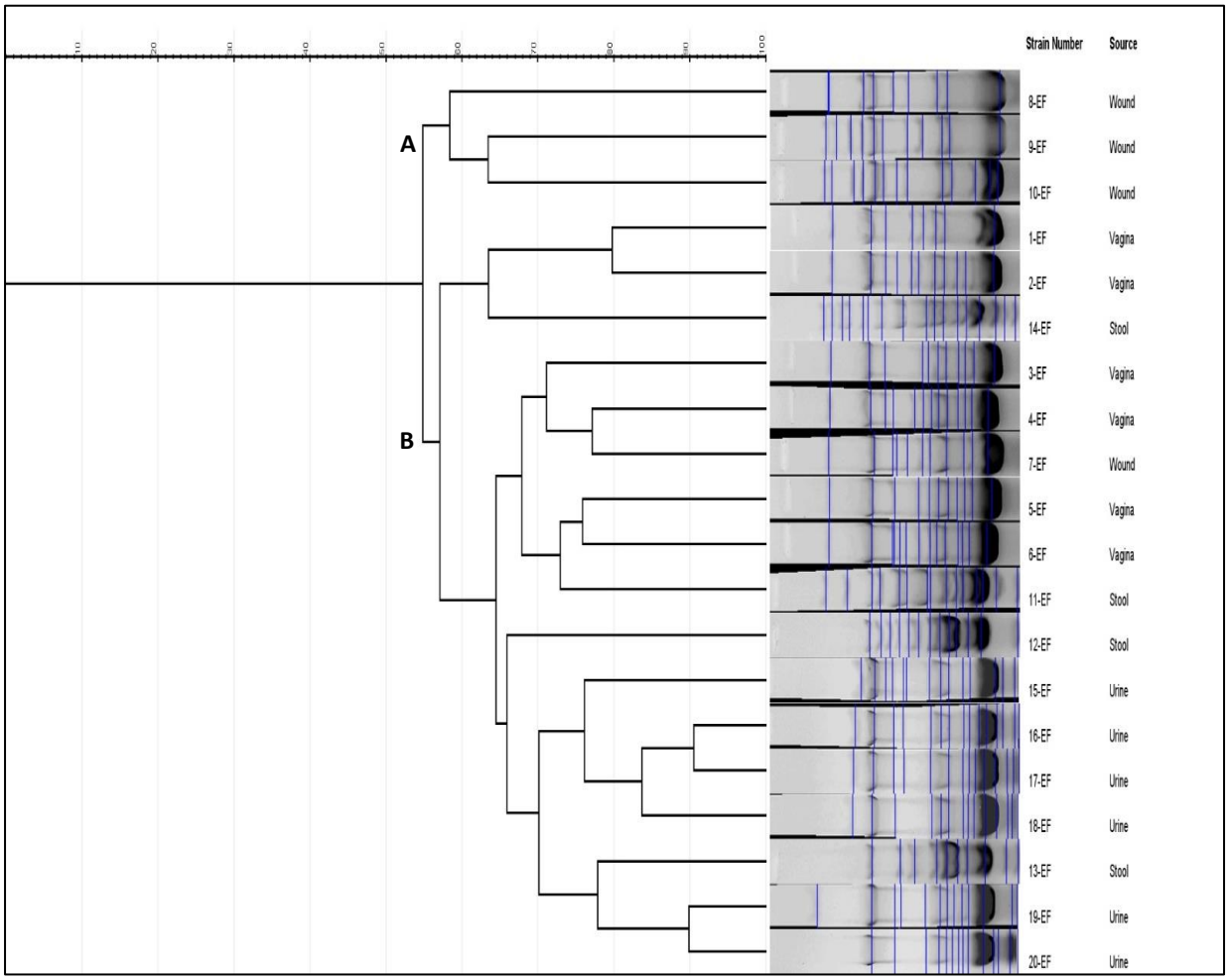


Fig. (3-8): The ERIC-PCR-derived cladogram representing the relationship among 20 of *E. faecalis* in patients with different specimens sources. Bar (above) represents the distance values. This cladogram was generated by Unweighted Pair Group Method with Arithmetic mean (UPGMA).

Correlation matrix analysis with heat map as shown in figure (3-9) was utilized to detect association the genotyping feature and origin of strain.

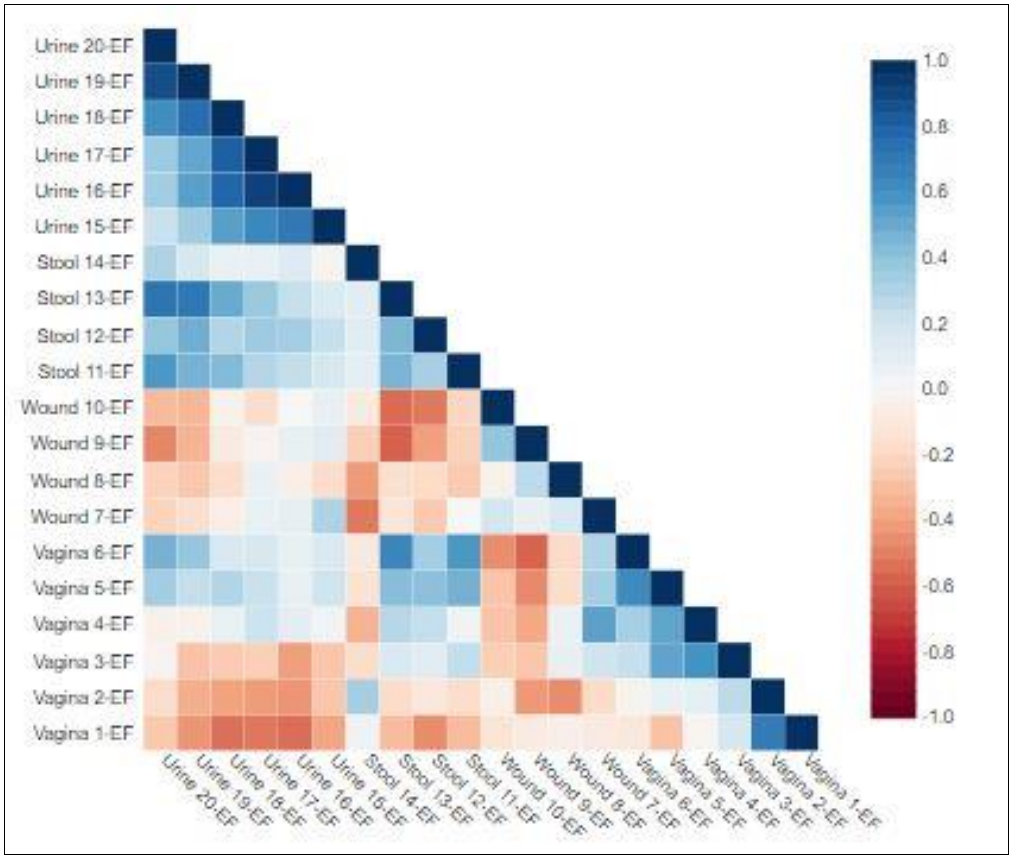


Fig. (3-9): A Heat map represent the Genetic correlation matrix among the studied 20 of *E. faecalis* isolates using ERIC-PCR assay.Red sequares indicated significant negative correlation and blue sequares show significant positive correlation.

The genome of the *Enterococcus* has a large number of repetitive sequences that are randomly distributed over DNA. In ERIC-PCR, a separated patterns for each strain and is considered a separated type .

However, the cluster analysis program were generated some genetically distant isolates with a differences at the gene level from different bacterial source such as urine, stool, wound, and vagina infection based on the ERIC-PCR pattern similarity. The genetic diversity of the chromosomal DNA may be explained by differences in the banding patterns among the isolates. In this study, ERIC-PCR results provided a better overview of *E. faecalis* diversity. Most of collected *E.*

faecalis isolates in the same area belong to the same cluster, which agrees with the result of a previous studies (Martin-Platero *et al*.,2009).

The Enterobacterial Repetitive Intergenic Consensus (ERIC) PCR is a simple, sharp and cost effective genotyping technology for discriminating different types of strains. Indeed, ERICs are recognized as mobile DNA particles in association with Miniature Inverted Transposable Elements (MITEs) (Behzadi *et al*., 2015).

The most common way to analyze the complex patterns obtained by electrophoretic separation is to compare the bands between isolates, obtain a similarity matrix and then analyze by UPGMA cluster methods. This method is a score of the presence or absence of variability in determining when an isolate is related can be partly attributed to be have the same ERIC sequence (Wijetunge *et al*., 2012).

Enterobacterial Repetitive Intergenic Consensus (ERIC) sequence is the sequence length is about 124-127bp, which contain about 44bp highly conservative core sequences in its center, primarily presents with multiple copies in genomes of Enterobacteraciae and vibrios (Wilson and Sharp, 2006). This technique has been applied for genotyping of *E. faecalis* isolates (Martin-Platero *et al*., 2009).It is situated in noncoding transcribed regions of the chromosome (Waturangi *et al*., 2012).

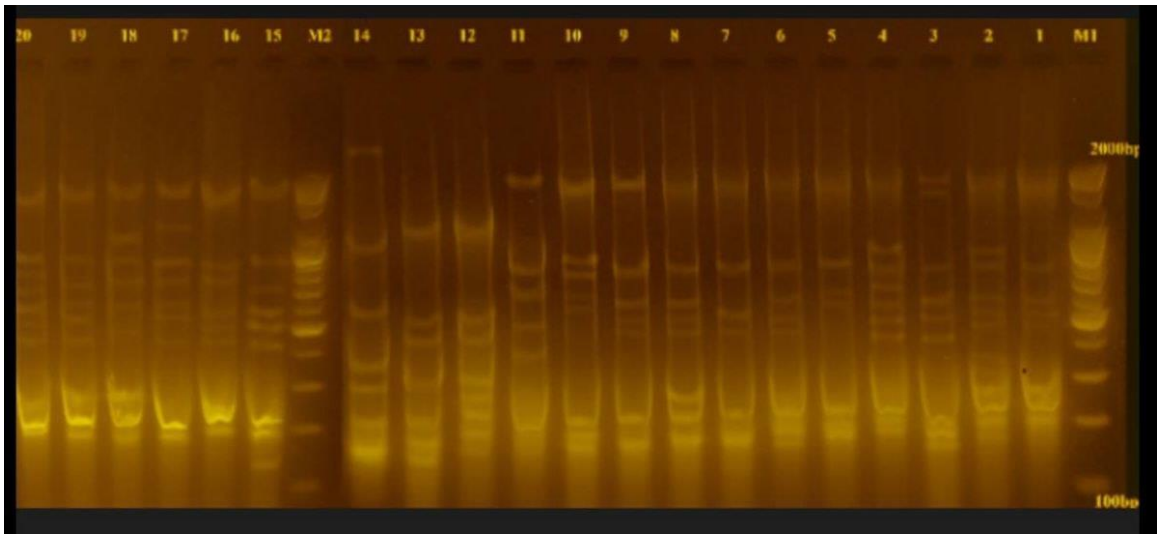
The performed investigations confirm the rapidity and simplicity of ERIC-PCR as a cheap advanced molecular technology for different strains of a bacterial species (Ranjbar *et al*., 2013).

The epidemiological purposes the use of ERIC-PCR with ERIC primer in comparison with other methods applied such as Pulsed-field gel electrophoresis(PFGE) and plasmid profile analysis yields a benefit because of its time-and cost- consuming aspects. In addition, the ERIC-PCR with ERIC

primer uses the total DNA which guarantees good reproducibility of results contrary to the plasmid profile analysis (Jurkovič *et al*., 2007).

3.6.2 Detection of poly trinucleotide $(GTG)_5$ fingerprint analysis:

$(GTG)_5$ PCR is a type of repetitive extragenic palindromic (rep)-PCR which amplified the $(GTG)_5$ repetitive element that lays throughout the bacterial genome. In the present study, the distribution of repetitive DNA sequence in *E faecalis* have bands were assigned a number in relation to their migration distance within the get since it was employed complex banding patterns between clinical isolates of *E. faecalis* for each of 20 isolates analyzed. The size of bacterial isolates $(GTG)_5$ gene from all location are within the range 150-3000pb of all 20 isolates which yielded different banding patterns to produce between 7-12 bands that can be differentiated by agarose electrophoresis as shown in Figure (3-10).



figure(3-10) 1.5% Agarose gel electrophoresis image at 75V for 1 hour that showed DNA fingerprinting patterns of 20 isolates of *E. faecalis* with (150-3000bp)by using (GTG)5 primer and Lane (1-20) showed (1,2,3,4,5,6 vagina , 7,8,9,10 wound , 11,12,13,14 stool , 15,16,17,18,19,20 urine) .

The 20 *E. faecalis* isolates could be divided in to 13 genotypes by (GTG)5 the dendrogram constructed from (GTG)5 by using (UPGMA)revealed two major cluster as shown in figure (3-11). The first cluster A contained only one isolate (11EF) and the second cluster divided the 19 isolates in to two sub cluster, the first contained one isolates(14EF), whereas the second subsequently divided to two branches, the first branch divided in to two sub branch and contained (4) isolates among which isolates (5EFand 10EF)with similarity (85%) while isolates (15EFand 16EF) with similarity (76%).

Moreover, the second branch divided more to two sub branches and contained 14 isolates among which isolates(4EFand 6EF), (1EFand 8EF) shown similarity at rate (88%) and (84%)respectively. While the isolates (3EF and 9EF) was shown similarity at rate (79%) and the isolates (12EF and 13EF) shown similarity at rate (91%). Also the isolates (19EFand 20EF) show similarity at rate (79%).

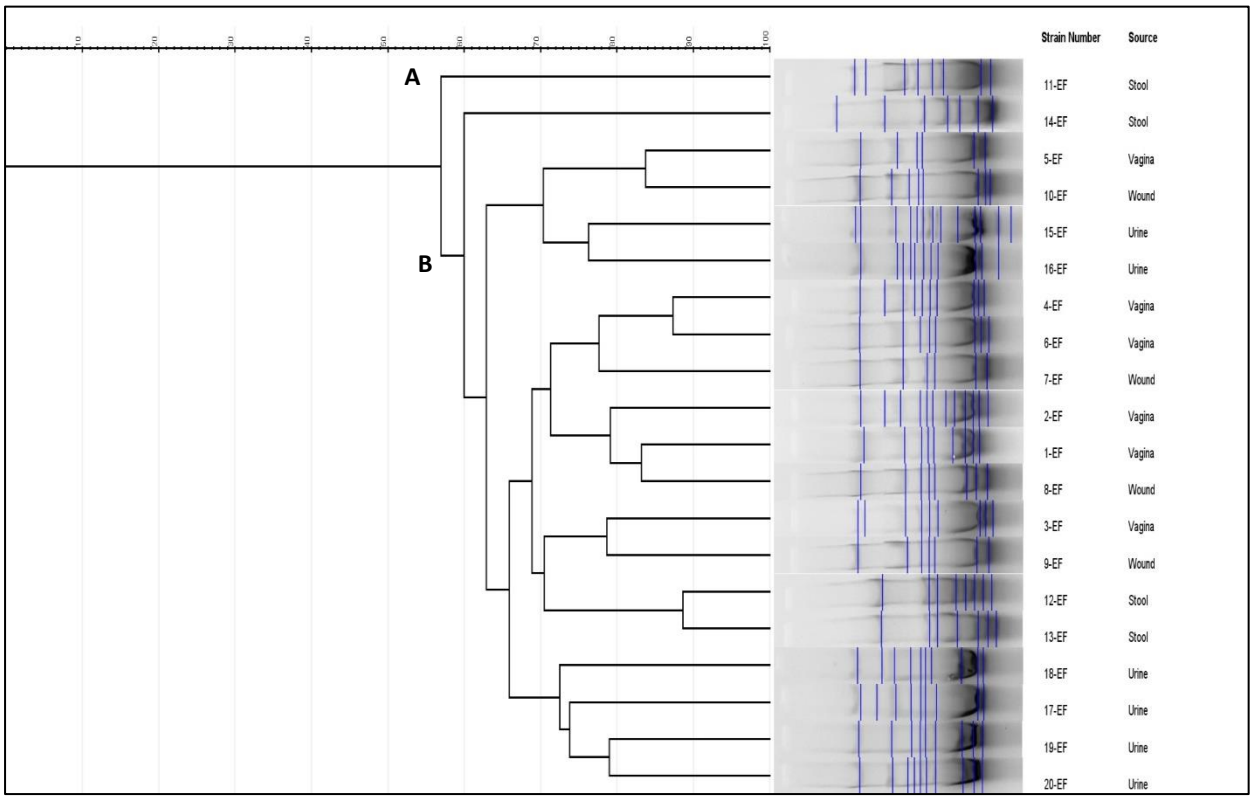


Fig. (3-11): The (GTG)5-PCR-derived cladogram representing the relationship among 20 of *E. faecalis* in patients with different sample sources. Bar (above) represents the distance values. This cladogram was generated by Unweighted Pair Group Method with Arithmetic mean (UPGMA).

Correlation matrix a lysis heat map as shown in figure (3-12) was used to determine association between the genotyping traits and source of the isolates.

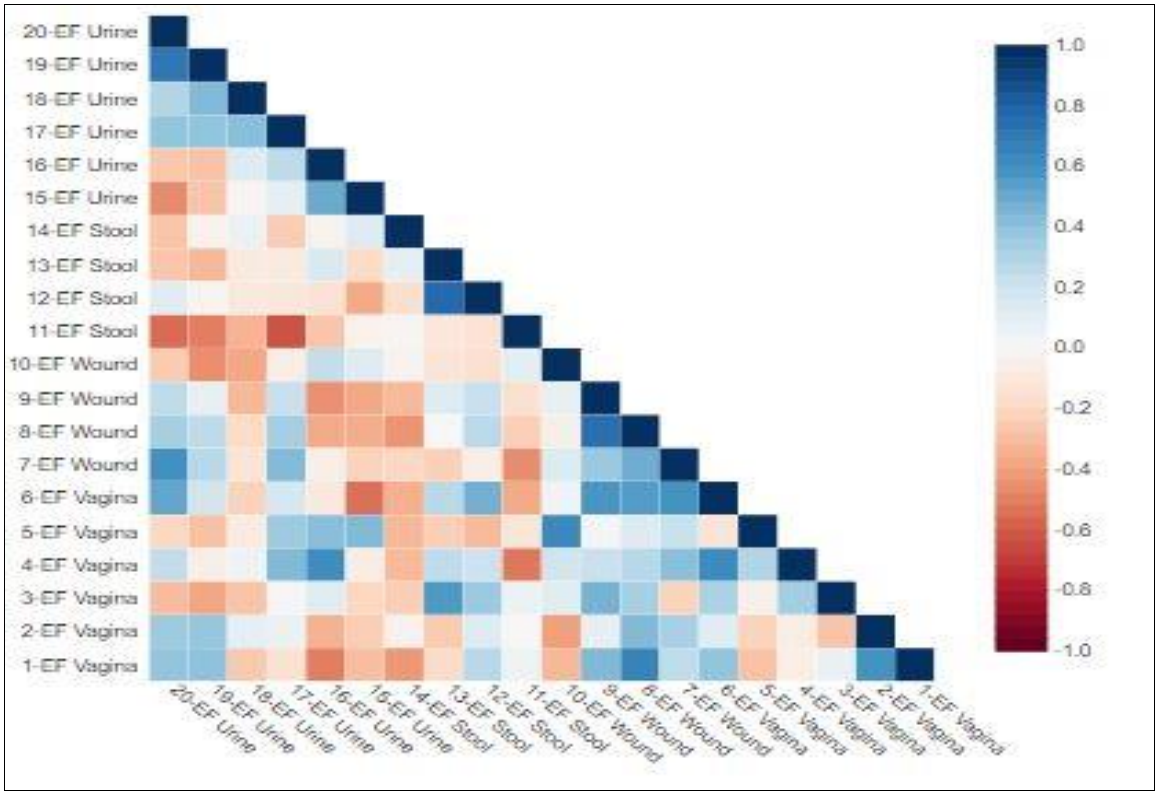


Fig. (3-12): A Heat map represent the Genetic correlation matrix among the studied 20 of *E. faecalis* strains using (GTG)5-PCR assay. Red sequares indicated significant negative correlation and blue sequares show significant positive correlation.

(GTG)5 typing of 20 *E. faecalis* isolates were genetically diverse consisted of heterogeneous within test species and their recovery source, all generated DNA patterns are relatively complex.

The study was shown that the $(GTG)_5$ analysis give different size of bands as conserved sequences of each isolates.

However, according to UPGMA analysis, the results converted in to dendrogram were analysis. Cluster analysis program were generated with the tree option to estimates the extent of similarity among isolates, which are obtained from different source such as urine, stool, wound, and vagina infection.

Although, the isolates located in the same cluster are isolated from different sources but the results of $(GTG)_5$ give one important evidence on the closed relation of these isolates. Screening of clonal isolates using $(GTG)_5$ PCR technique is cheap and easy. It also has high discrimination power when compared to other DNA fingerprint. Using of $(GTG)_5$ PCR fingerprinting to assess bacterial source tracking in the field and analysis of genetic diversity *E. faecalis* according to strain considerable. This method is wildly used for DNA typing analysis and has been shown to successfully differentiate between different related or unrelated strain compared with other methods.

Based on the $(GTG)_5$-PCR dendrogram constructed, it was found that 20 isolates were genetically diverse and heterogenous. The clustering of strains from different sampling sites together is suggestive of an evolutionary relationship. The repetitive DNA sequences were dispersed in *E. faecalis* chromosome in different orientations and separated by various distance. These dispersed repetitive sequences could be used as primer binding sites and PCR amplification between them would yield distinct patterns of DNA fragments varying in size when separated by agarose gel electrophoresis. So, the $(GTG)_5$ sequence provides a fast and discriminatory tool for characterizing taxonomic diversity and phylogenetic structure.

All results obtained in this short study dealing with bacterial isolates isolated from different clinical specimens of patients. It was demonstrated that the $(GTG)_5$-PCR fingerprinting be a good tool for identification of *E. faecalis* isolates. This study demonstrated that $(GTG)_5$ primer could be applied due to its easy and quick performance and low expense for fast screening of multiple bacterial isolates and for reliable *E. faecalis* identification, which is essential in clinical and epidemiological as well as taxonomic studies. Repetitive sequence based PCR polytrinucleotide $(GTG)_5$ sequence is based on fact that outwardly facing primers, complementary to interspersed repeated sequences, enable the amplification of different size DNA fragments consisting of sequences lying between these elements.

This method is effective in screening a large number of bacterial isolates and it is beneficial for intra species differentiation and identification of bacterial genomes. Indeed, (GTG)5-PCR is a type of repetitive extragenic palindromic (rep)-PCR that amplifies the (GTG)5 repetitive element which lays throughout the bacterial genomes(Gevers *et al*., 2001). This PCR-based method can be used for a broad range of Gram-negative bacteria and a narrow range of Gram-positive bacteria (Gevers *et al*., 2001; Kathleen *et al*., 2014).

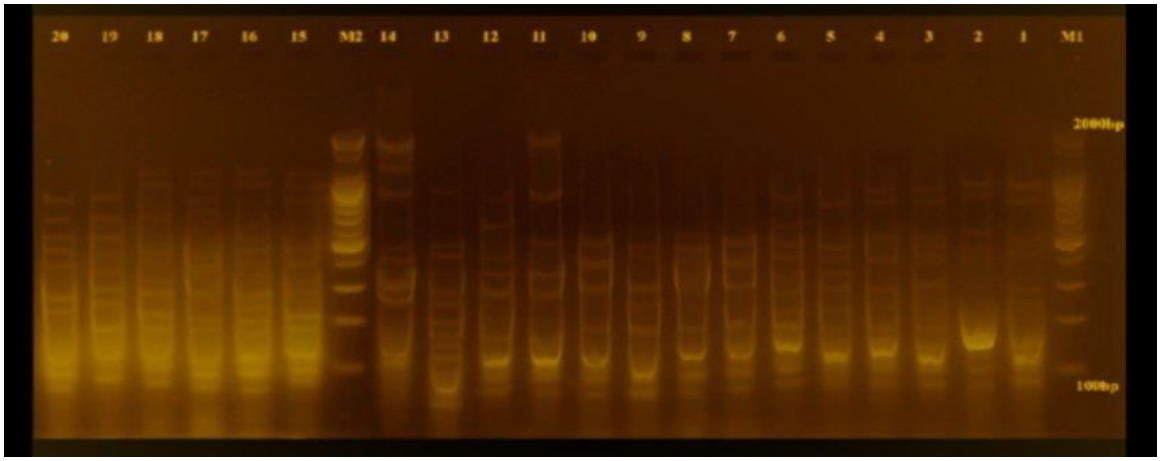
Many phenotypic and molecular methods have been described for the identification of enterococci (Domig *et al*., 2003). The rep-PCR with (GTG)5 primer has also been presented as a reliable method for species identification of all enterococci strains which grouped clearly into well-separated clusters and representing single species (Švec *et al*., 2005).

3.6.3 Detection of Box-Elements fingerprint in *E. faecalis*:

A highly conserved repeated DNA element has been identified in the chromosome of *E. faecalis* is Box repetitive element. This demonstrated of the

presence of such a repetitive DNA moiety in a gram positive bacterial species. So, the development of a methodology to identify the source of bacteria is important for assessing of the risk pose to public health associated with bacteria evolution to determine degree of genomic diversity and comparison with $(GTG)_5$ and ERIC-PCR. The Box genomic fingerprinting methods was assessed for their potential in differentiation 20 clinical *E. faecalis* isolates under study.

Based on the results of clusters and discriminate function analysis, the length sequence and composition of these sequence in the genome are variable and often unique for each isolate, it was indicated fingerprints for all *E. faecalis* isolates using BOX A1R primer from *E. faecalis* performed the complex DNA patterns. Molecular typing of *E. faecalis* isolates using BOX A1R primer generated 8-12 bands ranging from 90-2100bp. The phylogenetic tree presented in Figure (3-13) illustrated that automated data analysis can be performed and that data generated in this way are congruent with those obtained by visual inspection.



figure(3-13) 1.5% Agarose gel electrophoresis image at 75V for 1 hour that showed DNA fingerprinting patterns of 20 isolates of *E. faecalis* with (90-2100bp)by using BOX elements primer and Lane (1-20) showed (1,2,3,4,5,6 vagina , 7,8,9,10 wound , 11,12,13,14 stool , 15,16,17,18,19,20 urine) .

The relationships among BOX PCR profiles of the 20 isolates are represented in the dendrogram as shown in figure (3-14).

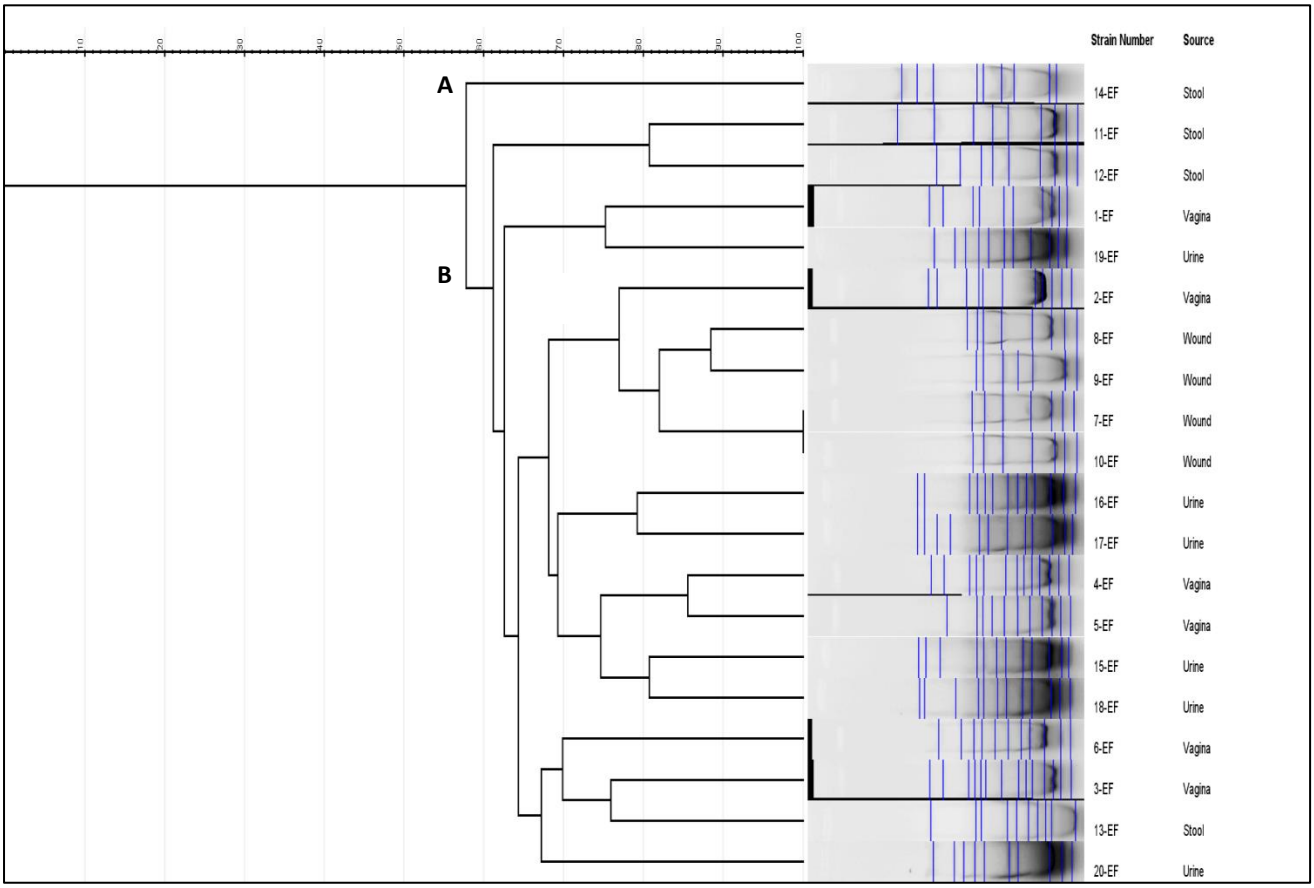


Fig. (3-14): The BOXAIR-PCR-derived cladogram representing the relationship among 20 of *E. faecalis* in patients with different sample sources. Bar (above) represents the distance values. This cladogram was generated by Unweighted Pair Group Method with Arithmetic mean (UPGMA).

The 20 isolates subjected to BOX PCR analysis, 12 patterns were observed . Genetic similarity between each pairs of the twenty *E. faecalis* was performed on the basis of BOX amplified fragments.

The constructed UPGMA dendrogram showed two main clusters, the first cluster (A) contained one isolate (14EF) and the second cluster (B) contained (19) isolates. This cluster divided the (19) isolates in to two sub cluster, the first

sub cluster contained two isolates (11EFand 12EF) with similarity (82%), whears the second sub cluster contained two branches, the first branch contained two isolates (12EFand 1EF) with similarity (77%). However, the second branch divided more to two sub branches, one contained (11) isolates among which isolates No. (8EFand 9EF) with similarity (89%), isolates (7EFand 10EF) revealed highest similarity (100%), also isolates (16EFand 17EF) with similarity at rate (81%), while the isolates (4EFand 5EF) and(15EFana 18EF) show similarity at rate (87%)and (82%) respectively. On the other hand, the second showed four isolates among which the isolates (3EFand 13EF) show similarity (78%).

The correlation matrix analysis with heat maps shown in figure (3-15) was utilized to detect the genotype trait and source of isolates.

The clusters showed a remarkable diversity among the *E. faecalis* isolates since differences among its banding patterns were observed.

The current study showed that the prokaryotic genome include dispersed repeat sequences that are relatively short non-coding, and dispersed in the bacterial genome. BOX-PCR primer is complementary to these repeat sequences and allow for dedicated binding and unique BOX-PCR fingerprint patterns with reproducibility capability.

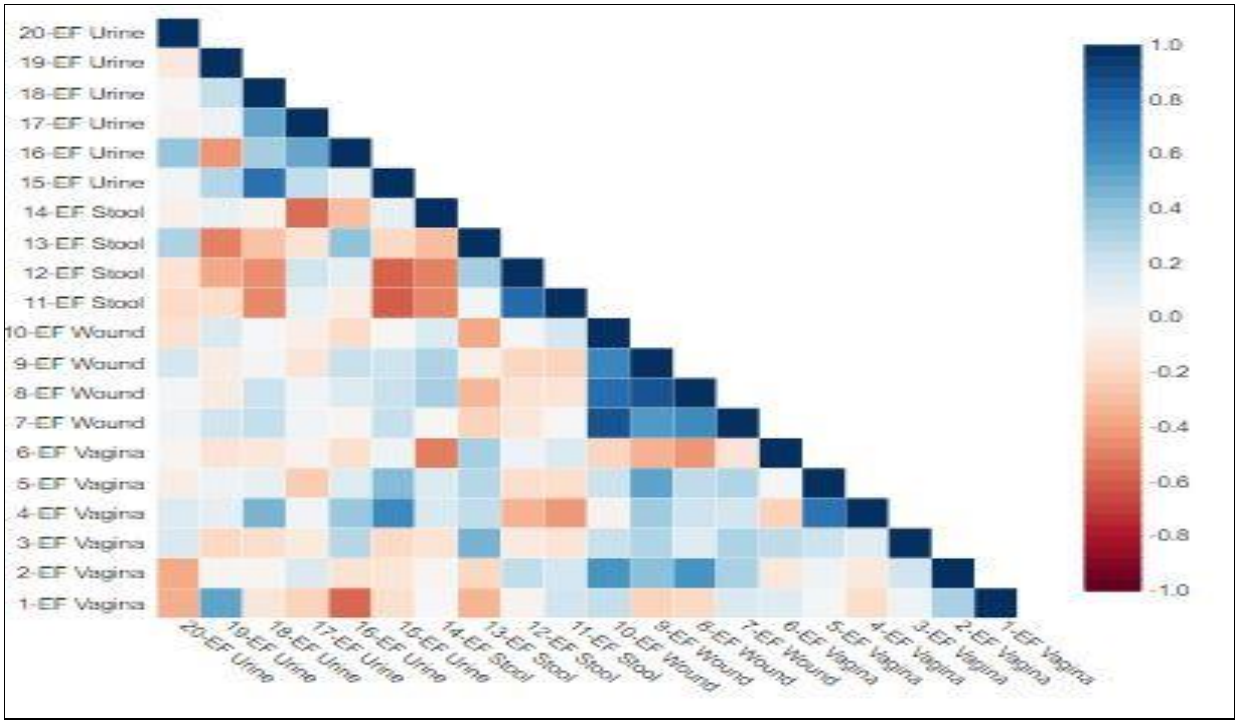


Fig. (3-15): A Heat map represent the Genetic correlation matrix among the studied 20 of *E. faecalis* isolates using BOXAIR-PCR assay. Red sequares indicated significant negative correlation and blue sequares show significant positive correlation.

In the present study, the BOX A1R primer showed that DNA polymorphism among *E. faecalis* isolated from clinical specimens, either in the occurrence of amplified or in the variable genetic similarities of each isolates with others, despite the fact that, they should display narrow and low variation due to genomic structure of *E. facials* and the structure of Box A1R primer. Eventually, the fluctuation of genetic similarity value each of the 20 isolate with other using the BOXA1R primer evidently revealed the divergent genetic background of such isolates with their DNA polymorphism patterns. However, Box analysis revealed that, there were some bands which are common in all the samples and some were not evident.

BOX elements are mosaic repetitive elements comprised of different combinations of three subunit sequences. These three subunit sequences are boxA, boxB, and boxC which are 59, 45, and 50 nucleotides long, respectively (Dombek *et al*., 2000).

In fact, the infectious agent that causes an outbreak often result from clones that are genetically identical or related to the source of infection. In other word, it may have the same biochemical traits, virulence factors and genomic characteristics (Olive and Bean, 1999). DNA-based typing methods have been widely used to demonstrate the epidemiology and molecular characterization of most pathogens (Ranjbar *et al*., 2013).

3.6.4 Assign of isolates to cluster groups according to ERIC, BOX1A and $(GTG)_5$ DNA fingerprinting analysis:

The present study employed the BOX1A , ERIC and $(GTG)_5$ fingerprint it was prove to be useful for determine the genetically closely related *E. faecalis* isolates or the degree of relatedness distance between individual according to conservation repetitive sequence recognizable homogeneity in gene content and sequence similarities in bacterial genome .

BOX1A, ERIC, and (GTG)5-PCR DNA fingerprinting analysis revealed extensive genetic diversity among *E. faecalis* isolates even from the same sampling sites. So the differences in PCR fingerprinting profile existed among isolates when use three mention techniques, so it was demonstrated that the diversity of *E. faecalis* isolates were high through applied dendrogram cluster analysis.

In general, 20 isolates characterized using the three molecular techniques had comparable number of bands with some degree of polymorphism. *E. faecalis* isolated from same source were clustered into different groups, and had cross with other isolates source. Clustering using ERIC, (GTG)5 and BOX1A

generated two main cluster. The cluster group analysis of BOX1A can be compare with (GTG)5 or ERIC fingerprint patterns and can be recorded some differences in the distribution of some conservation sequence in local isolates of *E. faecalis*, the cluster that contain the isolates (7&10)isolated from wound at 100% similarity level through using BOX-PCR profile while this isolates generated different pattern of cluster using ERIC and (GTG)5.

However, the isolates have a different clusters pattern when used methods (BOX1A, ERIC and $(GTG)_5$) and there have no similarity between the methods in the distribution of isolates in the same pattern. On the other hand, the results revealed that some isolates from a different source do not share patterns with any isolates in cluster style which can be classified in distinct mode.

In order to compare the capability of each molecular typing method to differentiate among all *E. faecalis* isolates, Discriminatory indices(DI)was calculate from each constructed phylogenetic tree.

Moreover, the results was shown that the DI were calculate for (GTG)5, ERIC and BOX-PCR by using simpsons index of diversity which were 0.4199, 0.2353, and 0.3578 respectively which is acceptable confidence value for interpreting the level of discrimination. The discrimination power of these methods and their composite forms used in this study revealed the robustness of the methods and promises a high potential for molecular typing, genetic relationship analysis and epidemiological purpose in Babylon *E. faecalis* isolates.

However, according to the value of DI suggested (GTG)5 as a better molecular typing method than ERIC and BOX-PCR in their capability to distinguish among closely genetically related *E, faecalis* isolated from hospitalized patients. Also it was demonstrated to be the most suitable

molecular typing method to group into clusters of similar genetic relatedness among *E. faecalis* isolates from the same source and from different source.

The explanation that (GTG)5 is considered as a good discriminatory, this study involves groups of *E. faecalis* isolates. It was noted in phylogenetic tree that there was closeness between groups of *E. faecalis* isolates. The more groups were closer, the more random was the gene hit; thus, the gene become less important. It is noted that gene (GTG)5 has less closeness among different isolates.

Furthermore, this study showed that the isolates responsible for infection in the hospital wards have a shared genetic origin and are genetically related. The similarity between patterns from different hospitals can be explained by the transfer of patients between hospitals in AL-Hilla. The results also indicate that the (GTG)5-PCR technique can be the best approach to distinguishing *E. faecalis* isolatesand an appropriate way of control and prevention of bacterial spread. Hopefully, this molecular method would be essential for hospitals in the future.

In the current study, found that, the genetic diversity in *E. faecalis* depend on the source of isolates and occurrence of mutants. However, the data acquired in the present work confirm the wide genotypic diversity of *E. faecalis* from various clinical samples. It is interesting to note that, there was no evident correlation between the observed isolates variability and the sample from which the isolates originated. Although, the isolates located in the same cluster were isolated from different source but the results give important evidence on the closed relative of these isolates. So, the present study revealed that *E .faecalis* was not specific for infection. This clearly showed that, one cannot use the same drug for a particular infection caused by *E. faecalis* because of their variation in DNA polymorphism.

The presence or absence of bands in some isolates under some ERIC, BOX and (GTG)5 fingerprinting may be attributed to the presence of frame shift mutation (insertion and deletion) in that locus . So, the diversity from *E. faecalis* isolates depend on the appearance or disappearance band in gel electrophoresis these markers were able to distinguish bacterial isolates into different genotype groups showing that the techniques had the requisite discriminative power to differentiate closely related bacterial isolates. Isolates from different sources were found to cluster together showing that the isolates still showed some genetic similarities that have remained unchanged throughout the evolutionary pathway.

This study recovered the presence of BOX, ERIC and (GTG)5 like sequence in *E .faecalis* populations thus DNA fingerprinting techniques can be used in genetic studies to elucidate the population structure of the pathogen and generate knowledge to be employed in disease management. DNA fingerprinting by BOX, ERIC and (GTG)5 techniques has an excellent potential as a tool for tracking the evolution and population dynamics of pathogen . The three fingerprinting techniques generated characteristic banding profiles that can be used in clustering and grouping the pathogen isolates. The banding profiles were different with the different techniques, which can be explained by the fact that these markers make use of dispersed repetitive sequences and there exists some differences in the consensus sequences (De Vuyst *et al*., 2008).

This study, based on an extended and diverse selection of *E. faecalis* isolates and field isolates covering all currently described *E. faecalis*, showed that (GTG)5-PCR fingerprinting is a suitable, reliable and fast identification tool for enterococci. All species constitute separate entities. In general, the taxonomic resolution at the strain-level was limited as strains from different

ecological niches and geographic locations revealed analogous (GTG)5-PCR fingerprint patterns.

current results revealed that all the isolates produced bands after amplification by BOXIA-PCR, ERIC-PCR and (GTG)5-PCR which implied the complete typeability of *E. faecalis* isolates using those three molecular typing tools.

These markers were able to distinguish bacterial isolates into different genotype groups showing that the techniques had the requisite discriminative power to differentiate closely related bacterial isolates. Isolates from the different source were found to cluster together showing that despite the habitat separation, the isolates still shared a same genetic similarities that have remained unchanged throughout the evolutionary pathway.

On other side, considering molecular typing methods are ease of interpretation reproducibility type ability, and discriminatory power, so, it will make the interpretation of the overall performance of the method used as well as the interpretation of the resulting data useful in understanding the genetic relatedness of strains for the same species.

In the present study the genetic patterns of repetitive sequences can be reproduced by the rep-PCR method Then, strain relationships can be determined by analyzing their dendrograms. Hence, the knowledge of genetic diversity of bacterial strains associated with a region is important for finding the source of infection in the cases of epidemics and nosocomial infections.

Saeidi *et al*.,(2017) also detected identical patterns within the hospitals, which indicates the spread of bacteria among various sectors. These results were in line with the studies of Djahmi *et al*.,(2012) and colleagues performed in Algeria, where they obtained patters of typing indicated that the infection prevailing in a hospital had the same origin High genetic diversity among

isolates may contribute to the survival of various enterococci strains in hospital. Hospital procedures or equipment might have led to spread of these strains among admitted patients.

It demonstrated that rep-PCR which include (ERIC, BOX and GTG5) has considerably better discriminatory power than restriction analysis of the 16S rRNA gene (Appuhamy *et al*., 1997) the 16S-23S spacer region (Vila *et al*., 1996) multi locus enzyme electrophoresis (Woods *et al*., 1992), biochemical characterizations (Clarridge *et al*., 1995), and ribotyping (Snelling *et al*., 1996).

3.7 Antibiotic resistant of *E. faecalis*:

All the identified *E. faecalis* isolates from urine, fecal, wound, vagina samples were subjected to in *vitro* antibiotic susceptibility test by Kirby - Bauer (disc diffusion method). Selective antibiotics were used to show their effect on *E. faecalis* isolates such as Vancomycin, Teicoplanin, Imipenem, Piperacillin, Ciprofloxacin, Kanamycin, Levofloxacin, Erythromycin, and Clindamycin. The results are shown in Table (3-13) figure(3-16). The results compare according to Clinical Laboratory Standard Institute guidelines (CLSI, 2019).

Highest rate of resistant was seen to almost antibiotics used in present study, 20(100%) isolates were resistant to Clindamycin, Ciprofloxacin and Kanamycin for each, 18(90%) isolates were resistant to Imipenem, 17(85%) isolates were resistant to Erythromycin and Vancomycin for each, 2(10%) isolates were resistant to Levofloxacin, 1(5%) isolates were resistant to Teicoplanin and Piperacillin.

Table (3-13): Percentage of antibiotics resistance by *E. faecalis* against 9 different antibiotics by agar diffusion method according to CLSI, 2019

N	Antibiotics	Resistance of tested isolates (n=20)
1	Vancomycin	17(85%)
2	Teicoplanin	1(5%)
3	Imipenem	18(90%)
4	Piperacillin	1(5%)
5	Kanamycin	20(100%)
6	Levofloxacin	2(10%)
7	Erythromycin	17(85%)
8	Clindamycin	20(100%)
9	Ciprofloxacin	20(100%)

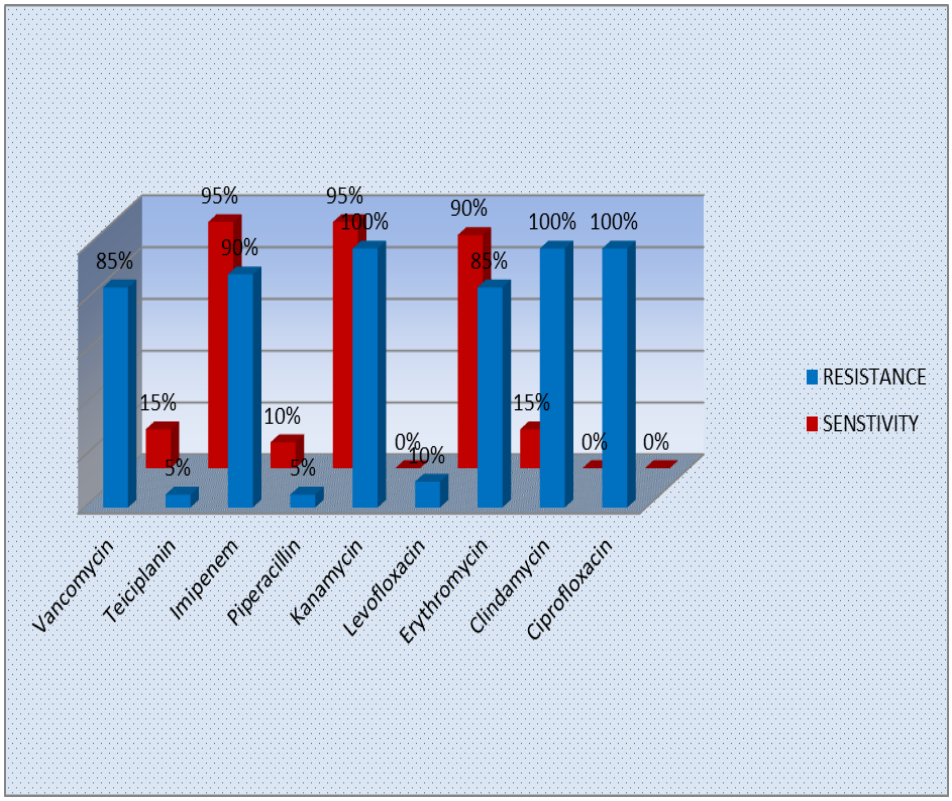


Figure (3-16)percentage of Antibiotic Susceptibility Profile of *E. faecalis* isolates Detected by DDT (n=20)

The present study revealed that 17(85%) of *E. faecalis* was resistant to Vancomycin, that were disagreement with results obtained by Khalid, (2016) who found that, all *E. faecalis* isolated from urine specimen were sensitive to Vancomycin at rate (96%). Seenaa (2020) was found that (6.4%)of isolates were resistance to Vancomycin when take samples from urine, stool, vagina, wound.

Perumal and Venkatesan, (2017) found that isolates of *E. faecalis* isolated from fecal specimens were sensitive to Vancomycin at rate (100%). Hasan *et al*., (2011) revealed that the highest susceptibility of enterococcal isolates from UTI was toward the Vancomycin (90.4%).

This study revealed 1(5%) of *E. faecalis* was resistant to Teicoplanin, Al-Dahmoshi *et al*.,(2019)were shown that *E. faecalis* showed different resistance percentage as erythromycin (85.71%), ciprofloxacin (60.71%) vancomycin (46.43%), and teicoplanin (25%) when take isolates of *E faecalis* from diarrhea . (Heidari *et al.*,2017).

Glycopeptides play an important role in the management of healthcare associated infections caused by β-lactam-resistant Gram positive cocci. Resistance to teicoplanin in CoNS has been reported, and resistance to vancomycin and teicoplanin has emerged in *Enterococcus* spp, Glycopeptide resistance in clinical enterococci is attributable to the acquisition of the gene clusters vanA and vanB, although the intact vanB operon is not induced by teicoplanin (García *et al*.,2019).

The main mechanism of glycopeptide resistance (e.g., vancomycin) in enterococci involves the alteration of the peptidoglycan synthesis pathway, specifically the substitution of D -Alanine -D -Alanine (D -Ala -D -Ala), to either D -Alanine -D -Lactate (D -Ala -D -Lac) or D - Alanine -D -Serine (D -Ala -D -Ser) (Reygaert, 2018). Vancomycin resistance is an acquired resistance

mediated by transposons or plasmids in bacteria, and these can develop in hospital wards that regularly use the drug and initiate serious infections (Giulieri *et al*., 2020). Vancomycin resistant enterococci (VRE) have been reported as a leading cause of outbreaks of hospital acquired infections and in(Intensive Care Units) ICU-hospitalized patients (Said & Abdelmegeed, 2019).

Current study revealed that (85%)of *E. faecalis* isolated from the clinical samples vagina, wound, stool, urine was resistant to Erythromycin.

AL-Khafaji (2021) showed that all *E. faecalis* isolated from stool and urine were sensitive at 100% to Erythromycin. These results were identical with results of Kim *et al*., (2018) mentioned that the resistance rate of *E. faecalis* to erythromycin was (76.9%), Osman *et al*., (2019) who mentioned that the *E. faecalis* strains had partial resistance to erythromycin (78.9%).

Naqid *et al*.,(2020) show antibiotic susceptibility test showed that *E. faecalis* was highly resistant to levofloxacin, erythromycin and clindamycin. These findings were in contrast to the results of a previous study conducted in Iraq, recruiting 151 subjects of both genders, which reported that *E. faecalis* was the second most common infectious agent causing UTIs (23.4%) . These differences could be attributed to the differences in sample size, study design, inclusion, and exclusion criteria. The small number of samples was one of the limit.

Generally, there are three mechanisms involved in the resistance to macrolides ; (a) By modification of the target site through methylation or mutation which prevents the binding of the antibiotic to its ribosomal target, (b) through efflux of the antibiotic, and (c) drug-inactivated macrolides have low levels of activity against Enterobacteriaceae associated with poor

membrane penetration of these antimicrobials, which prohibit their use in the treatment of Enterobacteriaceae (Gomes *et al*.,2017).

Resistance to the macrolides drugs that bind to the 50S ribosomal subunit, drugs *ErmA* gene that encode for erythromycin resistance methylases are acquired via plasmids. These enzymes methylate a site on the ribosome resulting in a conformational change. This decreases the ability of these drugs to bind to the ribosome (Klimienė *et al*., 2015; Castilho, 2018).

Current study revealed that (90%) of *E. faecalis* isolated from the clinical specimensvagina, wound, stool, urine was resistant to Imipenem.

Sharqi *et al*.,(2021) showed that *E. faecalis* isolated from varies clinical specimens (urine, faeces, burns, wound and sputum)was resistance to ciprofloxacin, Imipenem and Piperacillin (6.67%),(13.33%), and (80%) respectively.

Al-Sa'ady, (2019) found fully sensitivity (100%) was reported against imipenem (IPM) for all isolates of *E. faecalis* . This very high sensitivity can be attributed to the fact that carbapenems (IPM) are the effective antibiotic because it is broad-spectrum antibiotic, and it has β-lactam ring that has resistance to hydrolysis by most β-lactamases. On the other hand, low levels of resistance (14.3%) were shown against ciprofloxacin (CIP) and Clindamycin (DA).

However the present study revealed that (100%) of isolates of *E faecalis* from clinical specimens was resistance to Ciprofloxacin.

Bi *et al*., (2018),Tateda *et al*.,(2019) found that the resistance rate of *E. faecalis* to ciprofloxacin was (90%).UTIs due to ciprofloxacin resistant bacterial strains have continued to increase at an worry rate. Furthermore, ciprofloxacin have been inappropriately used to treat *E. faecalis* associated

UTIs. Ciprofloxacin is no longer a recommended therapy for *E. faecalis* from complicated UTI with risk factors(Gagetti *et al*., 2019; Naha *et al*., 2020).

Ciprofloxacin has also been associated with hemolytic anemia . Ciprofloxacin is a second-generation fluoroquinolone that is active against many Gram-negative and Gram-positive bacteria. It acts through inhibition of bacterial DNA gyrase and topoisomerase IV. There has been no cross-resistance reported for CIP and other fluoroquinolones; therefore, it is of high clinical value(Nawaz *et al*.,2021).

The quinolones(Ciprofloxacin , Levofloxacin) target two of the enzymes responsible for this process, DNA gyrase and topoisomerase IV. Both enzymes are tetramers composed of two different subunits: gyrA and gyrB form the DNA gyrase complex, Enterococci demonstrate low levels of intrinsic resistance to the quinolones, but can acquire high-level resistance through several mechanisms. Mutations in the target genes, specifically gyrA, Externalization of the antibiotic through efflux pumps is another mechanism of quinolone resistance(Miller *et al*.,2014).

The present study revealed(100%) of *E .faecalis* was resistance to each of Clindamycin and Kanamycin.

Sattari *et al*.,(2019) found that *E. faecalis* have high frequency of resistance to clindamycin (100%), erythromycin (98.5%) and ciprofloxacin (80.5%) was observed among *E. faecalis* isolates.

Li *et al*.,(2021) showed that *E. faecalis* isolates harboured the highest resistance rate towards erythromycin (100%), and clindamycin (69.6%) antimicrobials. *E. faecalis* isolates showed resistance towards vancomycin (10.7%).

Lincosamide (ex. Clindamycin) inhibit protein synthesis by interacting with the 50S subunit of bacterial ribosomes. *E . faecalis* resistance to Lincosamide

antimicrobial agents can be a result of the acquisition of endogenous mutations or horizontally transmitted resistance genes. The mechanisms commonly fall into three categories: enzymatic inactivation, active efflux, and/or structural changes at the ribosomal target site(Crowe *et al*.,2021).

Enterococci display intrinsic resistance (manifested by the lack of bactericidal activity) to the aminoglycosides(Kanamycin). This phenomenon seems to be mediated by two main factors: poor uptake of the antibiotic requiring higher concentrations to promote entrance into the intracellular space and inactivation by covalent modification of the hydroxyl or amino groups of the aminoglycoside molecule carried out by naturally occurring enterococcal enzymes, creating a steric hindrance and decreasing the binding to the ribosomal target(Miller *et al*.,2014).

On the other hand , the current result was showed that(10%) of *E. faecalis* isolates were resistant to Levofloxacin.

Boccella *et al*.,(2021) Find when take specimens from urine cultures wound swabs, vaginal swabs , blood cultures, catheters and others find *Enterococcus faecalis* showed a high resistance to imipenem (1.7%), teicoplanin (98.5%), and vancomycin (98.2%). Moreover, *E. faecalis* exhibited a lower rate of sensitivity to levofloxacin (65.1%).

Tollu and & Ekin,(2021) found all the examined *E. faecalis* types were found to be sensitive to imipenem, and vancomycin, while 42.5% were resistant to erythromycin, 17.5% to ciprofloxacin, and 95% to clindamycin.

The present study revealed(5%) of *E .faecalis* was resistance to Piperacillin. Carbapenem (Piperacillin) resistance may also be mediated by the loss or alteration of porin channels, the expression of efflux pumps, or penicillin-binding protein (PBP) modification (Kotov *et al* .,2021).

Many studies reported that more than (70%) of *E. faecalis* are resistant to macrolides, fluoroquinolones, and aminoglycosides. This high level of resistance not only hinders successful therapy but also allows the microorganisms to persist in the hospital, expanding its reservoir (Bortolaia *et al*., 2018).

E. faecalis has gradually become a leading cause of health care-associated infections because it has developed resistance to multiple clinically used antibiotics, such as macrolides, aminoglycosides, and glycopeptides, including vancomycin, which was previously used as the antibiotic of last resort for enterococcal infections. The emergence of vancomycin resistance has made the treatment of infections with *E faecalis* a major challenge for clinicians, because it means that few or no treatment options are available(Bernardi *et al* 2021).

Carbapenem (Piperacillin) resistance may also be mediated by the loss or alteration of porin channels, the expression of efflux pumps, or penicillin-binding protein (PBP) modification (Kotov *et al* .,2021).

Enterococci exhibit a variety of mechanisms of intrinsic and acquired resistance to the major classes of antibiotics of clinical use, as well as efficient genetic exchange mechanisms that facilitate the dissemination of antibiotic resistance genes (Rehman *et al*., 2017) .

Conclusions and Recommendations

Conclusions:

The study has reached at the following conclusions:

1. Detection of *E. faecalis* using D-alanine D-alanine ligase gene more specific than biochemical test.
2. *E. faecalis* showed different behavior of resistance against to one or more of antibiotic.
3. The presence of some virulence factors genes in *E. faecalis* as, *srt*, *cps1*and *cps5* at high percentages which increase the pathogenicity of these pathogens while The *fsr*, *cps2* and *Atl* showed the lowest prevalence among *E faecalis* isolates.
4. The capsule locus polymorphism showed that *cps1* was the most common type followed by *cps5* and the low prevalent *cps2*.
5. The indication of genetic diversity among clinical isolates which was constitutes with high level of polymorphism could be certified with study of genotyping method (ERIC , BOX and(GTG)5)that gave a significant assumption for evolutionary ancestral gene.
6. The polytrinucleotide(GTG)5 is considered the suitable method for molecular typing of *E. faecalis*

Recommendations:

Depending on the finding of this study the recommended include:-

1. Direct and rapid identification of *E. faecalis* in clinical samples through using molecular technique which minimize the mixed growth.
2. Using Real-time PCR to detect pathogen as the main causative agent depending on the copy number.
3. Detection of mutation rate in *E. faecalis* through using DNA microarray.
4. Re- evaluation of antibiotics currently used to treat *E. faecalis*, especially after the bacteria have developed resistance to most of them.
5. Further global investigations covering more isolates and methods like whole genome sequencing would advisable.

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