Republic of Iraq
Ministry of Higher Education and Scientific Research
University of Babylon
College of Medicine

Molecular characterization of CRISPR-cas system and Multilocus sequence typing of *Enterococcus faecalis* isolates 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 of Science in Medical Microbiology

By

Shymaa Abdul Jabbar Saeed Alhussainy

BSc. Microbiology / Collage of Science
Baghdad University (2008-2009)

Supervised
by

Dr. Lamees Abdul-Razzaq Abdul-Lateef

2023 A.D **1445 H.D**

بِسْمِ اللهِ الرَّحْمنِ الرَّحِيمِ

((يَرْفَعِ اللَّهُ الَّذِينَ آمَنُوا مِنكُمْ وَالَّذِينَ أُوتُوا الْعِلْمَ دَرَجَاتٍ))

صدقَ اللهُ العليُّ العظيم

المجادلة : 11

Supervision Certification

I certify that this thesis, entitled "**Genotyping diversity and CRISPR-Cas genes of *Enterococcus faecalis* isolates in Babylon province**" has been prepared under my supervision by "**Shymaa Abdul Jabbar**" at that college of medicine, university of Babylon, as a partial requirement for the degree of Master of Science in Medical Microbiology.

Professor

Dr. Lamees Abdul-Razzaq Abdul-Lateef

Babylon University

College of Medicine

/ / 2023

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

Professor

Dr. Hayam K. Al-Masuodi

Head of department of microbiology

Babylon University

College of Medicine

/ / 2023

Dedication

Even though my father and martyr brother don't exist alive anymore , their spirits are still inspire me and guide me. I will always be grateful for the time we spent together. Having them in my life was a blessing, brought me love, joy and sweet memories that I will appreciate forever.

To the woman who raised me and overwhelmed me with her kindness, my mother the precious and noble.

To my sisters, my brother, my nephews and my nieces for their continuing efforts to support and encourage me finishing my study.

To my husband and my children. Your love and support means the world to me. I am grateful for all the love and happiness you bring into my life.

Special thanks to my friends who were always there for me, who answered my phone calls at the day or even to a late time at night, who helped without expecting any personal gains and especially Khalood Abdul Mageed

Shymaa 2023

Acknowledgments

At first of all , my great thanks to ***Allah*** *for enabling me to finish what I started and for helping me to present this work . The prayers and peace upon our prophet the master of the creatures* ***Mohammed*** *, and his genial modest family and his companions .*

Special thanks with the respect and appreciation to my supervisor Prof. ***Dr. Lamees Abdul-Razzaq Abdul-Lateef*** *for her continuous support , valuable advices and for providing all require facilitates to perform this work .*

A word of thanks to the Ministry of Higher Education and Scientific Research in Iraq , also special thanks to the Dean of our college of medicine mentioning Prof. ***Dr. Mohend Abbass Al-shalah*** *and the whole teaching staff of the Department of Microbiology especially Prof. Dr.* ***Hayam K. Al-Masoudi*** *for their precious help, My sincere thanks to all members in collage of medical , University of Babylon for their help and providing most of the requirement for this project .*

With my great appreciate and love.

Shymaa 2023

Summary:

A total of 105 clinical samples were collected during this study which obtained from patients suffering from different infection such as UTI, vaginitis, wound infection, bacteremia who admitted to two main hospitals of AL-Hilla City: Al-Hilla Surgical Teaching Hospital and Imam Sadiq Education Teaching Hospital during a period extending from (August 2022 to November 2022).

All specimens were subjected to aerobic culturing on different media and it was found that out of the total 105 specimens, 70 (66.6%) specimens showed positive bacterial culture. No growth was seen in other 35 (33.3%) specimens. Among (70) positive culture were culturing on chromogenic agar medium as (selective media), only 15 (14.2%)positive samples were identified as *E. faecalis.*

These isolates then subjected to molecular detection method using specific primer based on *D-alanine D-alanine ligase* gene *ddl* as a genetic marker to confirm the identification of *E. faecalis* by PCR, the results revealed that 15(100%) were positive for *ddl* .

All the identified *E. faecalis* isolated from different sources are subjected in vitro to antibiotic susceptibility test by modified Kirby-Bauer disc diffusion method. It has been found that *E. faecalis* isolates are 15 (100%) isolates were resistant to Clindamycin, Ciprofloxacin and Kanamycin for each, 14(93.3%) isolates were resistant to Imipenem, 13 (86.6%) isolates were resistant to Refampin and Nitrofurantoin,12 (80%) isolates were resistant to Erythromycin ,11 (73.3%) isolates were resistant to Vancomycin, 2(13.3%) isolates were resistant to Levofloxacin, and 1(6.6%) isolates were resistant to Teicoplanin and Piperacillin.

The phylogenetic diversity between 15 *E. faecalis* isolates from different clinical specimens using RAPD-PCR was performed . Molecular typing of *E.*

faecalis using M13 primer showed polymorphism among the isolates generated 7-14 bands, ranging between 95 to 3000 bp.

RAPD-PCR analysis yeiled 10 distinct genotype among 15 investigated isolates .The cladogram of genetic distance among all the isolated based on fragment polymorphism generated by RAPD – PCR after using the primer M13 by using UPGMA. The cladogram divided all isolates (15) in to two clusters . First cluster (A) conteined only one isolate (EF17) and the second cluster (B) contained 14 isolates, divided the 14 isolates in to two sub clusters, the first sub cluster divided in to two branches, the first branch divided more to two sub branches . one contained two isolates (EF 12 & EF 13). On the other hand the second contained eight isolates (EF6,EF7,EF8,EF9,EF10,EF11,EF14,&EF15).The second branch contained only one isolate (EF16) .Moreover ,the second sub cluster contained three isolates (EF18,EF19,EF20).Cluster (B) showed close genetic linkage &the same genetic distance between some isolates therefore displaying clonal dissemination. Their close genetic relationship based on the dissimilarity coefficient indicated a low genetic variability between them.

In this study, multilocus sequence typing method (MLST) were used to investigate the discriminatory ability, reproducibility, and the genetic relationship between 15 *E. faecalis* isolates. The sequence data obtained for MLST for determining the population structures analyzing the extent of linkage disequilibrium between alleles for phylogenetic relationships between 15 isolates, depended on the five housekeeping genes frequently used for MLST analysis of *E. faecalis* (*pepC, recA, clpX , rpoB, groEL*).

The mean GC content of sequnces of five gene fragments ranged from 35%(*pepC*) to 43%(*recA*):Trimmed fragment size of the five selected loci ranged from 479 bp (*groEL*)to 619 bp (*clpX*).The nucleotide diversity ranging from 0.00048

to 0.00999 pergene . Moreover, the number of polymorphic site per locus ranged from 2 (*recA*) to 22(*rpoB*) and harbored a total of 45 SNP.

the d_N/d_S ratios which indicated negative selection were determined to be less than 1 for the *pepC & recA* genes while were determined to be more than 1 for the *clpX, rpoB* &*groEL* genes which indication positive selection .

According to allelic profile it was found that the presence of allelic variant (SNP, insertion, or deletion) between isolates. In the case *rpoB* was more variant or mutant than other 4 housekeeping genes, contrary to the *recA* which was the least variant. The gene chosen for the present MLST scheme seem to be representatives of the general polymorphism seen in housekeeping genes of *E. feacalis*.

Fifteen sequence types (STs) were identified through MLST analysis of the *E. feacalis* isolates. The phylogeny of these ST, an MLST phylogenetic tree of all the *E. feacalis* strains was inferred maximum like hood approach from concatenated sequence. All *E. feacalis* isolates showed polyphyletic lineage and revealed three distinct clusters, cluster A contain one isolate (13EF), cluster B contain one isolate (14EF) and cluster C contain 13 isolates, and this cluster was divided into subclusters.

The split graphs for *clpX , groEL & rpoE* revealed network like with parallelogram structures, while the split graphs of *pepC &recA* are tree like structures and the split decomposition analysis of combined five MLST Loci display network like structure with rays of different length .

MLST of 15 isolates from different clinical specimens revealed 15 different sequence typed that grouped into two major clonal complex (cc1 & cc2) by use of eBURST. Among the 2 cc, cc1 was the largest and comprised 8 link ST, namely ST1, ST3, ST2, ST5, ST7, ST11, ST13 & ST6.

Summary

In this study, it was determined the occurrence of CRISPR loci in *E. faecalis* isolated from different infectious source (urine ,vagina &wound) . The distribution & the frequency of three different CRISPR- cas elements in *Enterococcus faecalis* isolates. Over all, CRISPR2 was identified in 8(53.3%) of the isolates followed by CRISPR3- cas and CRISPR1 – cas (33.3 & 20% respectively).

The presence of CRISPR loci among the UTI *E. faecalis* (8) isolates was higher than for vagina (6) isolates & wound (2) isolates which were multidrug resistance isolates. The reason of higher presence of CRISPR loci among UTI isolates is not clear but could confer antibiotic resistance to them.

The correlation between the presence of CRISPR – cas & phenotypic antibiotic susceptibility was also studied . The CRISPR – cas positive *E. faecalis* showed lower resistance rates against almost antibiotic in comparision with CRISPR – cas negative *E. faecalis* isolates. Overall, CRISPR2 was predominant in almost antibiotic susceptible isolates . The presence of CRISPR – cas is associated with the absence of some antibiotic resistance acquired by horizontal gene transfer.

List of contents

Item No.	subject	Page No.
	Summary	**I-IV**
	Lists of contents	**V-IX**
	Lists of tables	**IX-X**
	Lists of Figures	**X-XI**
	Lists of abbreviation	**XII-XV**
	Lists of appendices	**XV**
Chapter One: Introduction and Literatures Review		
1.1	**Introduction**	**1-4**
	The Aims of the study	**4**
1.2	**Literature Review**	**5**
1.2.1	**Charactreistics of *Enterococcus faecalis***	**5-8**
1.2.2	**Pathogenesis of *Enterococcus faecalis***	**8-11**
1.2.3	**Clinical factures of *Enterococcus faecalis***	**11**
1.2.3.1	**Urinary Tract Infections (UTIs)**	**12-14**
1.2.3.2	**Bacteremia**	**15-16**
1.2.3.3	**Wound Infections**	**16-18**
1.2.3.4	**Vagina Infections**	**18-19**
1.2.4	**Antibiotics profile in *Enterococcus faecalis***	**19-25**
1.2.5	**Randomly Amplified Polymorphic DNA Polymerase Chain Reaction (RAPD-PCR)**	**25-28**

1.2.6	**Multilocus sequence typing (MLST)**	**28-32**
1.2.7	**Clustered regularly interspaced short palindromic repeats (CRISPRs)**	**33-35**
Chapter Two: Material and Methods		
2.1	**Materials**	**36**
2.1.1	**Laboratory instruments and equipment**	**36-37**
2.1.2	**Chemical and Biological Materials**	**38**
2.1.3	**Culture Media**	**39**
2.1.4	**Antibiotics Disks**	**39**
2.1.5	**Commercial Kite**	**40-41**
2.2	**Methods**	**41**
2.2.1	**Preparation of Reagents and solutions**	**41**
2.2.1.1	**Reagents**	**41**
2.2.1.1.1	**Oxidase Reagent**	**41**
2.2.1.1.2	**Catalase Reagent**	**42**
2.2.1.2	**Solutions**	**42**
2.2.1.2.1	**Normal Saline Solution**	**42**
2.2.1.2.2	**Agarose Gel**	**42**
2.2.1.2.3	**Sugar Solutions**	**42**
2.2.2	**Preparation of Culture Media**	**42-45**
2.3	**Subjects of the Study**	**45-46**
2.3.1	**Ethical approval**	**47**
2.3.2	**Isolation and identification of *Enterococcus faecalis***	**47**
2.3.2.1	**Clinical specimens**	**47**

2.3.2.1.1	**Urine samples**	**47**
2.3.2.1.2	**Vaginal swabs**	**47-48**
2.3.2.1.3	**Blood samples**	**48**
2.3.2.1.4	**Wound swabs**	**48**
2.4	**Laboratory Diagnosis of *Enterococcus faecalis***	**48**
2.4.1	**Colonial Morphology and Microscopic Examination**	**48**
2.4.2	**Biochemical Tests**	**49**
2.4.2.1	**Catalase Test**	**49**
2.4.2.2	**Oxidase Test**	**49**
2.4.2.3	**Bile-Esculine Hydrolysis Test**	**49**
2.4.2.4	**NaCl Tolerance Test**	**49**
2.4.2.5	**Motility Test**	**50**
2.4.2.6	**Sugar Fermentation**	**50**
2.4.2.7	**Growth at 10ºC and 45ºC**	**50**
2.4.2.8	**Growth at alkaline pH (9.6)**	**50**
2.5	**Hemolysin production**	**51**
2.6	**Antimicrobial Susceptibility Test: Disk Diffusion Test (DDT)**	**51**
2.7	**Genotyping Assays**	**52**
2.7.1	**DNA Extraction**	**52-53**
2.7.2	**Estimation of DNA Concentration**	**53**
2.7.3	**The mixture of PCR reaction**	**54**
2.7.4	**Primer Sequences**	**54**
2.7.5	**Agarose gel documentation**	**54-55**

2.8	**Detection of Amplified Products by Agarose Gel Electrophoresis**	**55-56**
2.9	**Gel Analysis**	**56**
2.10	**Multi locus sequence typing *(*MLST)**	**56**
2.10.1	**PCR amplification**	**56-57**
2.10.2	**Sequencing of PCR products**	**57**
2.10.3	**Bioinformatic analysis**	**58**
2.11	**CRISPR identification**	**58-59**
Chapter Three: Results and Discussion		
3	**Results and discussion**	**60**
3.1	**Isolation of *E. faecalis***	**60-61**
3.2	**Distribution of *E. faecalis* isolated from different clinical specimens**	**61-64**
3.3	**Identification of *E. faecalis***	**65-66**
3.4	**Confirmed detection of *E. faecalis* by D-alanine D-alanine ligase gene by PCR technique**	**66-68**
3.5	**Antibiotic resistant of *E. faecalis***	**68-76**
3.6	**RAPD-PCR genotyping of *Enterococcus faecalis* isolates**	**77-85**
3.7	**Multilocus sequence typing of *Enterococcus faecalis***	**85-101**
3.8	**Clustered regularly interspaced short palindromic repeats (CRISPRs)**	**101-108**
	Conclusion	**110**
	Recommendation	**111**
	References	**112-169**

	Appendices	**170-172**

List of Table

Table No.	Title	Page No.
Chapter two		
2.1	Laboratory instruments and equipment	36-37
2.2	Chemicals and Biological materials used in the laboratory work	38
2.3	Culture Media	39
2.4	Antibiotics Disks (Bioanalyze / Turkey)	39
2.5	Commercial kits used in the present study	40
2.6	DNA extraction kit (Geniad/ UK).	40
2.7	DNA ladder	41
2.8	Master Mix Used in PCR (Promega/USA).	41
2.9	Contents of the Reaction Mixture	54
2.10	Primers sequences and PCR condition of *ddl* gene for identification of *E. faecalis*	55
2.11	The primers used for the amplification of a fragment gene	55
2.12	Genes and sequences used	57
2.13	CRISPR identification primers	59
Chapter three		
3.1	*E. faecalis* isolated from different sites of infection	61

3.2	**Diagnostic Features of *E. faecalis***	**66**
3.3	**Similarity Matrix of 15 studied EF isolated according to RAPD assay. Similarity differences were illustrated above by color of conservation. (6,7,8,9,10 wound, 11,12,13,14 vagina, 15,16,17,18,19,20 urine)**	**81**
3.4	**Nucleotide and allelic diversity of the 5 housekeeping genes evaluated**	**87**
3.5	**Allelic profiles based on 5 housekeeping genes from all isolates of *Enterococcus faecalis* evaluated**	**89**
3.6	**Identification of Crisper I, Crisper II and Crisper III of *E. faecalis* in all studied samples**	**101**
3.7	**Identification of Crisper 1cas, Crisper 2 and Crisper 3 cas genes of *E. faecalis* in patients with different sample sources.**	**104**
3.8	**Association between Antibiotic sensitivity and the occurrence of CRISPR-I, II, III in *E. faecalis***	**106**

List of Figure

Item No.	Title	Page No.
2.1	**Experimental design**	**46**
3.1	**prevalence of *E. faecalis* among other etiological agents associated with isolated sample**	**61**
3.2	**Agarose gel electrophoresis image at 75 volts for one houre that displayed PCR results analysis of D-alanine D- alanine ligase gene in E. faecalis isolated from clinical samples.**	**67**

3.3	**Levels (%) of antibiotic resistance demonstrated by *Enterococcus faecalis* isolates.**	**69**
3.4	**The RAPD-PCR gel products representing the relationship among 15 of *Enterococcus faecalis* in patients with different sample sources.**	**78**
3.5	**The RAPD-PCR-derived cladogram showing the relationships among 15 different strains of *Enterococcus faecalis* in patients with various sample sources The distance values are shown in the bar (above). Unweighted pair group method with arithmetic mean was used to create this cladogram (UPGMA).**	**79**
3.6	**agarose gel electrophoresis for housekeeping genes for 15 *E. faecalis* isolates.**	**87**
3.7	**Circular phylogenetic analysis based on concatenated sequences of 5 housekeeping genes from 15 *Enterococcus faecalis* isolates by maximum likelihood method, and comparison with the concatenated Reference sequences**	**91**
3.8	**Split-decomposition analysis based on concatenated sequences of 5 housekeeping genes from 15 *Enterococcus faecalis* isolates and comparison with the concatenated Reference sequences (NC_004668) .**	**92**
3.9	**Split-decomposition analysis based on *cplx* , *groEL, pepC, RecA, rpoB* gene sequences of 5 housekeeping genes from 15 *Enterococcus faecalis* isolates.**	**93**
3.10	**Comparative eBURST analysis showing the clonal assignment and the relative distances of the identified STs**	**95**
3.11	**Identification of CRISPR1 – cas genes of *E. faecalis* in patients with different sample sources.**	**102**
3.12	**Identification of CRISPR2 – cas genes of *E. faecalis* in patients with different sample sources.**	**102**
3.13	**Identification of CRISPR3 – cas genes of *E. faecalis* in patients with different sample sources.**	**103**

List of Abbreviations

Abbreviation	Complete term
°C	**Celsius degree**
AADC	**Aromatic amino acid decarboxylase**
ABU	**Asymptomatic bacteriuria**
Ace	**Adhesion to collagen from *E. faecalis***
Ace gene	**Angiotensin – converting enzyme**
AV	**Aerobic vaginitis**
BHI	**Brain heart infusion**
BSI	**Bloodstream infections**
CAUTI	**Catheter associated urinary tract infection**
CCs	**Clonal complexes**
CDC	**Centers for Disease Control**
CIP	**Ciprofloxacin**
ClpX	**ATP- dependent protease subunit**
CLSI	**Clinical Laboratory Standard Institute guidelines**
CRISPRs	**Clustered regularly interspaced short palindromic repeats**
crRNA	**CRISPR RNAs**
CSF	**Cerebrospinal Fluid**
DA	**Clindamycin**
ddl	**D-Alanine D-Alanine Ligase**
DNA	**Deoxyribonucleic acid**
DSBs	**Double –stranded DNA breaks**

DW	**Distilled water**
DRC	**Dentil –root canal**
E	**erythromycin**
E. faecalis	***Enterococcus faecalis***
Ebp pili	**Endocarditis and biofilm-associated pili**
EDTA	**Ethylene diamine tetra acetic acid**
erm gene	**Erythromycin resistance methylase gene**
ESP	**Enterococcal surface protein**
FDA	**Food and Drug Adminstration**
GI	**Gastroenteritis**
Gm	**Gram**
groEL	**Chaperonin GroEL**
GTF	**Glycosyl transferases**
HA	**Hyaluronic acid**
HAI	**Hospital-acquired infections**
HGT	**Horizontal gene transfer**
ICU	**Intensive Care Units**
IgA	**Immunoglobulin A**
IPM	**imipenem**
K	**Kanamycin**
L -dopa	**Levodopa**
LEV	**Levofloxacin**
LPS	**Lipopolysaccharide**
LTC	**Long term care**

MDR	**Multidrug resistant**
mef gene	**Macrolide Efflux Genes**
MGE	**Mobile genetic elements**
MHA	**Muller Hinton Agar**
Ml	**Milliliter**
MLST	**Multilocus sequence typing**
msr gene	**Macrolide Resistance genes**
NGG	**Next Generation Genotyping**
Nitro	**Nitrofurantoin**
NNRTA	**Non – nucleoside reverse transcriptase**
PAM	**Protospacer adjacent motif**
pb	**Base pair**
PBP	**Penicillin Binding Protein**
PCR	**Polymerase Chain Reaction**
pepC	**Aminopeptidase C**
PFGE	**Pulse Field Gel Electrophoresis**
PMNs	**Polymorphnuclear leuckocyte**
PRL	**Piperacillin**
RAPD	**Random amplified polymorphism**
RecA	**Recombinase protein A**
REF	**Rifampin**
RFLP	**Restriction Fragment Length polymorphism**
rpoB	**RNA polymerase b subunit**
SNP	**Single nucleotide polymorphism**

ST	**Sequence type**
TBE	**Tris- Borate EDTA buffer**
TEC	**Teicoplanin**
UPGMA	**Unweighted pair group method with arithmetic**
UTIs	**Urinary tract infections**
VAN	**Vancomycin**
VRE	**Vancomycin resistant enterococci**
WHO	**World health organization**
µl	**Microliter**

List of appendices

Appendex	contents
Appendex 1	Phylogram analysis based on (*pepC, rpoB, groEL, recA, clpX)* sequences from 15 *Enterococcus faecalis* isolates by maximum likelihood method.

Chapter One

Introduction and Literature Review

1.1 Introduction:

Enterococci are Gram-positive facultative anaerobic cocci in short and medium chains, first discovered in 1899 in the human gastrointestinal tract. They were recognized as a separate genus from streptococci by DNA hybridization and 16S rRNA sequencing in 1984 (Fiore *et al*.,2019). They are the first of the ESKAPE organisms (*Enterococci* spp., *Staphylococcus aureus*, *Klebsiella pneumoniae*, *Acinetobacter baumannii*, *Pseudomonas aeruginosa*, *Enterobacter* spp.) highlighted by the WHO as rising causes of nosocomial and antibiotic-resistant infections in the last few decades threatening public health (Zhen *et al*.,2019).

There are about 58 species recognized of enterococci so far, the most important and common being *E. faecalis* and *E .faecium* (García-Solache *et al*.,2019). Enterococcal colonization of the GI tract is the main predisposing factor for severe infections, which occur through gut translocation. Enterococci are phagocytosed and transported across the intestinal wall and resist killing by the lymph system (Fernández *et al*.,2019).

E. faecalis strains cause serious infections, including gastrointestinal and urinary tract infections, meningitis, bacteraemia, and periodontitis (Abat *et al*., 2016; Said *et al*., 2021). The severity and pathogenicity of diseases caused by these bacteria are higher in the presence of well-defined virulence factors and toxins (Goh *et al*., 2017;Wu *et al*., 2020).

Infections caused by *E. faecalis* are mainly hard to treat by common antimicrobials (Shiadeh *et al*., 2019). Therefore, the assessment of antimicrobial resistance of *E. faecalis* strains can directly introduce the most suitable antimicrobial agents for further therapeutic options (Perera *et al*., 2020).The ability of enterococci to resist the action of many antibiotics used played an important role in increasing the rate of prolonged enterococcal infections (Hollenbeck *et al*.,2012).

Enterococcus species is known for both intrinsic and acquired resistance to many antimicrobials. The most common mechanism for intrinsic resistance is due to the presence of many resistance genes against the various antimicrobials. The acquired resistance of enterococci is due to DNA mutation or acquiring of other new genes through different methods of gene transfer. This leads to the development of resistance against many antibiotics like vancomycin, tetracycline, macrolides, fluoroquinolones, and others (Esmail *et al*.,2019). Multidrug resistant isolates are those isolates which are resistant to three or more different classes of antimicrobial agents (Falagas *et al*.,2008).

The randomly amplified polymorphic DNA (RAPD) polymerase chain reaction (PCR) to detect the amount of genetic diversity among the *Enterococcus* group, pulse field gel electrophoresis for comparative profiling analysis and 16S rDNA ribotyping to identify the strains to species level (Healy, *et al.*, 2005).The applied RAPD-PCR method was also reported to be sensitive and practical for the molecular typing of clinical isolates of *E. faecalis* (Banerjee, 2013; Emaneini *et al*., 2016).

The multiple locus sequence typing (MLST) have been globally recognized as highly discriminative standard strategies in modern epidemiological studies (Ghaderi *et al.*, 2015). MLST, which is a typing method based on the sequencing of housekeeping genes and characterizes isolates on the basis of variation in nucleotide sequences of each locus of the selected genes. The different sequence at each locus are assigned with specific allele numbers and each unique combination of alleles, often called as allelic profile is assigned a sequence type (ST), which is the unambiguous descriptor of the strain (Sharma *et al.*, 2016).

Five gene multi-locus sequence typing (MLST) is a widely used classification system for categorizing bacteria. It can be used to quickly rule an isolate in or out of an outbreak and knowing a sequence type (ST) can often allow for general characteristics of a bacteria to be inferred (Gardy and Loman 2018).

Multilocus sequence typing (MLST) is a commonly used method of assessing subspecies phylogenetic relationships. In one *E. faecalis* MLST employing five housekeeping gens: *pepC* (Aminopeptidase C), *rpoB* (RNA polymerase β subunit), *groEL* (Chaperonin GroEL) ,*recA* (Recombinase protein A) and *clpX* (ATP-dependent protease subunit) are amplified by PCR, sequenced, and compared to a global database such that an allele variant number can be assigned to each gene(Ruiz-Garbajosa *et al*.,2006). Based on the combination of allele numbers, a sequence type (ST) is assigned to a strain. Analysis of whole genome sequences has also been used to assess phylogenetic relationships among *E. faecalis* strains (Bourgogne *et al*.,2008 ; Palmer *et al*.,2010).

The Clustered Regularly Interspaced Short Palindromic Repeats (CRISPR)-associated (Cas) system is an acquired immune defense system with immune memory in prokaryotic genomes, which can effectively resist the acquisition of foreign mobile gene elements such as plasmids or phages to maintain the stability of bacterial genomes (Xu and Li , 2020; Haider *et al*.,2022). CRISPR-Cas system is one of the factors limiting the development and evolution of bacterial antibiotic resistance (Gholizadeh *et al*.,2020). CRISPR-Cas is considered as a natural barrier to horizontal gene transfer and transmission of antibiotic resistance (Wheatley *et al*.,2021). The CRISPR-Cas system consists of a CRISPR array and the Cas gene family, in which the CRISPR array is composed of highly conserved repeat sequences and spacer sequences, with upstream leader sequences responsible for their transcription (Guo *et al*.,2022).

CRISPR-Cas systems constitute a multi-step adaptive immune response that defends prokaryotes against foreign invading genetic material. CRISPR-Cas systems consist of 2 classes and six types (types I, III, and IV belong to class 1 and types II, V and VI belong to class 2) (Shabbir *et al*.,2018;Kamruzzaman & Iredell .,2020).

Aim of study: -

This study aimed to detection of CRISPR-cas system and Multilocus sequence typing of *Enterococcus faecalis* isolated from different clinical samples

Objectives

1. Isolation and detection of *E. faecalis* from different clinical samples (urine, vagina swab, blood, wound swab)
2. Molecular diagnosis of *E. faecalis* by using specific primer
3. Study antibiotic susceptibility test on bacteria
4. Study of genotyping in *E. faecalis* isolated from different clinical samples by using MLST and RAPD-PCR
5. Detection of CRISPR-cas system of *E. faecalis* by PCR technique

1.2 Literature Review

1.2.1 characteristics of *Enterococcus faecalis*:

Enterococcus faecalis – formerly classified as part of the group D *Streptococcus* system – is a Gram-positive, commensal bacterium inhabiting the gastrointestinal tracts of humans (de Almeida *et al*.,2018). Like other species in the genus Enterococcus, *E. faecalis* is found in healthy humans and can be used as a probiotic (Panthee *et al* .,2021).

Enterococcus is a commensal bacterium of the gastrointestinal tract, but it can also become an opportunistic pathogen. It may colonize the female genital tract and vaginal colonization increases following antibiotic treatment or in patients with aerobic vaginitis. *E. faecalis* is associated with a wide spectrum of infections, particularly in immunocompromised states and when there is change in the host microbiota. There is increasing evidence which links enterococci with bacterial vaginosis and aerobic vaginitis (Alhajjar *et al*.,2020).

Enterococcus faecalis is an opportunistic pathogen that can cause urinary tract infections (UTI), catheter associated urinary tract infections (CAUTI), wound infection, tissue infection, sepsis, meningitis, life-threatening bacteraemia and endocarditis (Vu and Carvalho 2011; Gawryszewska *et al*. 2016; Ch'ng *et al*. 2019). Infections by this opportunistic pathogen can be difficult to treat due to their propensity to form biofilms, as well as their frequent and multiple antibiotic resistances (Miller *et al*. 2016).

The enterococcal species most frequently isolated from human clinical specimens is *Enterococcus faecalis* followed by *Enterococcus faecium* (Weng *et al*., 2013). Up to 90% of nosocomial enterococcal infections of humans are due to *E. faecalis* (Kuch *et al*., 2012).

Enterococci were formerly classified as group D streptococci (Lancefield,1933) but were given genus status in 1984 (Schleifer & Kilpper-Balz ,1984) based on nucleic acid hybridization studies that showed a more distant relationship to the streptococci. The genus *Enterococcus* contains over 50 species, and appears to have branched from its last common ancestor approximately 425 million years ago (Lebreton *et al*.,2017). Members of this genus are endowed with intrinsic properties that confer the ability to survive host defenses and compete in the intestinal tract, and then persist and spread in the natural environment or hospital, leading to colonization of new hosts. Among these traits are the ability to grow over wide-temperature and pH ranges, survive desiccation, and grow in the presence of 6.5% NaCl and 40% bile salts (Lebreton *et al*.,2014).

Enterococcus species are non-spore-forming facultative anaerobes tolerant to a wide range of environmental conditions (Bondi *et al.*,2020). *Enterococcus faecalis* is an aerotolerant, Gram-positive bacteria that is distributed widely in the natural environment, and in the gastrointestinal tracts of humans, animals, and insects.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).

Samples were culture on different media such as MacConkey agar the 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 slightly convex smooth edges, white or creamy color and convert media color into black (Al-Halaby *et al*., 2017).

Enterococcus can express alpha, gamma, or beta hemolysis on blood agar. Haemolysin producing strains of enterococci have been shown to be virulent in a human infections, and to be associated with increased severity of infection.

Trypticase soy agar or Columbia agar with 5% (v/v) defibrinated sheep blood may be used to assess the haemolysis produced by enterococci. If human or horse blood is used, haemolysis is based on cytolysin activity and may cause haemolytic reaction, 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, colonies appear blue –green color (Atlas and Snyder, 2015). 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).

E. faecalis is a nonmotile microbe; it ferments glucose without gas production, and does not produce a catalase reaction with hydrogen peroxide. It produces a reduction of litmus milk, but does not liquefy gelatin. 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, agmatine, and many keto acids. Enterococci survive very harsh environments, including extremely alkaline pH (9.6) and salt concentrations. They resist bile salts, detergents, heavy metals, ethanol, azide, and desiccation. They can grow in the range of 10 to 45 °C and survive at temperatures of 60 °C for 30 min. (Stuart *et al*.,2006)

However *E. faecalis* contains a tyrosine decarboxylase enzyme capable of decarboxylating L-dopa, acrucial drug in the treatment of Parkinson's disease. The primary medication used to treat Parkinson’s disease is levodopa (L-dopa). To be effective, L-dopa must enter the brain and be converted to the neurotransmitter dopamine by the human enzyme aromatic amino acid decarboxylase (AADC). If L-dopa is decarboxylated in the gutmicrobiome, it cannot pass through the blood-brain

barrier and be decarboxylated in the brain to become dopamine (Maini Rekdal *et al*.,2019).

Enterococci have a high capacity to acquire and transfer genetic elements that confer resistance to antimicrobial agents (Hegstad *et al*., 2010). As in human medicine, the use of antimicrobial drugs in veterinary medicine creates a selective pressure for the emergence of multidrug-resistant bacterial strains (Kuch *et al*., 2012; Ste¸pien´ -Pys´niak *et al*., 2016; Nowakiewicz *et al*., 2017). An important factor in the spread of multi-resistant bacteria is microbial contamination of the environment by manure from intensive livestock production (Radhouani *et al*., 2014).

1.2.2 Pathogenesis of *Enterococcus faecalis*:

The pathogenicity of *Enterococcus faecalis* requires the general colonization of mucosal surfaces with these organisms (Peters *et al*., 2012). *Enterococcus* employs a variety of microbial mechanisms for the colonization. In addition, many factors are influence colonization of one species over another in the intestinal tract. However, it is known that pathogenicity of this bacterium involves a number of steps (Stecher *et al*., 2011):

a. Adherence factors.

b. Evasion of host immune system defenses.

c. Secretion of cytolysins and other toxins that breach cellular membranes.

The most notable enterococcal human pathogens are *Enterococcus faecalis* and *Enterococcus faecium*. When pathogenic enterococci gain access to niches in the human body beyond their commensal habitat of the GI tract, such as the urinary tract, skin (via wounds/burns), bloodstream and the heart, they have a high potential to cause persistent infections and subsequent serious disease (Fiore *et al*., 2019). These types of infections are more common in people with underlying risk factors that

include immunosuppression, renal/liver disease, diabetes mellitus or abdominal and oral surgery (Billington *et al*., 2014, Kajihara *et al*., 2015).

E. faecalis possesses the ability to survive for up to 72 hours in peritoneal macrophages. Intestinal *E. faecalis* overgrowth by antibiotic treatment, and observed organisms adhering to epithelial surfaces of the ileum, cecum, and colon. 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).

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 *et al*., 2016).

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).

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).

The first step in the pathogenesis of enterococcal infection appears to be persistence on inanimate surfaces, followed by colonization of the GI tract and amplification of resistant enterococcal numbers. Initial colonization of the GI tract appears to result from the acquisition of small numbers of organisms from inanimate surfaces within the hospital, including thermometer handles, bed rails, health care providers, and other sources (Ramsey *et al*., 2014).

Colonization of the patient's GI tract as a staging ground for disseminated infection, can be divided into four groups: 1) Factors that overcome secreted, non-specific defenses, such as bile, 2) Factors that promote colonization in the presence of overt selection, such as antibiotic resistance determinants, 3) Factors that enhance or alter the ability of enterococci to attach to surfaces within the GI tract, such as adhesins, and 4) Factors that enable invading enterococci to displace resident enterococci or other species contributing to colonization resistance, including bacteriocins and other secreted antimicrobial products (Garsin *et al*., 2014).

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).

The final step in the pathogenesis of enterococcal infection is the damage that results to vital tissues as the result of the presence of enterococci. Factors that would enhance the steps of the pathogenesis of enterococcal infection include those that

induce overt tissue damage, or factors that enhance bystander damage resulting from inflammation (Da Costa *et al*., 2013).

It is important to note that the presence of virulent strains among *E. faecalis* alone is not predictive of infection as there may be other mediators of pathogenicity that have to be elucidated (Stecher *et al*., 2011). The pathogenicity is also related to the ability of virulent strains to grow in high densities in the intestinal tract and spread to other sites in the body. Host factors, such as predisposing medical conditions, immune status, and exposure to antibiotics, are also thought to play a role in the ability of enterococci to establish infection (John *et al.*, 2015).

1.2.3 Clinical factures of *E. faecalis*:

Enterococcus faecalis (E. faecalis) is a species of bacteria that lives harmlessly in the digestive tract, although it can be found in the oral cavity or vaginal tract. It can be resistant to antibiotics. When people are immunocompromised or have an underlying disease, *E. faecalis* can become pathogenic. For this reason, *E. faecalis* is considered an opportunistic pathogen—one that takes advantage of the body when immune defenses are low. *E. faecalis* can enter the body during surgery, insufficiently cleaned medical devices and equipment, improper hand hygiene, or ingesting contaminated foods and fluids. Of the many species of Enterococcus, *E. faecalis* and *Enterococcus faecium* are pathogenic, with *E. faecalis* being the most prevalent in infections. (Anderson *et al*.,2015)

Common *E. faecalis* infections include urinary tract infections (UTIs), wound infections, intra-abdominal and pelvic infections, bacteremia (infection in the blood), and endocarditis (inflammation of the heart). If these infections become systemic (widespread), they can cause serious to life-threatening symptoms. Approximately 85% to 90% of Enterococci infections are caused by *E. faecalis*, and are typically nosocomial (hospital-acquired) (Jabbari shiadeh *et al*.,2019).

1.2.3.1 Urinary Tract Infections (UTIs)

UTIs are urinary system infections of the urethra, ureters, kidneys, or bladder. They can be caused by various species of bacteria, including *E. faecalis*. UTIs caused by Enterococcus species account for greater than 30% of all cases in hospitalized patients (Lin *et al*.,2012).

Risk factors for bladder infections include previous UTI, sexual activity, older age, bladder emptying issues, an obstruction in the urinary tract (urinary stone or enlarged prostate), or use of a urinary catheter. (National Institute of Diabetes and Digestive and Kidney Disease) In a nosocomial population surveyed between 2011 and 2014, enterococci were the most commonly isolated gram-positive bacteria from catheter-associated urinary tract infections (CAUTI), with over 20,000 cases reported to the CDC National Healthcare Safety Network between 2011 and 2014 (weiner *et al*.,2016). Of these cases, just over 50% were caused by *E. faecalis*, followed by "other Enterococcus species" (~30%) and *E. faecium* (~20%). Most worrisome was the fact that ~85% of *E. faecium* isolates were vancomycin-resistant, with the fraction of resistant isolates increasing each year (weiner *et al*.,2016).

enterococci consistently rank within the top three uropathogens in regards to infection incidence, with most enterococcal UTIs being associated with *E. faecalis* (Fisher *et al*.,2009).

First observed as pathogens 100 years ago, *Enterococcus* sp. have since established themselves as a major threat to human health (van Harten *et al*.,2017). In addition to their pathogenic potential, enterococci are commensals of the gastrointestinal tract, with *E. faecalis* being the most ubiquitous among species of animals, birds, insects, mammals, and reptiles. In humans, 98% of people are colonized by enterococci (de Lastours *et al*.,2017). Commensal *E. faecalis* are also considered to offer a health benefit to the host; *E. faecalis* has been used as a

commercial probiotic for over 50 years, and commensal *E. faecalis* produce a pheromone capable of killing multi-drug and vancomycin-resistant *E. faecalis* in addition to inhibiting both Gram-positive and negative pathogens (Phumisantiphong *et al*.,2017).

Enterococcal pathogenesis in the bladder is severely understudied relative to other uropathogens. Most studies of UTI use *E. coli* as a model pathogen, which is understandable given it's standing as the most prevalent uropathogen. Using *E. coli* as a guide, enterococcal adherence to and biofilm formation on urothelial cells has been well documented using mouse models of UTI and is thought to be primarily mediated through the Ebp pili (Endocarditis and biofilm-associated pili), as well as the adhesins, Ace (Adhesion to collagen from *E. faecalis*) and Esp (enterococcal surface protein) (Flores-Mireles *et al*.,2015). Invasion of the bladder epithelium by *E. faecalis* was only recently reported via *ex vivo* imaging of shed human urothelial cells (Horsley *et al*.,2013). This invasive capability was then confirmed *in vitro* using a human bladder epithelial cell line. Unlike *E. coli*, the most common cause of UTI, *E. faecalis*, generally, does not induce severe symptoms of UTI. On the contrary, many *E. faecalis* UTIs present as low-grade discomfort and urine counts below the classic standard threshold for UTI of 105 CFU/mL. However, it should be noted that groups have suggested this standard is too high for *E. faecalis* and that true enterococcal UTI may be caused by a lower bacterial count (Colodner *et al*.,2006). An alternative explanation is that most enterococci do not produce potent pro-inflammatory toxins and this is supported by research demonstrating minimal induction of bladder inflammation by *E. faecalis* in a murine cystitis model (Kau *et al*.,2005).

Further, *Enterococcus* is prevalent in polymicrobial UTI (Seno *et al*.,2005), given that most polymicrobial isolates are dismissed as contamination, enterococcal UTI may be under-diagnosed.

Bitsori *et al*.(2005) found in children receiving antibiotic therapy for UTI; recurrence rates were higher for those with enterococcal UTIs compared to those with Gram-negative UTIs.

Enterococcus faecalis can not only adhere to epithelial cells in the urinary tract, but also invade cells, leading to formation of intracellular bacterial communities in the bladder. The combination of multiple virulence mechanisms, the ability to survive in harsh conditions, and both intrinsic and acquired resistance to many antibiotics explains the high frequency of infection by *Enterococcus faecalis* (Horsley *et al*.,2013).

Enterococci species are part of the healthy human gut microbiota (Kao *et al*.,2019) and sometimes can also colonize in the urinary tract, causing different clinical settings from asymptomatic bacteriuria (AB) to UTIs. Bacteriuria caused by enterococci has a deleterious effect on the urinary tract, which promotes innate immune suppression and increases the risk of infection by other uropathogens (Tien *et al*.,2017), suggesting that the treatment of AB can be successful in terms of the prevention of the recurrence of urinary tract infection. Nevertheless, paradoxically, it has been shown that antibiotic treatment of AB caused by Enterococcus, in patients with recurrent infection, increases the risk for recurrent urinary infection by other uropathogens (Cai *et al*.,2012). Regarding UTIs, Enterococcus spp. is the cause of cystitis, prostatitis and epididymitis (Gilmore *et al*.,2014). Experimental studies in mice have shown that enterococci can also affect the higher urinary tract, thus causing pyelonephritis (Pinholt *et al*.,2014), which is one of the most frequent pathogens involved in complicated UTIs. Enterococci was the cause in 7–25% of

patients with severe urinary sepsis and UTIs (Wang *et al*.,2016).Despite pyelonephritis caused by Enterococcus spp. is not usually associated with bacteremia (Artero *et al*.,2019).

1.2.3.2 Bacteremia

Bacteremia occurs when bacteria get into the blood, often from a wound site or untreated UTI (Higuita *et al*.,2014). If left untreated, bacteremia can lead to life threatening conditions, such as endocarditis, sepsis (a systemic reaction to a blood infection), and septic shock (low blood pressure in severe sepsis that can cause organ failure). Common symptoms of bacteremia include: Fever and chills , Nausea or vomiting ,Rapid heart rate ,Lightheadedness or confusion ,Skin rashes, Enterococcal bloodstream infections (BSI) are associated with a high level of mortality. In survey study of Canadian hospitals (Billington *et al*.,2014), the incidence of enterococcal BSI of hospitalized patients was 6.9 per 100,000, with most cases due to *E. faecalis* (4.5 per 100,000). Incidence of enteroccocal BSI increased between 2008–2014 (9–14 per 100,000) in Switzerland (Buetti *et al*.,2017), suggesting that enterococcal BSI are on the rise. Overall mortality from nosocomial enterococcal BSI is quite high, ranging from 25–50% (Pinholt *et al*.,2014).

Most cases of enterococcal BSI are thought to result from translocation of enterococci from the gut into the bloodstream. Other routes of infection include along intravenous lines, endocarditis, urinary tract infections and other abscesses (Arias & Murray, 2012). The risk factors for mortality associated with enterococcal bacteremia include severity of illness (based on APACHE II scores), patient age, and use of third-generation cephalosporins or metronidazole (Stroud *et al*.,1996). Huycke *et al*., (1991) found that patients infected with hemolytic, gentamicin-resistant *E. faecalis* strains had a five-fold increased risk for death within three weeks compared to patients with nonhemolytic, gentamicin-susceptible strains. Moreover,

mode of treatment was not associated with outcome, discounting the direct contribution of aminoglycoside resistance. Mortality associated with high-level gentamicin resistance (29%) was not significantly different from gentamicin-susceptible strains (28%). Taken together, these two studies suggest that high-level aminoglycoside resistance itself does not explain clinical outcome, and that the presence of other factors, such as the *E. faecalis* cytolysin (hemolysin) appear to contribute enhanced lethality (Caballero-Granado *et al*.,1998).

1.2.3.3 Wound Infections

Wound infections occur when cuts, scrapes, animal bites, sutured wounds, and puncture wounds get infected, which typically happens 24 to 72 hours after the event. Signs of wound infections include: Pus , red area or streak that's spreading, Increased pain and swelling , fever ,& swollen lymph node.

Wound infections caused by *E. faecalis* are harder to treat than those caused by other types of bacteria. The following factors are associated with higher chances of death, especially in developing countries: A longer hospital stay , A repeated surgical procedure ,Prior antibiotic therapy, An Intensive Care Units (ICU) stay (Dan Brennan.,2021).

Wound infections affect between 7% and 15% of hospitalized people globally (World Health Organization, 2016). *Enterococcus faecalis* is one of the most frequently isolated bacterial species across all types of wounds, including diabetic foot ulcers, burns, and surgical sites (Dowd *et al*.,2008). In surgical site infections, *E faecalis* is the third most commonly isolated organism (Gjødsbøl *et al*.,2006). *E faecalis* infections are increasingly difficult to treat due to their intrinsic and acquired resistance to a range of antibiotics (Hollenbeck ,2012). Despite the high frequency of *E faecalis* in wound infections, little is known about its pathogenic strategies in this niche.

Enterococci are one of the most frequently isolated bacterial genera from wound infections (Dowd *et al*., 2008 ; Heitkamp *et al*., 2018); however, their pathogenic mechanisms enabling persistence in this niche are not well understood. Chong *et al.,* (2017) have previously shown in a mouse excisional wound infection model that *E. faecalis* undergo acute replication and long term persistence, leading to delayed wound healing, despite a robust innate inflammatory response at the wound site. These data suggest that *E. faecalis* possess mechanisms to evade the innate immune response, and indeed, we have also shown that extracellular *E. faecalis* can actively suppress NF-κB activation in macrophages (Tien *et al*., 2017). In addition, *E. faecalis* can persist within a variety of eukaryotic cells including macrophages (Zou and Shankar,2014; Zou and Shankar,2016), osteoblasts (Campoccia *et al*., 2016; Tong *et al*., 2016), monocytes (Baldassarri *et al*., 2005), endothelial cells (Millan *et al*., 2013), and epithelial cells (Bertuccini *et al*., 2002; Horsley *et al*., 2018). However, the mechanisms mediating intracellular persistence have not been reported.

However *E. faecalis* persist within epithelial cells and intracellularity contributes to pathogenesis. Using a mouse model of wound infection, we found viable *E. faecalis* within both immune and non-immune cells at the wound site up to 5 days after infection. Using an in vitro model of keratinocyte infection, we show that *E. faecalis* is taken up into these cells via macropinocytosis, whereupon they traffic through the endocytic pathway to late endosomes, and ultimately undergo intracellular replication. Interestingly, *E. faecalis* infection results in a marked reduction of Rab7 expression, a small GTPase required for late endosome-lysosome fusion, providing a potential mechanism for intracellular survival. Finally, we show that *E. faecalis* derived from the intracellular niche are primed to more efficiently reinfect new keratinocytes. Together, our data are consistent with a model in which

a subpopulation of *E. faecalis* are taken up into epithelial cells during wound infection, providing immune protection and a replicative niche, which may serve as a nidus for chronically infected wounds (Wei Hong *et al*.,2021).

1.2.3.4 Vagina infection :

Aerobic vaginitis (AV) is a lack of balance of the vaginal flora and was first described in 2002 by Donders *et al*., (2002) and is characterized by abnormal vaginal flora containing aerobic and intestinal pathogens, varying degrees of vaginal inflammation and immature epithelial cells (Donders *et al*.,2017). The causes of AV that are responsible for inflammatory changes are: *E. faecalis*, *Escherichia coli*, *group B streptococcus and Staphylococcus aureus* (De Seta *et al*.,2019; Tao *et al*.,2019; Wang *et al*.,2020). The most common isolated pathogen of AV is *E. faecalis* in 32% (Sangeetha *et al*.,2017). The pathogenic effect of aerobic microorganisms such as *E. faecalis* has been shown to cause spontaneous abortion, premature birth, puerperal sepsis, abscesses, and urinary tract infections (Bolocan *et al*.,2019).

Immunoglobulin A (IgA) is significantly reduced in the vaginal mucosa during colonization by various bacteria (Abitzsch *et al*.,2001; Hassan *et al*.,2020). It is likely that *E. faecalis* in these places is exposed to other factors by the organism such as age, immune status, hormonal status, influence of various antibiotics, etc. (Jett *et al*.,1994). The basic mechanisms of pathogenicity of this microorganism are enabled: ability to colonize mucous membranes second, to produce pathological changes in the host by its toxic activity by inducing an inflammatory process and to avoid the host's immune defense mechanisms (Weinstock *et al*.,2007).

Colonization of the vaginal mucosa with *E. faecalis* is also enabled by the reduced number or disappearance of lactobacilli, natural colonizers of the vaginal mucosa (Fisher *et al*.,2009). These microorganisms can prevent the colonization of

the vaginal mucosa with *E. faecalis* and other microorganisms in various ways. The use of broad spectrum antibiotics or spermicidal ointments leads to a reduction in the number or destruction of lactobacilli and thus allows the growth of the vaginal mucosa with *E. faecalis* and/or other bacteria (Arias *et al*.,2010).

Propagation of *E. faecalis* from the vaginal mucosa to the uterus, fallopian tubes and ovaries can lead to inflammation of these organs. This microorganism can cause pelvic abscesses and bacteraemia as complications after caesarean section, endometritis or salpingitis (Fan *et al*.,2013). Also, *E. faecalis* can propagate to the urethra and ascendantly lead to inflammation of the urethra, bladder, and renal pelvis (Lamichhane *et al*.,2013; Hassan *et al*.,2020). In a study of AV in 2021, (Shazadi *et al*.,2021) stated that *E. faecalis* is the cause of histological changes in cells in the form of cell damage, increased cell thickness and peeling. Jahic *et al*.,(2007) states that under the influence of *E. faecalis* there is an increase in the pH of the vaginal environment above 4 with a decrease in the number of lactobacilli.

1.2.4 Antibiotics 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).

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). *Enterococcus faecalis* is known to produce biofilms which makes it resistant to the different antimicrobial agents (Kumar *et al*.,2019).

E. faecalis where ampicillin and vancomycin resistance are less frequently reported. Antibiotics such as linezolid, daptomycin, tigecycline and quinupristin-dalfopristin are commonly used to treat MDR enterococcal infections, however resistance to these drugs has also been observed (Kristich *et al*., 2014, Mercuro *et al*., 2018). *E. faecalis* strains were resistant to linezolid (Malisova *et al*., 2021).

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).

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).

The risk of VRE colonization and infections is associated with previous antibiotic exposure disrupting normal gut microbiomes, especially vancomycin and cephalosporins use. In addition, prolonged hospitalization, ICU stays, residence in long term care (LTC) facilities, hemodialysis patients, diabetes, cancer, and transplant patients, stomach acid suppression, use of invasive devices, and exposure

to contaminated surfaces including shared medical equipment can also predispose individuals to get enterococci/VRE infections (Ostrowsky *et al* 2001 ; Zheng *et al*.,2017;Chanderraj *et al*.,2019).

Ciprofloxacin(CIP) is a large spectrum fluoroquinolone antibiotic that is widely used for the treatment of numerous G+Ve bacterial infections in joints, bones, skin, tooth, gastrointestinal, and urinary and respiratory tracts (Herizchi *et al*.,2016).

It is very important to know that Aerobic vaginitis (AV) cannot be treated with metronidazole as bacterial vaginosis is treated, because it will not cure but will lead to increased infection, due to the natural resistance of *E. faecalis* to this drug (Han *et al*.,2015). If it is a pregnancy, it is better to opt for clindamycin. Fluoroquinolones such as ciprofloxacin can be used in the treatment of AV because they have little effect on the normal vaginal flora leading to rapid recovery from AV (Han *et al*.,2015; Oerlemans *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).

Beta-lactam antibiotics, such as penicillin, amoxicillin and Imipenime are uniformly effective against most strains of *Enterococcus*, these antibiotics have activity against this bacteria, and they are commonly used together with beta-lactamase inhibitors (Cho *et al*., 2014). Beta-lactam antibiotics are one of the most commonly prescribed drug classes with numerous clinical indications, and was effected on *Enterococcus* spp. (Prescott, 2013).

Enterococcus is developing resistance against the most commonly used anti-enterococcal antibiotics like ampicillin and high-level aminoglycosides, besides being inherently resistant to many others like cephalosporins and clindamycin. This

makes the treatment of these infections a real challenge for clinicians (Gupta *et al*.,2015).

Enterococci are intrinsically resistant to clindamycin, aminoglycosides (García-Solache & Rice 2019). They also gain antibiotic resistance through their ability to acquire and transfer resistance-related mobile genetic elements (MGE) via various mechanisms like plasmids, conjugation, and transposons (Fiore *et al*.,2019).

Improved usage of antimicrobials that are effective for the treatment of Enterobacteriaceae or anaerobes infections and to which Enterococci has natural tolerance or just poor sensitivity such as Cephalosporins, Clindamycin or Quinolones can lead to selection and improved occurrence of *Enterococococci* or this genus can lead to superinfection (Sartelli *et al*., 2016).

According to one study combined drug therapy has shown some efficacy in cases of severe infections against susceptible strains of *E. faecalis* . Ampicillin and vancomycin-sensitive *E. faecalis* (lacking high-level resistance to aminoglycosides) strains can be treated by gentamicin and ampicillin antibiotics. A less nephrotoxic combination of ampicillin and ceftriaxone may be used alternatively for ampicillin-susceptible *E. faecalis* (Pamer & Eric, 2018).

The incidence of UTIs due to *Enterococcus faecalis* has risen steadily over the years, and infections due to multiple-drug-resistant strains present a significant medical problem and are a common cause of chronic or recurrent UTIs, especially those associated with structural abnormalities and instrumentation (Shankar *et al*.,2001).

Due to the rapid adaptation of bacteria, antimicrobial treatment for the management of urinary tract infections is becoming increasingly difficult, causing the antimicrobial profile of bacterial uropathogens to change over the years, despite having better antimicrobial agents. Resistance to these has increased significantly

due to their incorrect use, limiting therapeutic options (Almeida *et al*.,2020). Currently several bacterial strains are resistant to practically all known antibiotics. Such as carbapenems, macrolides, and penicillins (Almeida *et al*.,2020).
Levofloxacin and ciprofloxacin are antimicrobial agents and are expected to develop a widened use for its underlying effect in neuroinflammation modulating (Zusso *et al*., 2019), hematopoietic stem cell transplantation (Rambaran and Seifert, 2019).

Levofloxacin and ciprofloxacin are both recommended for clinical application in UTIs. Levofloxacin shows advantage over ciprofloxacin in terms of efficacy, disease reoccurrence and adverse event (Zhang *et al*., 2012). On the contrary, microbiology evidence shows that the uropathogen is more sensitive to ciprofloxacin (Afriyie *et al*., 2018; Humphries *et al*., 2019). Ciprofloxacin and levofloxacin have marginal activity against enterococci, and their use is restricted to the treatment of urinary tract infections due to susceptible strains. High-level resistant strains have been shown to contain mutations in both gyrA and parC (el Amin *et al*.,1999). Some strains have mutations in only parC, suggesting that this topoisomerase may be the primary target of fluoroquinolones in enterococci. There has been suggestion in some studies that efflux pumps are also involved in enterococcal fluoroquinolone resistance, but specific efflux pumps have not been identified (Oyamada *et al*.,2006). Erythromycin and Levofloxacin were more resistance in Enterococcus isolated (Ibtisam *et al*.,2020).

Nitrofurantoin is an antibiotic medication that is used for the treatment of uncomplicated lower urinary tract infections. It is effective against most gram-positive and gram-negative organisms. The Food & Drug Administration (FDA) approved nitrofurantoin in 1953 to treat lower urinary tract infections. Nitrofurantoin is a synthetic antimicrobial created from furan and an added nitro group and a side change containing hydantoin (Calderaro *et al*.,2021). Nitrofurantoin's primary use

has remained in treating and prophylaxis of urinary tract infections. Nitrofurantoin is advantageous as it concentrates in the lower urinary tract while maintaining a low serum concentration and does not significantly affect bowel flora. Nitrofurantoin is bactericidal against most common urinary tract pathogens, including *Escherichia coli*, *Enterococci*, *Klebsiella* (Gardiner *et al*.,2019).

Rifampicin is rapidly bactericidal against many Gram-positive bacteria and displays good tissue penetration, but the rapid development of resistance precludes its use as monotherapy (O'Driscoll *et al*.,2015), leading to this old agent often being considered specifically for use in combination therapy (Holmberg *et al*.,2012).

Rifampicin can be synergistic against enterococci, but this is not a consistent finding among enterococci. Although traditionally used in the investigation of antibacterial combinations for synergistic activity (Kirsty et al.,2017).

Erythromycin and tetracycline are therapeutic agents used for treatment of enterococcal infections. Undoubtedly, the appearance of resistant strains is the result of the extensive use of these antibiotics (Tian *et al*.,2019). Methylation of 23S rRNA encoded by *erm* genes which reduces macrolide ability for ribosome binding is one of the most often associated erythromycin resistance mechanisms. Also, export of antibiotics mediated by genes encoding efflux pumps (*mef* and *msr*) is of the other mechanisms involved in macrolide resistance (Choi *et al*.,2018).

The high prevalence of high-level resistance to both erythromycin and tetracycline was documented. The distribution pattern of *erm* (A-C) genes was unexpectedly different between enterococci and staphylococci from the same geographic region. Concurrent resistance mechanisms were more involved in resistance to erythromycin versus tetracycline (Nikta *et al*.,2021).

Teicoplanin is widely used in Europe and the Asia because teicoplanin possesses more potent *in vitro* activity against enterococci than vancomycin, along

with a longer half-life, which enables a once-daily dose (Svetitsky *et al*.,2009). A retrospective study has demonstrated the advantage of teicoplanin in terms of effectiveness in treating enterococcal infective endocarditis (Escolà-Vergé *et al*.,2019).Teicoplanin is noninferior to vancomycin in clinical treatment success and to determine if teicoplanin is less nephrotoxic than vancomycin (Ryo Yamaguchi *et al*.,2023).

Imipenem is a carbapenem antibiotic normally administered with cilastatin to treat a variety of infections. Imipenem is a semisynthetic thienamycin that has a wide spectrum of antibacterial activity against gram-negative and gram-positive aerobic and anaerobic bacteria, including many multiresistant strains. It is stable to many beta-lactamases (Brunton *et al*.,2018).

Imipenem is commonly used in combination with cilastatin and is now available in a triple-drug product with cilastatin and relebactam which was approved by the FDA. Imipenem was first approved by the FDA in November 1985 as the combination product Primaxin marketed by Merck & Co. (Brunton *et al*.,2018).

1.2.5 Randomly Amplified Polymorphic DNA polymerase Chain Reaction (RAPD-PCR)

Polymerase chain reaction (PCR) is a laboratory technique for rapidly producing (amplifying) millions to billions of copies of a specific segment of DNA, which can then be studied in greater detail. PCR involves using short synthetic DNA fragments called primers to select a segment of the genome to be amplified, and then multiple rounds of DNA synthesis to amplify that segment (Mike , 2023).

The polymerase chain reaction (PCR) is used to amplify specific regions on a gene. PCR is used to amplify random regions along the genomic DNA of the *Enterococcus* species .The PCR procedure can be done when at least one short DNA

segments on each side of the region of known interest. The PCR reaction requires synthetic oligonucleotides complementary to these known sequences to prime enzymatic amplification of the Penicillin Binding Protein (PBP) segment DNA in a test tube (Wilkie & Simon, 1991).

RAPD analysis is a technique to rapidly detects genomic polymorphisms. Whilst many methods are available for molecular profiling microorganisms, RAPD-PCR approaches are powerful, rapid, have low cost and are accessible. Furthermore they can be applied in many research environments and with minimal equipment. As such, RAPD-PCR methods have often been considered as 'gold standards' in molecular typing and analysis by this method is widely accepted and regarded as a reliable tool for differentiating and identifying enterococci (Al-Badah *et al.*,2015).

RAPD analysis involves an unknown target sequence. The primer used is about ten base pairs in length and will bind randomly along the genome and will amplify that particular sequence. The PCR product expected will be a high number of bands. In a large genomic DNA is subjected to RAPD PCR primers like that of *Enterococcus* isolates (Snustad & Simmons, 2000).

Randomly amplified polymorphic DNA is PCR reactions that amplify segments of DNA at random. A normal PCR will amplify a known DNA sequence. The primers designed in a normal PCR flanks the gene of interest and amplifies that particular gene. A particular product will be expected, i.e., a single band. The primers used in RAPD PCR are designed to bind randomly to segments of DNA along the *Enterococcus* genome (Kearns, *et al.,* 2002).

RAPDs are PCR -based molecular markers that may substantially reduce time, labour, and cost required for molecular mapping. RAPDs involve the use of a single DNA primer to direct amplification under PCR based amplification of random

sequences. RAPD PCR is very useful in different objectives. These are assessment of genetic variation in populations and species, to study the phylogenetic relationships among species and subspecies, to construct and understand genetic linkage maps, gene tagging, and identification within species such as *Enterococcus*, any fingerprinting application to characterize a particular species (Abhita,2007).

RAPD analyses are a technique for rapidly detecting genomic polymorphisms (Williams *et al*.,1990). These techniques differ with regard to their DNA amplification conditions, the length of the primers used and the resolution of the products obtained. Arbitrary primers are usually not more than 7 – 10 bases in length, with an arbitrary sequence not directed toward any known target sequence of the bacterial genome. Therefore, the amplification of random segments of genomic DNA is directed by a single oligonucleotide primer of arbitrary sequence (AP-PCRthus generating a characteristic spectrum of short DNA products of various complexities. Polymorphisms can be detected by variations in the length of the obtained amplified sequences, commonly known as RAPD, which can be used to compare bacterial strains (Domig *et al*.,2003).

Genomic fingerprints are increasingly used to study relationships at the intra- or even the inter-specific level. Differences in these fingerprints between individuals are interpreted as genetic distances (Scheidegger *et al*.,2009). However, the applied methods should provide the appropriate level of discriminatory power and be relatively efficient and cost effective. Several methods are available for DNA finger printing, including restriction fragment length polymorphism (RFLP) and randomly amplified polymorphic DNA (RAPD-PCR), which have been described as powerful molecular typing methods for microorganisms and have become the .gold standard. For molecular typing (Krawczyk *et al*.,2006 ; Nieto-Arribas *et al*.,2011; Nieto-Arribas *et al* .,2013).

There are many advantages to RAPD PCR technology. There are more polymorphisms than restriction fragment length polymorphism, fast and simple, a large number of bands produced per primer and differentially amplifies DNA samples based on mutations. There are some disadvantages to RAPD PCR technology. Detection of polymorphisms are still limited, reproducibility of results is inconsistent, poor profile resolutions of RAPDs on agarose gel resulting in very few bands, only detects dominant markers (Abhita,2007).

1.2.6 Multilocus sequence typing (MLST) :

Multilocus sequence typing (MLST) is an unambiguous procedure for characterising isolates of bacterial species using the sequences of internal fragments of (usually) five house-keeping genes. Approximately 450-500 bp internal fragments of each gene are used, as these can be accurately sequenced on both strands using an automated DNA sequencer. For each house-keeping gene, the different sequences present within a bacterial species are assigned as distinct alleles and, for each isolate, the alleles at each of the seven loci define the allelic profile or sequence type (ST). Each isolate of a species is therefore unambiguously characterised by a series of seven integers which correspond to the alleles at the seven house-keeping loci (Maiden *et al.*, 2013).

In MLST the number of nucleotide differences between alleles is ignored and sequences are given different allele numbers whether they differ at a single nucleotide site or at many sites. The rationale is that a single genetic event resulting in a new allele can occur by a point mutation (altering only a single nucleotide site), or by a recombinational replacement (that will often change multiple sites) weighting according to the number of nucleotide differences between alleles would erroneously consider the allele to be more different than by treating the nucleotide changes as a single genetic event. Most bacterial species have sufficient variation

within house-keeping genes to provide many alleles per locus, allowing billions of distinct allelic profiles to be distinguished using five house-keeping loci. For example, an average of 30 alleles per locus allows about 20 billion genotypes to be resolved. MLST is based on the well-established principles of multilocus enzyme electrophoresis, but differs in that it assigns alleles at multiple house-keeping loci directly by DNA sequencing, rather than indirectly via the electrophoretic mobility of their gene products (Jolley *et al*., 2018).

the relationship among the *E. faecalis* isolates from different sources is becoming more important. various genotyping methods were used to identify isolates or to further track their sources, including pulsed field gel electrophoresis, multiple variable number tandem repeat analysis, and multilocus sequence typing (MLST). Among them, MLST is a popular one. Nallapareddy *et al*., (2002) first evaluated the discriminatory power of MLST compared with pulsed field gel electrophoresis for *E. faecalis* and showed that sequence-based typing had potential to differentiate the isolates at the subspecies level and identify outbreak isolates. Subsequent studies confirmed the potential of MLST as an excellent tool for isolate characterization and long-term epidemiologic analysis in the related species *E. faecium* (Homan *et al*., 2002), Although some studies have been done on the properties and epidemiological characteristics of *E. faecalis*, few studies are available on the relationship between properties of *E. faecalis*, particularly whether they are pathogens, probiotics, or otherwise useful in the food industry, and their original source.

Multilocus sequence typing (MLST) is a commonly used method of assessing subspecies phylogenetic relationships. In one *E. faecalis* MLST employing five housekeeping gens: *pepC* (Aminopeptidase C), *rpoB* (RNA polymerase β subunit), *groEL* (Chaperonin GroEL), *recA* (Recombinase protein A) and *clpX* (ATP-dependent protease subunit) are amplified by PCR, sequenced, and compared to a

global database such that an allele variant number can be assigned to each gene (Ruiz-Garbajosa *et al*.,2006). Based on the combination of allele numbers, a sequence type (ST) is assigned to a strain. Analysis of whole genome sequences has also been used to assess phylogenetic relationships among *E. faecalis* strains (Bourgogne *et al*.,2008; Palmer *et al*.,2010).

To validate epidemiological linkages and disclose possible transmission events among humans, animals, food, and the environment, discriminatory high-resolution typing is of the utmost importance. The high genetic plasticity of *E. faecalis* is the result of wide exchanges of genetic material, which in turn complicate molecular investigations (Paulsen *et al*.,2003). In contrast to the second most important *Enterococcus* species in hospital settings, *Enterococcus faecium*, for which hospital-adapted lineages have already been identified, most *E. faecalis* genotypes do not show extended host or context specificity (Leavis *et al*., 2006). For instance, Buhnik-Rosenblau *et al*.(2013) demonstrated, on the basis of multilocus sequence typing (MLST), that no specific genetic groups could be assigned to a colonization- or infection-associated origin.

Classical MLST provides an international and expandable typing nomenclature, but the method provides only a moderate typing resolution. the Multilocus Sequence Typing (MLST) is an ordinary typing method that is based on the characterizing bacterial species via sequencing of internal fragments of multiple housekeeping genes (Kalia *et al*.,2001). In this process, each sequence of internal fragments compares with the other alleles that they were already characterized. Then, each sequence classified at one of those seven housekeeping genes category. Ultimately, by the combination of obtained data, the allelic profile will be construct and each distinct profile consider as absolute sequence type.

MLST is a more appropriate typing technique for long-term epidemiology, which is currently also widely used for subspecies differentiation. MLST, based on the allelic variations in sequences of multiple loci, unambiguously types strains (Maiden *et al*.,1998) and offers an advantage over other techniques used for typing, such as PFGE, since the data are objective and easily stored, compared, and shared via the Internet. Two different MLST schemes have been used successfully for differentiation of *E. faecalis* strains (Nallapareddy *et al*.,2002; Ruiz-Garbajosa *et al*.,2006). The first scheme, which assessed three antigenic genes and one house-keeping gene, found that the allelic profile of two antigenic genes (*ace* and *salA*) was sufficient to discriminate the 22 *E. faecalis* isolates included there in (Nallapareddy *et al*.,2002). The second MLST scheme, based on the allelic profiles of seven housekeeping genes, was used to type 110 isolates and provided insight into the population structure as well as long-term epidemiological relationships of *E. faecalis* strains (Ruiz-Garbajosa *et al*.,2006).

Two schemes exist for MLST typing of *E. faecalis*. Both have some alleles in common, but differ in selecting (a) explicitly only housekeeping genes (Ruiz-Garbajosa *et al*.,2006) or (b) mix housekeeping with virulence genes for allele pattern analysis (Nallapareddy *et al*.,2005). The latter approach was suitable to identify a clonal lineage highly prevalent in the US and characterized by beta-lactamase production (still rare in *E. faecalis*) and presence of the pathogenicity island. The scheme of Ruiz-Garbajosa *et al*. (2006) follows common rules for MLST allele selection (housekeeping genes only) and has been implemented into the central MLST database (http://efaecalis. mlst.net/; managed by: Rob Willems and Janetta Top (UMC Utrecht, NL, hosted by the Imperial College London and funded by the Wellcome Trust, UK) (Ruiz -Garbajosa *et al*., 2006; Sadowy *et al*.,2011; Kuch *et al*.,2012; Poulsen *et al*.,2012**;** Werner *et al*.,2012).

Over the past three decades, overlapping in specific sequence types (STs) and high-risk clonal complexes (CCs) between healthy humans and nosocomial patients suggested that inter-host-species transmission may play a role in the spread of multidrug resistant *E. faecalis* (Anderson *et al*.,2015; Hammerum *et al*., 2012;, Kuch *et al*.,2012). It is different to quantify the risk for *E. faecalis* of different host types in relation to human health.

E. faecalis lack a distinct structure in clades. Some clades related to outbreaks in hospitals were also found in the community. In some studies, specific genetic lineages (CC) of hospital-adapted *E. faecalis* strains had emerged (Guzman prieto *et al*.,2016; Ruiz-Garbajosa *et al* .,2006). *E. faecalis* is considered a generalist bacterial species depicting no prominent host specialization (Pontinen *et al*.,2021).

Population structure of *E. faecalis* appears somehow different from that of *E. faecium*. MLST analysis does not strictly differentiate hospital-associated lineages from colonizing variants. Nevertheless, certain sequence types and clonal complexes appear enriched in the nosocomial setting and which are often found to be multi-resistant (Ruiz-Garbajosa *et al*.,2006; McBride *et al*.,2007). The great advantage of MLST is that sequence data are unambiguous and the allelic profiles of isolates can easily be compared to those in a large central database via the Internet (in contrast to most typing procedures which involve comparing DNA fragment sizes on gels). Allelic profiles can also be obtained from clinical material by PCR amplification of the five house-keeping loci directly from CSF or blood. Thus isolates can be precisely characterised even when they cannot be cultured from clinical material (Maiden *et al*.,2013; Jolley *et al*.,2018).

1.2.7 Clustered regularly interspaced short palindromic repeats (CRISPRs):

The Clustered Regularly Interspaced Short Palindromic Repeats (CRISPRs) loci and CRISPR associated (Cas) protein-encoding genes known as CRISPR-Cas systems (Garcia –Solache *et al*.,2019). The CRISPR-Cas system consists of a CRISPR array and the Cas gene family, in which the CRISPR array is composed of highly conserved repeat sequences and spacer sequences, with upstream leader sequences responsible for their transcription (Guo *et al*.,2022).

CRISPR-Cas systems constitute a multi-step adaptive immune response that defends prokaryotes against foreign invading genetic material. A genomic locuin cluding the CRISPR and nonrepetitive spacer sequences, as well as 6 to 20 genes producing CRISPR-Cas related proteins, make up the system. CRISPR-Cas systems consist of 2 classes and six types (types I, III, and IV belong to class 1 and types II, V and VI belong to class 2) (Shabbir *et al*.,2018;Kamruzzaman & Iredell .,2020).

The CRISPR-Cas systems can be used for genome engineering. Also, they impose a strong selective pressure on pathogenic bacteria to acquire virulence traits and antibiotic resistance (Kamruzzaman and Iredell .,2020;Gholizadeh and Kosa *et al*., 2020).

Three TypeII CRISPR occur with variable distributions in the *E. faecalis* species, including two that possess *cas* genes (CRISPR1 and CRISPR3, which are Type II-A systems (Fonfara *et al*., 2014), and one orphan array (CRISPR2) that is ubiquitous but lacks associated *cas* genes (Bourgogne *et al*.,2008; Palmer and Gilmore, 2010; Hullahalli *et al*., 2015). The orphan locus possesses repeats identical to those in CRISPR1, but not CRISPR3. CRISPR1 and CRISPR2 both have seven repeats of a 37-bp palindromic motif that bears no resemblance to any of the 29-bp spacer sequences. Despite this, they likely originated from pheromone-responsive

type plasmids, plasmids integrated inside the *E. faecalis* V583 genome, and enterococcal prophage and phage, due to short spacer sequences (Gholizadeh *et al*., 2020).

All *E. faecalis*, however, possess an orphan CRISPR locus, known as CRISPR2, that lacks *cas* genes (Hullahalli *et al*.,2015). CRISPR1 and CRISPR3 are the functional CRISPR loci in *E. faecalis,* with a complete collection of type II *cas* genes upstream of the repeat- spacer array (Price *et al*.,2016).

The CRISPR array consists of 36 bp repetitive DNA elements (repeats) interspersed by 30 bp sequences usually derived from foreign DNA (spacers). The cognate spacer sequence present in foreign DNA, termed the protospacer, is usually located proximally to a conserved DNA sequence referred to as the protospacer adjacent motif (PAM). The final spacer in the CRISPR array (terminal spacer) is followed by a degenerated repeat (terminal repeat). During CRISPR interference, the Cas9 endonuclease is guided to DNA targets by CRISPR RNAs (crRNAs), which are processed transcripts derived from the CRISPR array. An active Cas9-crRNA targeting complex is also associated with a trans-activating crRNA (tracrRNA), which is partially complementary to the repeats of the CRISPR array. This targeting complex samples PAMs in DNA, and once it encounters a match to the affiliated crRNA spacer sequence, Cas9 creates a double-stranded break in the target DNA. Further, the consensus PAM sequences for CRISPR1-Cas and CRISPR are identical (NGG), while the protospacer adjacent motif PAM for CRISPR3 is distinct (NNRTA) (Price *et al*., 2016). Some *E. faecalis* encode Type II CRISPR-Cas systems, defined by the presence of *cas9,* and which consist of two main components: a CRISPR array and *cas* genes. The mechanism of Type II CRISPR-Cas systems has been well characterized (Anders *et al*., 2014; Jinek *et al*., 2014; Nishimasu *et al*., 2014; Marraffini, 2015). The *cas9* associated with CRISPR3 is

distinct in sequence and function from the CRISPR1 *cas9*. The CRISPR3-Cas9 cannot confer defense from conjugative plasmids using CRISPR2 spacers (Price *et al*., 2016). An inverse correlation between the occurrence of Type II CRISPR-Cas systems and antibiotic resistance has been reported for *E. faecalis* (Palmer and Gilmore, 2010). All *E. faecalis* isolates possess a CRISPR2, but most MDR strains lack the functional CRISPR1-Cas or CRISPR3-Cas systems (Palmer and Gilmore, 2010; Hullahalli *et al*., 2015).

CRISPR-Cas has widely been used as a genome editing tool (Jiang *et al*.,2015; Barrangou & van Pijkeren.,2016). CRISPR-assisted genome editing relies on the premise that targeting the chromosome, thereby inducing double-stranded DNA breaks (DSBs), is lethal, and can select for outgrowth of low-frequency variants or rare recombinants (Selle and Barrangou.,2015).

CRISPR-Cas function as a bacterial 'immune system' that evolves through integration of DNA recognised as foreign into a CRISPR array, which then become CRISPR guide RNAs. The guides target foreign DNA to be specifically degraded by nuclease encoded activity. The presence of CRISPR-Cas is generally more common in commensal enterococcal strains which prevent the uptake of foreign DNA such as the acquisition of drug resistance elements (Johnson *et al*., 2021). Conversely, MDR/clinical enterococcal strains either lack these defence systems or contain non-functional variants such as CRISPR2 (contains spacer array but no cas genes) (Johnson *et al*., 2021). Therefore, the MDR strains have increased genetic plasticity which can enhance survival compared to strains that lack genetic diversity due to the absence of MDR genes (Johnson *et al*., 2021).

Chapter Two
Materials and Methods

2.1 Materials:

2.1.1 Laboratory instruments and equipment:

Table (2-1): Laboratory instruments and equipment

No.	Name of Item	Company	Origin
1	Analytical Balance	KERN	Germany
2	Autoclave	Drawell	China
3	Automated Water distillator	K&K Scientific	South Korea
22	Bensen burner	Satorins	Germany
4	Centrifuge	Cypress Diagnostics	Belgium
23	Cooling box	Ningbo	China
24	Distillator	GFL	Germany
25	DNA extraction tubes.	Eppendorf	Germany
5	Gel Documentation System	Bioneer	South Korea
6	Gradient Thermal Cycler	Techne	UK
7	Horizontal electrophoresis system	Mupid	Japan
8	Hot plate magnetic stirrer	IKA	Germany
9	Incubator	Cypress Diagnostics	Belgium
10	Laboratory Electrical Oven	DAIHAN LabTech	Korea
11	Microcentrifuge	Hettich	Germany
12	Micropipette	Nanolytik	Germany

13	Microscope	Cypress Diagnostics	Belgium
14	Microscope camera	Omax	China
15	Microwave Oven	LG	Korea
26	Millipore filter (0.45mm)	Satorins membrane Filter Gm, BH, W.	Germany
27	Nano drop	Memmert	Germany
28	PCR thermocyling	Clever	USA
29	Plastic Test tubes 10ml.	AFCO	Jordan
30	Platinum wire loop	Himedia	India
16	Refrigerator	Concord	Korea
31	Screw capped bottles 30 ml	Hirschmann	Germany
32	Sensitive electron balance	A & D	Japan
17	Spectrophotometer	Chemglass Life Sciences	Finland
33	Sterile swab for streaking	Lab. Service	S.P.A
18	Upright Freezer	Bosch	UK
19	UV sterilization cabinet	SCI-PLAS	UK
20	Vortex mixer	DAIHAN LabTech	Korea
21	Water Bath	DAIHAN LabTech	Korea
34	Wooden sticks	Supreme	China

2.1.2 Chemical and Biological Materials:

Table (2.2): Chemicals and Biological materials used in the laboratory work.

No.	material	Company	Origin
2	Agarose	Bioneer	Korea
12	Carbohydrates (glucose, maltose, lactose)	Fluka chemika	Switzerland
13	Catalase reagent	Schuchariot	Germany
4	Decontamination nucleases solution	Bio-world	USA
5	DNA Loading Dye	Bioneer	Korea
6	Ethanol 96 %	Scharlab S.L	Spain
7	Ethanol 96 % spray	AMRESCO	USA
17	Ethidium bromide, Master mix	Promega	USA
15	Glycerol	Fluka	England
8	Gram stain reagents	Syrbio	S.A.R.
16	NaCl , NaOH	Merk Darmstadt	Germany
9	Nuclease-free water	Bioneer	Korea
14	Oxidase reagent	Himedia	India
10	Proteinase-K	Genaid	USA
1	TBE buffer (10X)	Bioneer	Korea
11	Tris EDTA (TE)	Bio basic	Canada

2.1.3 Culture Media:

Table (2-3) Culture Media

No.	Culture Media	Company	Origin
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

2.1.4 Antibiotics Disks:

Table (2-4) Antibiotics Disks (Bioanalyze / 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	5
6.	Erythromycin	E	15
7.	Imipenem	IMI	10
8.	Kanamycine	K	30
9.	Teicoplanin	TEC	30
10.	Nitrofuratoin	NOR	300
11.	Refampin	REF	5

2.1.5 Commercial kits:

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

No.	Name of Item	Company	Origin
1	100bp ladder	Bioneer	Korea
2	Presto Mini gDNA Bacteria Kit	Geneaid	USA
3	Go Taq®G2 Green Master Mix	Promega	USA
4	Gel/PCR DNA Fragments extraction kit	Geneaid	USA
5	Primers of *ddl*,*M13*,CRISPR-Cas &MLST	ALPHA DNA	Canada

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

No.	Component	Size
1	GT Buffer	30 ml
2	GT Buffer	40 ml
3	W1 Buffer	45 ml
4	Wash Buffer (add Ethanol)	25 ml (100 ml)
5	Elution Buffer	30 ml

Table (2-7) DNA ladder

No.	Materials
1.	Ladder consist of 13 double-stranded DNA with size 100-20 00 bp.
2.	Loading dye has a composition (15% Ficoll, 0.03% bromophenol blue, 0.03% xylene cyanol, 0.4% orange G, 10mM Tris-HCl (pH 7.5) and 50mM EDTA).

Table (2-8): Master Mix Used in PCR (Promega/USA).

	Materials
1.	DNA polymerase enzyme (Taq)
2.	dNTPs (400 µm dATP, 400 µm dGTP, 400 µm dCTP, 400 µm dTTP)
3.	$MgCl_2$ (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 freshly in a dark bottle by dissolving 1gm Tetramethyl Para-Phenylene diamine dihydrochloride in 100 ml distilled water (Forbes *et al*., 2007).

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 121oCfor 15 minutes, then kept at 4^{o}c (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 to 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.

No	Media	Purpose of use
1	Chromogenic agar medium	This 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	Bile-Esculin Agar Medium	This 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).
3	MacConkey Agar Medium	This 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).
4	Nutrient Agar Medium	This 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).
5	Blood agar medium	This medium has been prepared according to MacFaddin, (2000) by dissolving 40gm blood agar base in 1000 ml D.W. and autoclaved at 121^{o}C 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).
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).
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 121ºC for 15minutes. It was distributed in tubes. This medium was used to detect bacterial motility (MacFaddin, 2000).
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 then pouring to sterile test tubes and sterilizing by autoclave (MacFaddin, 2000).
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).
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).
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:

Specimens were taken from 105 patients who had been taken to such Al-Hilla Surgical Teaching Hospital and the Imam Sadiq Education Teaching Hospital with a variety of infections, such as UTIs, vaginitis, wound infections, and bacteremia at the period from (August 2022 to November 2022).

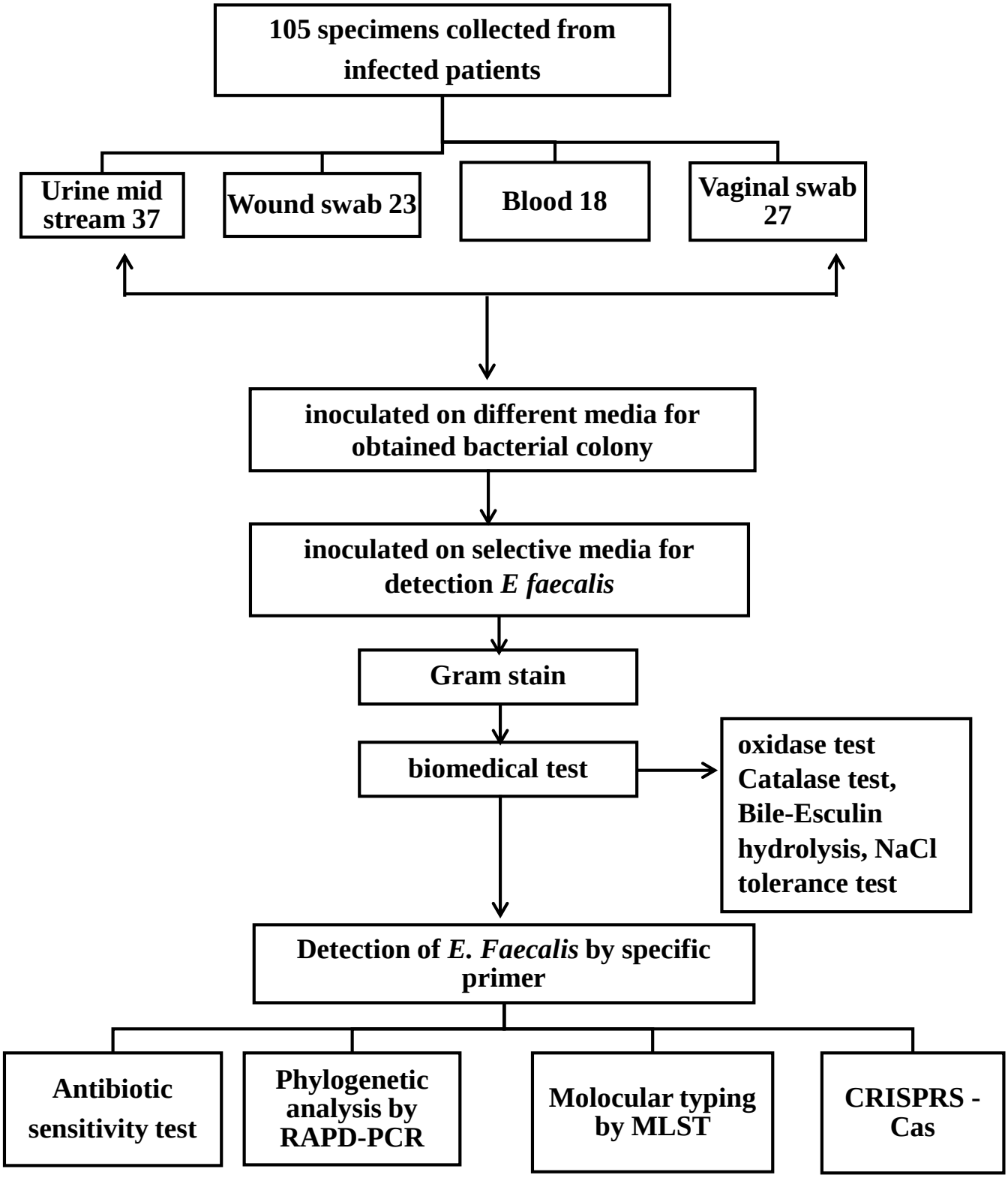


Fig. (2-1): Experimental design

2.3.1 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).

2.3.2 Isolation and identification of *Enterococcus faecalis*:

2.3.2.1 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).

2.3.2.1.1 Urine samples:

The specimens 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).

2.3.2.1.2 Vaginal swabs:

The samples were generally collected from women (pregnant and non-pregnant) suffering from vaginitis. The swabs were inserted into the posterior fornix, upper part of the vagina and rotated there before withdrawing them. A vaginal speculum was also used to provide a clear sight of the cervix and the swabs were rubbed in and around the introits of the cervix and withdrawn without contamination of the vaginal wall. Swab for culture should be placed in tubes containing normal

saline to maintain the swab moist until taken to laboratory. The swab was inoculated on culture media and incubated aerobically at 37°C for 24h.

2.3.2.1.3 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.

2.3.2.1.4 Wound swab:

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- 30ºC 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

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, 2023).

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 µl 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 µl 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 µl 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 µl 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 µl per sample) was pre-heated to 70^{o}C (for step 5 DNA Elution).

g. Following 70^{o}C incubation, 5 µl 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 µl 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 14000-16000 xg 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 µl 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 µl of wash buffer (ethanol added) was added to the GD column, centrifuged at 14000-16000 xg 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 14000-16000 xg 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 µl of preheated elution buffer or TE was added to the center of the matrix, centrifuged at 14000-16000 xg 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 (2-9) 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	2.5µl
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 10 gene, one for diagnosis, one for amplification , five for MLST and three for CRISPR , each one has specific nucleotide and product size. The primer sequences and PCR conditions that used are listed in **Table (2-10), (2-11),(2-12),(2-13).**

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 easy used to photograph the gel.

Table (2-10): Primers sequences and PCR condition of *ddl* gene for identification of *E. faecalis*

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

Table (2-11): The primers used for the amplification of a fragment gene:

Gene	primer sequence (5`-3`)	size bp	PCR condition	Reference
M13	GAG GGT GGC GGT TCT	variable	94^{o}C-5 min 1x 95^{o}C-60 s 42^{o}C-30 s 30x 72^{o}C-60 s 72^{o}C-5 min 1x	(Rossetti *et al*.,2005)

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 were added. 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

2.10 Multi locus sequence typing *(*MLST)**:**

In this study, The *Enterococcus faecalis* MLST scheme uses internal fragments of the following five house-keeping genes: *groEL, clpX, recA, rpoB, and pepC*. Primer and PCR conditions were based on MLST scheme obtained from X. Chen *et al.,* (2015).

2.10.1 PCR amplification:

PCR reactions (25µl) were carried out according to X. Chen *et al.,* (2015), by using the following cycling conditions 30 cycles of 94°C for 1 min, annealing at 50 to 60°C (optimal annealing temperatures for each locus were listed in Table (2-12) for 45 s, and 72°C for 1 min, and a final extension step at 72°C for 7 min followed by hold at 4°C. The PCR mixture was 25 µl volumes containing 1 µl of each primer, 12.5 µl of the master mix (GoTaq® G2 Green Master Mix, Promega, USA), 3 µl of DNA template, and 7.5 deionized water. A 5 µl of the PCR products were loaded into 2% agarose gels in 1 X TBE with loading dye, and run at 100 v in 1X TBE for 60 minutes. Images of the gels were captured using a gel documentation system.

Table (2-12):Genes and sequences of MLST &PCR condition :

Gene	Gene product	Primer [1]	Sequence (5′→3′)	Length(bp)	PCR condition
pepC	Aminopeptidase C	*pepC*/F	AGCAGCAACAGTTCCAAT	725	94ºC-4min 1x 94ºC-1min 30x 51.ºC- 45s 72ºC -1min 72ºC- 7min 1x
		pepC/R	ATCCACGCATCGCTCATA		
rpoB	RNA polymerase β subunit	*rpoB*/F	TCTTATGTTCGGATTGACC	708	94ºC- 4min 1x 94ºC-1min 30x 50.6ºC- 45s 72ºC -1min 72ºC- 7min 1x
		rpoB/R	TGAACGGATACGACGATT		
groEL	Chaperonin GroEL	*groEL*/F	GCGGATGATGTTGATGGG	612	94ºC- 4min 1x 94ºC-1min 30x 56.6ºC- 45s 72ºC -1min 72ºC - 7min 1x
		groEL/R	AGCGATTTGACGGATTGG		
recA	Recombinase protein A	*recA*/F	TTCTTTAGCGTTAGATGTTG	687	94ºC- 4min 1x 94ºC-1min 30x 49.9ºC- 45s 72ºC -1min 72ºC - 7min 1x
		recA/R	CCTTCTTGGGAAATACCTT		
clpX	ATP-dependent protease subunit Clp X	*clpX*/F	GATGAAGCAGTCCGAGAA	765	94ºC- 4min 1x 94ºC-1min 30x 51.1ºC-45s 72ºC -1min 72ºC – 7min 1x
		clpX/R	TACAGGTAAGCGTCCAAT		

1
F = forward; R = reverse.

2.10.2 Sequencing of PCR products

All PCR products obtained above were cleaned and submitted for sequencing as follows. The PCR product was cleaned of amplification primer using the Gel/PCR DNA Fragments extraction kit (Geneaid, USA) as per manufacturer's instructions. Purified DNA was sequenced at Macrogen company (Korea) with the sequencing primers for each gene as outlined in table . Bidirectional Sanger sequencing method was carried out on an Applied Biosystems 3730xl DNA Analyzer (Applied Biosystems, Foster City, CA, USA).

2.10.3 Bioinformatic analysis:

The raw Sequence data was trimmed and aligned to the control sequences. The standard sequences for alignment were taken from NCBI database. Multiple alignments were done by using Clustal W v2.0 (Thompson, *et al.* 1994) of Geneious Prime Software V2021.1 (Biomatters, Inc., North America). All unique sequences were assigned an allele number and every unique/unambiguous combination of 5 allele numbers (the allelic profile) was designated as a sequence type (ST). Identification of ST and allele profile was done by interrogation of gene sequences against each other and with the reference sequences.

Regarding the identification of phylogenetic relationships among *E. feacalis* isolates, the merged edited sequences were used to generate phylogenetic tree using the PhyML maximum likelihood by using MEGA X v10.0.5 (Kumar et al., 2019). Regarding recombination tree, Split decomposition analyses were performed with SplitsTree, version 4, by using LogDet distances, equal edge lengths, and 1000 bootstrap replicates. eBURST analysis was done according to Feil *et al*., (2004) and Ribeiro-Gonçalves, (2016).

2.11 CRISPR identification

Three separated PCR reactions (25µl) were carried out according to Gholizadeh *et al*., (2020) with some modifications (three PCR runs instead one multiplex PCR to avoid the interactions of PCR product), all primers were listed in Table (2-13). The PCR mixture was 25 µl volumes containing 1 µl of each primer, 12.5 µl of the master mix (GoTaq® G2 Green Master Mix, Promega, USA), 3 µl of DNA template, and 7.5 deionized water for CRISPR1 and CRISPR3, and 7.5 deionized water for CRISPR2. The amplification condition was carried out with the thermal cycling conditions. The PCR products were analyzed by electrophoresis

using a 1.5 % agarose gel in 1X TBE buffer and the stained gels were viewed using a standard UV transilluminator.

Table (2-13): CRISPR identification primers & PCR condition

CRISPR primer	Sequence	PCR product	PCR condition	Reference
CRISPR1-*cas* loci-F	GCGATGTTAGCTGATACAAC	315	95°C-10 min 1x	(Palmer& Gilmore.,2010)
CRISPR1-*cas* loci-R	CGAATATGCCTGTGGTGAAA		95°C-30 s 60°C -30 s 34x 72°C -45 s	
			72°C -5 min 1x	
CRISPR2 loci-F	CTGGCTCGCTGTTACAGCT	Variable		
CRISPR2 loci-R	GCCAATGTTACAATATCAAACA			
CRISPR3-*cas* loci-F	GATCACTAGGTTCAGTTATTTC	224		
CRISPR3-*cas* loci-R	CATCGATTCATTATTCCTCCAA			

Chapter Three
Results and Discussion

3. Results and discussion

3.1 Isolation of *Enterococcus faecalis*:

A total of 105 clinical specimens were collected during this study which obtained from patients suffering from different infection such as UTI, vaginitis, wound infection and bacterimia who admitted to two main hospitals of AL-Hilla City: Al-Hilla Surgical Teaching Hospital and Imam Sadiq Education Teaching Hospital during a period extending from (August 2022 to November 2022). All specimens were subjected to aerobic culturing on different media and it was found that out of the total 105 specimens,70(66.6%) specimens showed positive bacterial culture. No growth was seen in other 35(33.3%) specimens which indicate the presence of microorganisms that may be cultured with difficulty such as virus, fungi and other agent or may be due to difference in the size and nature of the specimens. Among (70) positive culture were culturing on chromogenic agar medium (selective media),only 15(14.2%) specimens show positive was identified as *E. faecalis* as shown in the **figure (3-1).**

These isolates then subjected to molecular detection method using specific primer based *D-alanine ligase* gene as a genetic marker for confirmed isolation of *E. faecalis* by PCR, the results revealed that 15(100%) were positive for PCR . However, this approach will enhance the accuracy, sensitivity specificity ,special and cost effectiveness in the detection of *E. faecalis* than culture technique and the PCR is the best choice for diagnosis of infection with *E. faecalis* .However , molecular technique has over convective methods ,it can provide results in 24 hr . where as routine culture followed by biochemical test need 36-48hr .

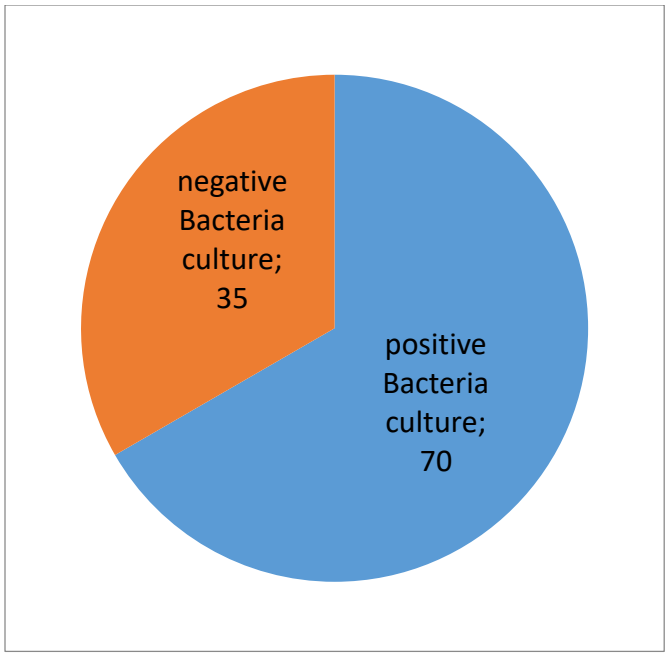


Fig. (3-1): prevalence of *E. faecalis* among other etiological agents associated with isolated sample

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

The result of this study was observed that 15 isolates were recorded related to *E. faecalis*, collected from the following site, 6 isolates (40.0%) obtained from urine specimens, 5 isolates (33.3 %) from wound, 4 isolates (26.6 %) from vagina. While no bacteria 0(0.0%) were isolated from blood specimens as shown in **table (3-1).**

Table (3-1): Percentage of *E. faecalis* isolated from different sites of infection

Site of infection	No. of specimens	*E. faecalis*	%
Urine	**37**	**6**	**40.0**
Wound	**23**	**5**	**33.3**
Blood	**18**	**0**	**0**
Vagina	**27**	**4**	**26.6**
Total	**105**	**15**	**100%**

This table indicated that *E. faecalis* are isolated from urine specimens at percentage (40.0%),wound specimens at percentage (33.3%) and from vagina specimens at percentage (26.6%).

Maha and Lamees.,(2022) in Babil/Iraq observed that 20 isolates were recorded 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, 6 isolates (30.0%) from vagina, While no bacteria 0(0.0%) were isolated from blood samples. AL-Khafaji,(2021) observed that 27 isolates were recorded related to *E. faecalis*, it was collected from the following site, 14(51.85%) positive culture for *E. faecalis* were isolated from urine, 10(37.03%) positive culture for this bacteria isolated from stool.

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

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

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.

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

Kandela, (2012) in Baghdad/ Iraq was found that 50 isolates belonged to *Enterococcus faecalis* of which, 23 isolates (46%) from stool, 27(54%) isolates from clinical cases distributed between 16 (32%) from urine, 6 (12%) from wounds and 5(10%) from vaginal.

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 sample size; all of these factors may combine and play an important role in inhibiting or stimulating bacterial resistance development.

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).

Enterococci normally colonize the gastrointestinal tract of humans. It is found in relatively large quantities in the faeces. It can be transferred from the gastrointestinal tract in different ways to other places. It can contaminate healthy skin and can colonize the vaginal mucosa and anterior urethra of women and men (Fisher *et al*.,2009). However, if it enters the systemic circulation it can lead to various diseases (Weinstock *et al*.,2007). Colonization of the mucous membrane is possible with a large number of factors such as: natural microflora, their pH and natural cleansing (Abitzsch *et al*.,2001).

Javed *et al*.,(2020)showed prevalence of *E. faecalis* (7% vs. 14%) in nonpregnant and pregnant females' respectively when take specimens from vaginal swab. Changes in vaginal microflora that show a critical role in promising vaginal colonization (Aiyegoro *et al*., 2007), and my hypothesis is that the reason intestinal bacteria are associated with urinary tract and vaginal infections is due to the close proximity of the anal opening to the vagina and urethra, so contamination from the anus can lead to bacteria being found in the vagina area, though this is a much less common occurrence (Inabo and Obanibi, 2006).

Enterococcus faecalis is the most common cause of infectious vaginitis, and is characterized by white, thin discharge with a fishy odor, and elevated vaginal pH that is often attributed to an imbalance of normal vaginal bacteria (Medeiros *et al*., 2014).

Asymptomatic bacteriuria is also a common finding in women, and sometimes it is followed by symptomatic urinary tract infection, these infections are usually caused by a broad spectrum of uropathogens like *Escherichia coli*, *Pseudomonas aeruginosa* and *Proteus mirabilis* followed by *Enterococcus faecalis* (Kline and Lewis, 2017).

Urinary tract infection in patients with indwelling devices, or infection of sutured surgical wounds, appear to result from external contamination, potentially by organisms that have amplified in the GI tract and become intimately associated with the patient upon hospitalization (Lebreton *et al*., 2014).

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).

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 (3-2).**

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.

Specimen 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 organism that had the capacity to ferment sugar, growth in the presence of crystal violet, Oxidase negative, esculin hydrolysis *Enterococcus* spp. is a gram-positive coccus catalase negative, and anaerobically facultative. It grows in 6.5% NaCl, 40% bile salts, 0.1% methylene blue milk and at pH 9.6. It grows at 10 and 45°C and resists 60C for 30 min (Nautiyal *et al*.,2016).

Table (3-2): Diagnostic Features of *E. faecalis*

Tests	Results
Colonies morphology	Small cocci, punctiform colony, convex with an entire margin
Gram Stain	gram positive
Catalase	Negative
Oxidase	Negative
Growth at 10 and 45s°C	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 color)
Type of hemolysis on blood agar medium	Beta, alpha, gamma hemolysis

However, some other characteristic of *E. faecalis* should be considered to confirm the identification of this bacteria through using specific markers via PCR techniques (Lüddeke *et al*., 2015).

3.4 Confirmed detection of *E. faecalis* by D-alanine ligase gene by PCR technique:

D-alanine ligase gene (ddI) is present in *E. faecalis* and this gene is specific for *E. faecalis*. These it can facilitated downstream analyses such as molecular detection. *E. faecalis* is an opportunistic bacterium considered as pathogen for significant infection to human.

To confirmed diagnosis for *E. faecalis* DNA was extracted from all suspected isolates that previously identified *E. faecalis* by selective media (Chromogenic agar

medium) conventional PCR was carried out using these DNA samples for the amplification of specific *ddl* primer.

The results recorded that all isolates 15 were produced the specific 941bp DNA fragment when compared with allelic ladder, as shown in **figure (3-2).**

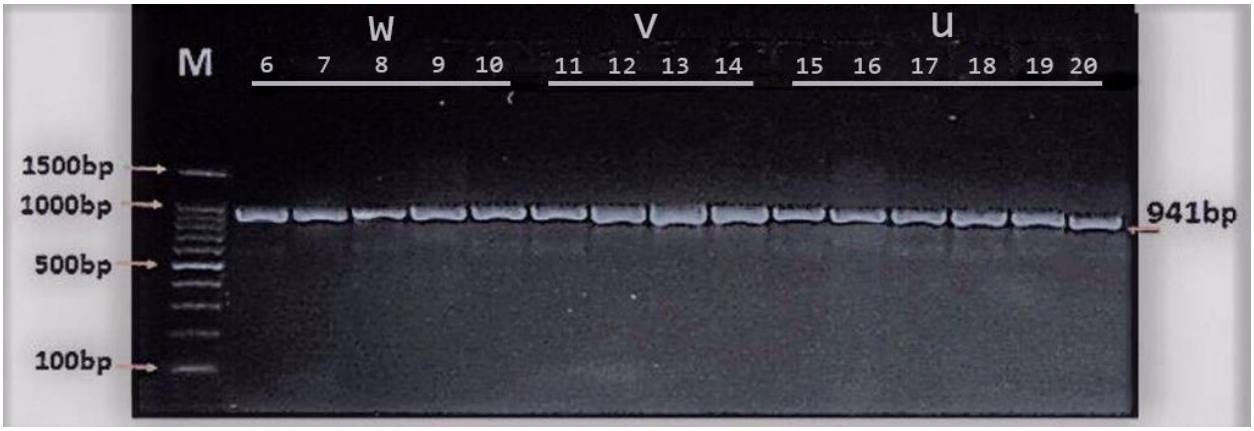


Figure (3-2): 1.5% Agarose gel electrophoresis image at 75 volts for one houre that displayed PCR results analysis of D-alanine D- alanine ligase gene *ddl* in *E. faecalis* isolated from clinical samples.Where M: (100-1500bp)and Lane (6-20) showed (6,7,8,9,10 wound, 11,12,13,14 vagina, 15,16,17,18,19,20 urine) at (941bp) .

To confirm the identity of *E. faecalis*, was 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).

Seenaa and Lamees (2020);Maha and Lamees (2022) found that all 47 &20 isolates of *E. faecalis* respecticaly isolated from different clinical specimens were confirmed by successfully amplification 941 bp of *ddl* gene was used as specific primer for detection of *E. faecalis* .

Nateghian *et al*., (2016) found that, out of the 200 enterococci studied by multiplex PCR, 180(90%) were identified as *E. faecalis*, While the result obtained by Kafil and Asgharzadeh, (2014) who were found that from (100) clinical isolates only (34) isolated *E. faecalis* using specific primer, López-Salas *et al*., (2013) who detected that (95%) from clinical isolates related to *E. faecalis*.

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 successful 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).

3.5 Antibiotic resistance of *E. faecalis* isolates:

The increasing use of antibiotics in medical treatments, contributed significantly to the emergence of multi-antibiotic resistant bacterial strains, which led to a severe health problem.

For the beta-lactams group, piperacillin antibiotic was chosen. Kanamycin was used as it belong to the aminoglycoside group. For the quinolones group, ciprofloxacin and levofloxacin were used. Others antibiotics were used in this study include, clindamycin (lincosamide), erythromycin (macrolides group), Imipenem (carbapenem), Rifampin (rifamycin) and nitrofuratoin (nityofurans). Vancomycin and Teicoplanin were selected as members of the glycopeptide group because to their significance in the treatment of severe, life-threatening infections caused by Gram-positive bacteria that are resistant to other antibiotics.

Fifteen *E. faecalis* isolates were subjected to screening its ability to resist eleven type of antibiotics. **Figure (3-3)** showed the percentage of antibiotics resistance among the *E. faecalis* isolates. These isolates were found to have increased resistance to conventional antibiotics such as, kanamycin, Ciprofloxacin & Clindamycin (100 %), Imipenem (93.3 %), Rifampin and Nitrofurantoin (86.6%),

erythromycin (80 %), Vancomycin (73.3%). The lowest percentage of antibiotic resistance was Teicoplanin & piperacillin (6.6 %) and Levofloxacin (13.3 %).

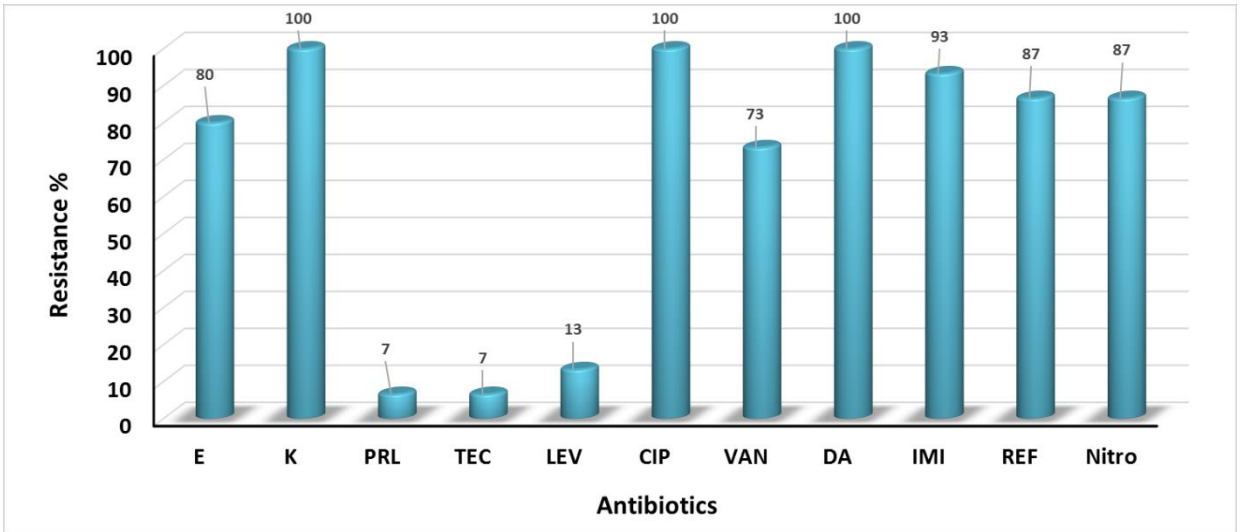


Figure (3-3): Percentage (%) of antibiotic resistance demonstrated by *Enterococcus faecalis* isolates.

The resistance mechanisms of the microorganisms are usually based on the delayed penetration of the antimicrobial agent (Donlan & Costerton, 2002). Furthermore, these organisms have developed resistance to virtually all antimicrobials currently used in clinical practice using a diverse number of genetic strategies (Miller *et al*., 2014).

However, the present study revealed that (100%) of isolates of *E faecalis* from clinical specimens were resistance to Ciprofloxacin. In a local study mentioned that the percentage of resistance of *Enterococcus faecalis* isolated from urine, fecal, wound, vagina and pus samples to Ciprofloxacin was 25.5% (Seenaa, 2020).

Alwan *et al*., (2018) that mentioned (100%) of *E. faecalis* isolates were resistance to Ciprofloxacin. Another studies by, (Bi *et al*., 2018; Tateda *et al*., 2019) were found that the resistance rate of *E. faecalis* to ciprofloxacin was (90%). Sattari *et al*., (2019) found that *E. faecalis* have high frequency of resistance to ciprofloxacin (80.5%).

UTIs due to ciprofloxacin resistant bacterial strains have continued to increase at a worry rate. Furthermore, ciprofloxacin has 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. In addition to improved antibacterial activity, Antibiotics, such as CIP, are an attraction because of their broad spectrum and lack of resistance development. *E. faecalis* has resistance acquisition capacity and this challenge can be sealed with CIP. 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).

However, there is a concern regarding the emergence and rapid expansion of resistance acquisition to fluoroquinolones by Enterococci, increase in the resistance to ciprofloxacin occurred (from 1% to 15%) among the *Enterococcus faecalis* strains (Higuita *et al*., 2014). Enterococci confers an unusual ability to acquire resistance to such diverse groups of drugs as aminoglycosides, beta-lactams, macrolides, fluoroquinolones and tetracyclines (Giedraitienė *et al*., 2011).

On the other hand, the result was shown that (13%) of *E. faecalis* isolates were resistant to Levofloxacin, this finding also recorded by another clinical study in Iraq (Maha, 2022). The quinolones (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, (Miller *et al*.,2014).

Enterococci demonstrate low magnitude of intrinsic resistance against the quinolones, but are capable of acquiring high-level resistance via diverse mechanisms. Mutations in the target genes, specifically *gyrA* and *parC*, have been reported in *E. faecium* and *E. faecalis* (Werner *et al*., 2012; Yasufuku *et al*., 2011). These changes affect the so-called 'quinolone resistance determining regions', which presumably distort the binding affinity of the antimicrobial agent. Externalization of the antimicrobial agent via efflux pumps is another well-known mechanism of quinolone resistance. A third mechanism of resistance, observed in *E. faecalis* is facilitated by *qnr* and encodes for a protein with a series of pentapeptide repeats similar to the plasmid-borne quinolone resistance genes described in Enterobacteriaceae. The presence of this protein is probably to protect DNA gyrase by reducing DNA binding of the quinolone and subsequently, the formation of the quinolone–gyrase complex (Miller *et al*.,2014).

However, the result was shown that (100%) of *E. faecalis* isolates were resistance to Clindamycin. This finding was also reported by Sattari *et al*., (2019) who found that *E. faecalis* have high frequency of resistance to clindamycin (100%). Li *et al*., (2021) showed that *E. faecalis* isolates harbored the highest resistance rate towards clindamycin. Another local study showed that 20(100%) the highest rate of resistant is seen to Clindamycin (Maha, 2022).

One more important clinically relevant finding was that the resistance of *E. faecalis* to Kanamycin was 100%, Alwan *et al*., 2018; Maha ,(2022) shown that the highest rate of resistant among *E. faecalis* isolated from different samples were high resistance to kanamycin (100%). Enterococci exhibit intrinsic tolerance against the aminoglycosides. This phenomenon appears to be facilitated by two major factors which are poor uptake of the antimicrobial agent requiring increased concentrations

to enable 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, thereby, creating a steric hindrance and reducing the binding to the ribosomal target (Costa *et al*., 1993).

Additionally, several clinical isolates also possess the enzyme APH (3´)-IIIa, which lead to resistance against kanamycin and amikacin via its phosphotransferase ability. Furthermore, enterococci are able to modify the ribosomal target through a ribosomal RNA (rRNA) methyltransferase referred to as *EfmM* (Galimand *et al*., 2011). This enzyme recognizes a specific cytidine at position 1404 of the 16S rRNA in *Enterococcus* sp., and methylation of this residue produce resistance against tobramycin and kanamycin.

In this study, the results revealed that 73 % of the isolates were resistance to Vancomycin Maha,(2022) was found that (85%)of isolates were resistance to Vancomycin when take samples from urine, stool, vagina, wound.While Seenaa, (2020) mentioned that the *E. faecalis* isolates was highly sensitive to Vancomycin in rate 93.6%. Another study showed that all *E. faecalis* isolated from urine sample were sensitive to Vancomycin at rate (96%) (Khalid, 2016). García-Solache & Rice (2019) were mentioned that enterococci are thought to be susceptible to vancomycin, and are considered intrinsically resistant to clindamycin, quinupristin-dalfopristin, cephalosporins and aminoglycosides. Hasan *et al*., (2011) revealed that the highest susceptibility of enterococcal isolates from UTI infection was toward the Vancomycin (90.4%).

Unfortunately, Enterococci are intrinsically resistant to many antimicrobials and easily acquire the high-level drug resistance via horizontal gene transfer. Resistant Enterococci species, especially vancomycin resistant enterococci may cause difficulties in treatment (Kang *et al*.,2019).

Enterococcus faecalis UTI are of particular concernin that they are intrinsically resistant to first-line antimicrobial agents, especially vancomycin (Zhenget *et al*.,2018).

Vancomycin is regarded as the main treatment option in resistant enterococci infections. However, we determined the Vancomycin resistance as 1.1% in *E. faecalis* isolates (Matheeussen *et al*.,2019). High Vancomycin resistance rates were associated with long hospital stays and extended use of antibiotics (Lee *et al*.,2018).

The main mechanism of glycopeptide resistance (e.g., vancomycin & teicoplanin) 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 ICU-hospitalized patients (Said and Abdelmegeed, 2019). Many studied 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*., 2016).

However, this study revealed (6.6%) of *E. faecalis* was resistant to Teicoplanin, this result agreed with another study by Maha, (2022). Kutlu, (2019) was shown that teicoplanin resistance was very low in *E. faecalis* (9%) was the most active agents against this species isolates. Al-Dahmoshi *et al*.,(2019) were shown that *E. faecalis* was shown different resistance percentage as ciprofloxacin (60.71%) vancomycin (46.43%), and teicoplanin (25%).

The resistance of *E. faecalis* against nitrofurantoin was 87%, this result is consistent with data obtained by Seenaa, (2020), the highest rate of resistant was to Nitrofurantoin, 89.3%. While, Kalid (2016) who found a high resistance of *E. faecalis* to Nitofurantoin (100%). Also the results by Dicks *et al*., (2011) who were found that strains of *E. faecalis* were resistant to Nitofurantoin in rate (100%). Nitrofurantoin's mechanism of action has remained poorly understood since its discovery in the 1940s. Nitrofurantoin uses several mechanisms to achieve an antimicrobial effect. Nitrofurantoin is taken up by bacterial intracellular flavoproteins that reduce nitrofurantoin to reactive intermediates. Intermediate metabolites resulting from this reduction then bind to bacterial ribosomes and inhibit bacterial enzymes involved in the synthesis of DNA, RNA, cell wall protein synthesis, and other metabolic enzymes (Giedraitiene *et al*.,2022). The broad-based mechanism of action may explain the lack of acquired bacterial resistance to nitrofurantoin. However, mutations in *nfsA* and *nfsB* are potential causes of nitrofurantoin resistance in *E. coli* (Wan *et al*.,2021) .

Due to this ability to recruit antibiotic resistance determinants, MDR enterococci display a wide repertoire of antibiotic resistance mechanisms including modification of drug targets, inactivation of therapeutic agents, overexpression of efflux pumps and a sophisticated cell envelope adaptive response that promotes survival in the human host and the nosocomial environment (Miller *et al*., 2014).

Erythromycin has been recommended as an alternative option for patients who are allergic to penicillin and are also widely used for antibiotic prophylaxis of endocarditis associated with dental procedures (Bagg *et al*., 2006). In this study, the results revealed that (80%) of the isolates were resistance to Erythromycin. A another study by Al-Dahmoshi *et al*., (2019) found that *E. faecalis* was shown resistance to erythromycin (85.71%).

The high number of strains resistant to erythromycin may suggest some of the mechanisms, the presence of efflux pumps specific for erythromycin, and suggests the greater resistance mechanism for the enterococcal genus, another mechanism could be mediated by erm methylases of the ermBermAM hybridization class that has been described in *Enterococcus* isolates (Huys *et al*.,2004).Enterococci have emerged as important nosocomial pathogens and emergence of resistance to many of the antimicrobials used for Gram-positive organisms has made the management of infections due to *Enterococcus* species difficult (Praharaj *et al*., 2013).

Our study revealed that (93%) of *E. faecalis* isolated from the clinical samples was resistant to Imipenem. Sharqi *et al*.,(2021) showed that *E. faecalis* isolated from varies clinical samples(urine, faeces, burns, wound and sputum)was resistance to ciprofloxacin, Imipenem and Piperacillin. 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. Imipenem acts as an antimicrobial through the inhibition of cell wall synthesis of various grampositive and gram-negative bacteria. This inhibition of cell wall synthesis in gram-negative bateria is attained by binding to penicillin-binding proteins (PBPs). imipenem has shown to have the highest affinity to PBP-2 PBP-1a and PBP-1b .This inhibition of PBPs prevents the bacterial cell from adding to the peptidoglycan polymer which forms the bacterial cell wall eventually leading to cell death (Nicolau *et al*.,2008).

Imipenem is active against aerobic and anaerobic Gram positive as well as Gram negative bacteria including Pseudomonas aeruginosa and the Enterococcus. It exerts a bactericidal effect by disrupting cell wall synthesis (Brunton *et al*.,2018).

The present study revealed (6.6 %) 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).

Finally,this study revealed (86.7%) of *E. faecalis* was resistant to rifampin . Astudy by Noroozi *et al*., (2022) where found that *E. faecalis* was shown resistance to rifampin (69.6%). While, Alduhaidhawi *et al*.,(2022) showed that *E. faecalis* isolated from varies clinical samples was resistance to rifampin in rate (9.4%). Mechanism of action Rifampin is thought to inhibit bacterial DNA-dependent RNA polymerase, which appears to occur as a result of drug binding in the polymerase subunit deep within the DNA/RNA channel, facilitating direct blocking of the elongating RNA (Campbell *et al*.,2001). This effect is thought to be concentration related (Boeree *et al*.,2015). The high propensity of enterococci to acquire and express new resistance determinants further enhances their ability to sustain antimicrobial resistance, promoting gastrointestinal colonization and nosocomial infections by antibiotic-resistant enterococci (Rice, 2001).

Many studies have confirmed that colonizing strains of enterococci serve as reservoir for antimicrobial resistance genes, which are capable of being transferred among enterococci or acquired by other bacteria (Schjørring and Krogfelt, 2011; Boehm and Sassoubre, 2014). High-density colonization by antimicrobial-resistant enterococci increases the risk of infections such as bacteremia (Lebreton *et al*., 2014; Tedim *et al*., 2015).

3.6 RAPD – PCR genotyping of *Enterococcus faecalis isolates :*

Genetic variation in microorganisms lead to several phenomena that are clinically very significant and demanding . In present study ,genomic DNA from the isolated *E. faecalis* (n=15)including 6 isolates from urine, 5 isolates from wound &4 isolates from vagina , were extracted ,& the purity of the DNA was confirmed using nanodrop .The purified bacterial DNA was used as the template for the analysis of *E. faecalis* clonal diversity by RAPD – PCR .

So ,the development of a methodology to identify the source of bacteria is important for assessing of the risk pose to public heath associated with bacteria evolution to determine degree of genomic diversity .

In this study , genotypic polymorphism and the relatedness of 15 *E. faecalis* isolates from urine ,wound & vagina were characterized by RAPD analysis with M13 primer.

Based on the result of clusters and discriminate function analysis, the length sequence & composition of the sequence in the genome are variable & often unique for each isolate ,since the PCR reaction it was indicated fingerprints for all *E. faecalis* isolates using M13 RAPD primer from *E. faecalis* performed the complex DNA patterns . molecular typing of *E. faecalis* isolates using M13 primer generated 7-14 bands ranging from 95-3000 bp can be differential by agarose electrophoresis as shown in **figure (3-4)** .

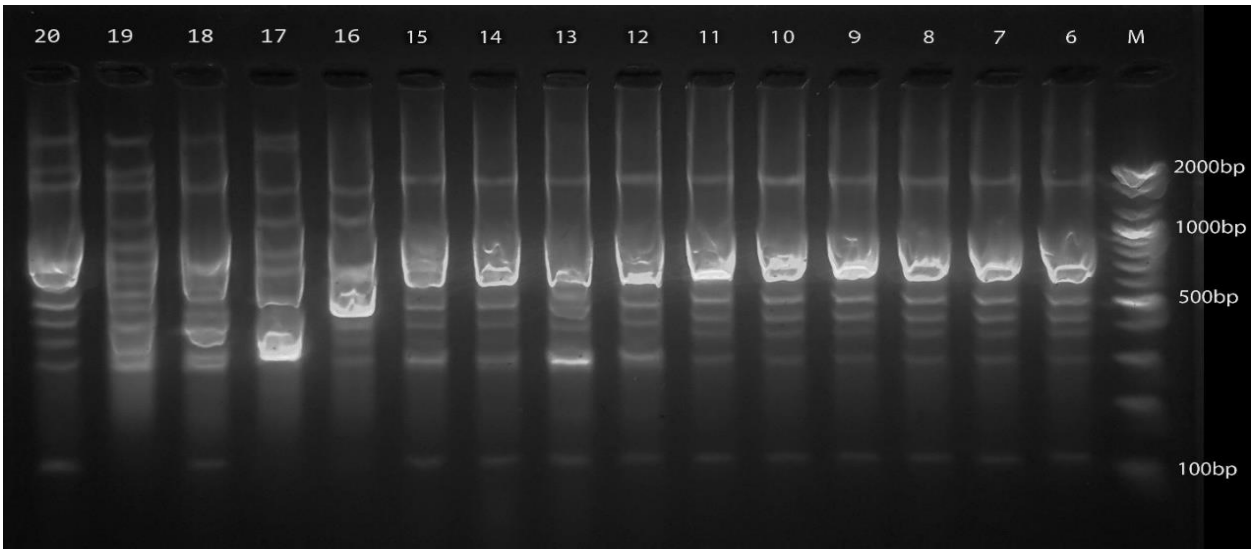


Fig. (3-4): The RAPD-PCR gel products representing the relationship among 15 of *Enterococcus faecalis* in patients with different sample sources. Lanes 6-20 are studied isolates, Lane M is 100bp DNA ladder .shown (6,7,8,9,10 wound, 11,12,13,14 vagina, 15,16,17,18,19,20 urine)

In the present study, the M13 RAPD primer showed that DNA polymorphism among *E. faecalis* isolated from clinical specimens, either in the occurrence of amplified fragments or in the variable genetic similarities of each isolate with the others. Despite the fact that they should display narrow and low variation due to the genomic structure of the *E. faecalis* species and the structure of the 10mer-RAPD primers. Eventually, the fluctuation of genetic similarity values each of the 15 isolates with others using the primer evidently revealed the divergent genetic backgrounds of such isolates with their DNA polymorphism patterns. The results revealed that the 15 isolates were genetically different, furthermore, primer M13 showed high polymorphism. However, the present study on RAPD analysis revealed that, there were some bands which are common in all the specimens and some were not evident.

The 15 *E. faecalis* isolated could be divided into 10 genotypes by M13 RAPD primer this finding may show different origins of *E. faecalis* isolates of the present survy .

The cladogram of genetic distance among all the isolated based on fragment polymorphism generated by RAPD – PCR after using the primer M13 by using UPGMA was shown in **figure (3-5)**.

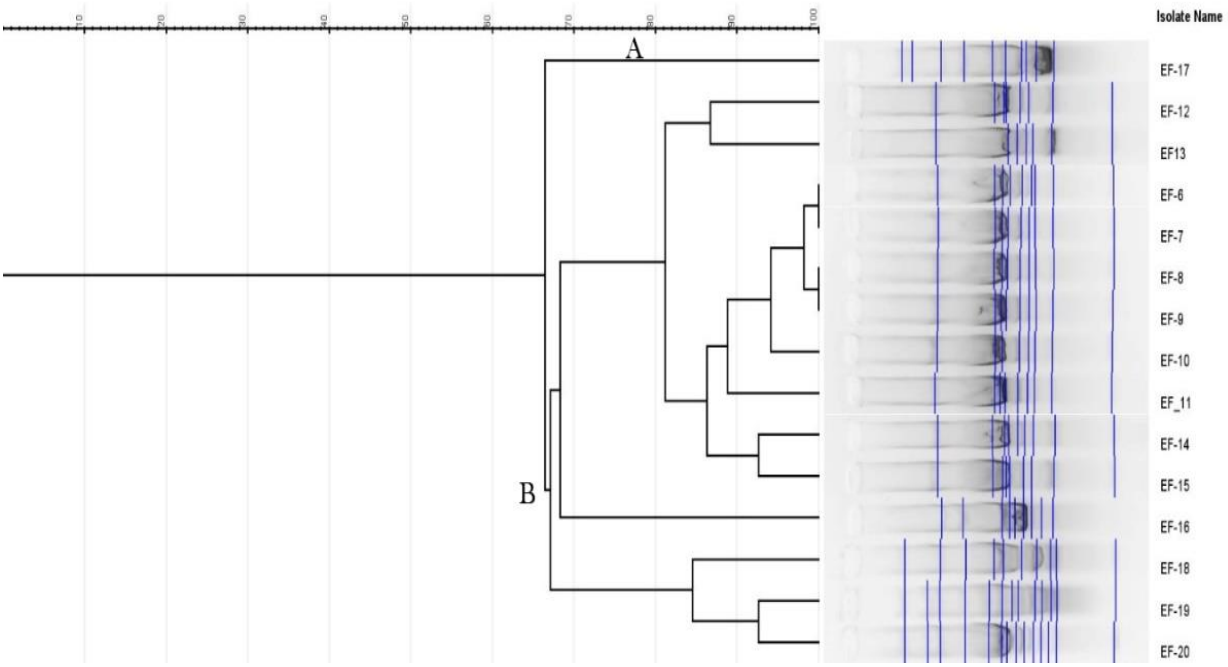


Fig. (3-5):The RAPD-PCR-derived cladogram showing the relationships among 15 different strains of *Enterococcus faecalis* in patients with various sample sources The distance values are shown in the bar (above). Unweighted pair group method with arithmetic mean was used to create this cladogram (UPGMA). (6,7,8,9,10 wound, 11,12,13,14 vagina, 15,16,17,18,19,20 urine).

The cladogram divided all isolates (15) in to two clusters . First cluster (A) conteined only one isolate (EF17) and the second cluster (B) contained 14 isolates , divided the 14 isolates in to two sub clusters , the first sub cluster divided in to two branches , the first branch divided more to two sub branches . one contained two isolates (EF 12 & EF 13).On the other hand the second contained eight isolates (EF6,EF7,EF8,EF9,EF10,EF11,EF14,&EF15).The second branch contained only one isolate (EF16) .Morever ,the second sub cluster contained three isolates (EF18,EF19,EF20).Cluster (B) showed close genetic linkage &the same genetic distance between some isolates therefore displaying clonal dissemination. Their

close genetic relationship based on the dissimilarity coefficient indicated a low genetic variability between them.

Cladogram branch lengths were proportional to genetic distance between isolates. Phylogenetic comparisons of *E. faecalis* isolates from different geographical regions have been useful in taxonomical and epidemiological investigations.

Genetic identify and genetic distance values among all isolated based on fragment polymorphisms generated by RAPD – PCR are present in table (3-3) .the highest similarity value (1:00) was found between (EF 6 &EF 7) ,(EF8 &EF 9) and the lowest value (0.35) was found between (EF 19 &EF 17),(EF 18 ,EF 13),(EF 10&EF 16). All the other show intermediate similiarity percentage between highest and lowest percentage. It should be noticed that some of isolates show lowes similarity percentage &this confirmed the different genetic background between 15 *E. faecalis* clinical isolates under the present study . The similarity between different isolates like vagina & urine, vagina &wound because nosocomial infection that taken from hospital patients.

Table (3-3) Similarity Matrix of 15 studied EF isolated according to RAPD assay. Similarity differences were illustrated above by color of conservation. (6,7,8,9,10 wound, 11,12,13,14 vagina, 15,16,17,18,19,20 urine)

Strain Number	EF-20	EF-19	EF-18	EF-17	EF-16	EF-15	EF-14	EF-13	EF-12	EF_11	EF-10	EF-9	EF-8	EF-7	EF-6
EF-20	1.00	0.89	0.75	0.50	0.61	0.52	0.52	0.57	0.36	0.61	0.45	0.52	0.43	0.52	0.43
EF-19	0.89	1.00	0.78	0.35	0.45	0.45	0.45	0.40	0.38	0.45	0.48	0.45	0.45	0.45	0.45
EF-18	0.75	0.78	1.00	0.60	0.42	0.53	0.63	0.35	0.56	0.53	0.67	0.63	0.63	0.63	0.63
EF-17	0.50	0.35	0.60	1.00	0.42	0.42	0.53	0.47	0.56	0.53	0.44	0.53	0.63	0.53	0.42
EF-16	0.61	0.45	0.42	0.42	1.00	0.56	0.67	0.63	0.47	0.44	0.35	0.44	0.56	0.56	0.56
EF-15	0.52	0.45	0.53	0.42	0.56	1.00	0.89	0.63	0.82	0.56	0.71	0.89	0.89	0.89	0.89
EF-14	0.52	0.45	0.63	0.53	0.67	0.89	1.00	0.75	0.71	0.78	0.71	0.67	0.89	0.78	0.89
EF-13	0.57	0.40	0.35	0.47	0.63	0.63	0.75	1.00	0.80	0.75	0.67	0.75	0.75	0.63	0.50
EF-12	0.36	0.38	0.56	0.56	0.47	0.82	0.71	0.80	1.00	0.71	0.75	0.71	0.82	0.82	0.71
EF-11	0.61	0.45	0.53	0.53	0.44	0.56	0.78	0.75	0.71	1.00	0.82	0.89	0.89	0.89	0.67
EF-10	0.45	0.48	0.67	0.44	0.35	0.71	0.71	0.67	0.75	0.82	1.00	0.94	0.94	0.82	0.94
EF-9	0.52	0.45	0.63	0.53	0.44	0.89	0.67	0.75	0.71	0.89	0.94	1.00	1.00	1.00	0.89
EF-8	0.43	0.45	0.63	0.63	0.56	0.89	0.89	0.75	0.82	0.89	0.94	1.00	1.00	1.00	1.00
EF-7	0.52	0.45	0.63	0.53	0.56	0.89	0.78	0.63	0.82	0.89	0.82	1.00	1.00	1.00	1.00
EF-6	0.43	0.45	0.63	0.42	0.56	0.89	0.89	0.50	0.71	0.67	0.94	0.89	1.00	1.00	1.00

The distribution of isolates with various origin was random in RAPD clusters. This may indicate some role of a disease host in selecting for specific types or some genotypes causing clinical infection .it is interesting to note that these isolates clustering in togather in RAPD analysis were isolated from patients suffering from different disease (UTI, vaginitis &wound infection).

The result suggest that the selected enterococcal isolates have a clonal distribution between them &there for have high & low genetic diversity & may

belong to the same source of infection. The result indicate that these isolates are very closely related &hence have clonal relationships between them.

The almost identical RAPD patterns indicate that the selected isolates are very closely related to each other or may belong to genetically similar isolates.

However, the cluster analysis program were generated some genetically distant isolates with a difference at the gene level from different bacterial source such as urine, vagina, &wound infection based on RAPD-PCR pattern similarity. The genetic diversity of chromosomal DNA may be explained by difference in the banding patterns among the isolates .

E. faecalis isolated from same source were clustered in to different groups ,and had cross with other isolates source .On the other hand ,the result revealed that some isolates in cluster style which can be classified in distint mode .

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 .

The presence of several isolates with the same pattern can be due to the various reason , such as transferring an isolate from one patient to another patient via hand contact or from contaminated environment by drinking dirty water or eating food that is contaminated by stool .

Furthemore , this study showed that the isolates responsible for infection in the hospital 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 hospital in AL-Hilla .High genetic diversiry among isolates may contribute to the survival of various enterococci isolate .Hospital procedures or equipment might have lead to spread of these isolates among admitted patients . Hence , the knowledge of genetic diversity of bacterial isolates associated with aregion is

important for finding the source of infection in the case of epidemic and nosocomial infection .

In the current study, it was found that the genetic diversity in *E. faecalis* depend on source of isolation and occurrence of mutants. However, the data acquired in the present work confirm the wide genotypic diversity of *E. faecalis* from various clinical specimens . It is interesting to note that, there was no evident correlation between the observed strain variability and the specimen from which the isolates originated.

So, the present study reveals that, *E. faecalis* is not specific for infections. This clearly shows that, one can't use the same drug for a particular infection caused by *E. faecalis* because of their variation in DNA polymorphism. Therefore further study on antibiotic sensitivity and sequence analysis would help to devise and prescribe a better drug for the future.

Genotypic methods are more accurate and faster than the phenotypic ones. RAPD-PCR is one of the known genotyping methods which has been widely used in various epidemiological investigations.

Random amplified polymorphic DNA PCR analysis in present study revealed that the isolates from similar or different hospital has high relatedness in their genetic linage as reflected in the cladogram clustering of the isolates, to certain extent, there may be an association between isolates in some of the genetic clusters, but most of respective isolates were distantly clustered, suggesting along linage of common origin. Although, the isolates located in the same cluster are isolated from different source but the results of RAPD give one important evidence on the closed relative of these isolates.

Although the number of isolates studied was not large, this work has shown that the specimens collected are highly diverse. This is due either to a dynamic

evolution of the local strains of the organism or to the continuous introduction of new isolates from abroad.

Although relatively few specimens were used in the study, the data suggest that RAPD typing is discriminatory; power for differentiation of *E. faecalis* isolates , it is easy to interpret and constitute a low-cost method to type the various *E. faecalis*. These observations revealed that RAPD profile could be best used for finding out the heterogeneity at the molecular level of *E. faecalis* isolates in combination with other molecular and phenotypic typing techniques.

The applied RAPD-PCR method was also reported to be sensitive and practical for the molecular typing of clinical isolates of *E. faecalis* (Banerjee *et al*., 2013; Emaneini *et al*., 2016).

RAPD-PCR analyses are a widely accepted and reliable tool for differentiating and identifying enterococci (Domig *et al*.,2003). For example, (Monstein *et al*.,1998) described its reliability for differentiating and identifying enterococci.

Many fingerprinting methods have been applied to study the microbial biodiversity. RAPD and RFLP have been shown to be reliable tools for microbial identification and typification (Sambrook *et al*.,2005; Martín-Platero *et al*., 2009). However, grouping of the same strains in different clusters based on the employed techniques has also been previously reported (Martín-Platero *et al*.,2009; Zoletti *et al*.,2011).

Ahmad *et al*., (2014) RAPD-PCR was used to characterize 35 selected strains of *E faecalis* producing a total of 23 RAPD unique profiles. This result indicated a high variability of enterococci sub-species diversity among the *E. faecalis* strains. The high variability observed is in concert with the findings of another study (Son *et al*., 1999) where 19 RAPD-types were reported from a total of 19 tested *E. faecum*

isolates. In a microbial source tracking attempt by (Martin *et al.*,2009), over ninety RAPD-types were reported albeit based on a library of 596 enterococci isolates. RAPD is an inexpensive, efficient, and sensitive alternative typing method for recognizing genetic differences between closely related bacteria.

However, its application has a number of limitations. Such problems with reproducibility and discriminatory power, are surmountable by precise optimization procedure allowing the achievement of reliable conditions for each species analyzed (Gzyl and Augustynowkz, 1998). Apart from initial species specific evaluation of the RAPD working conditions conducted in the study, two main primers (M13 and D8635) were screened and one retained based on the achievement of stable and informative amplification patterns for the purpose of discrimination among the tested *E. faecalis* strains.

A number of studies have also adopted RAPD-PCR as an important tool to indicate patterns of niche-specific associations of enterococci strains and to provide evidence that enterococci sub-species associate with specific environment (Son *et al*, 1999; Anderson, 2005; Rathnayake *et al*, 2011).

3.7 Multilocus sequence typing of *E. faecalis* :

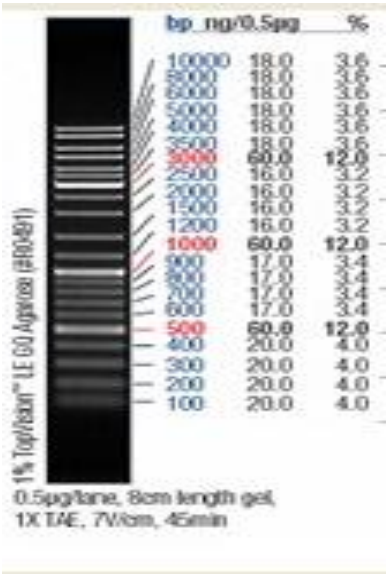
One of the new and valuable diagnostic techniques in molecular epidemiologic investigations is multilocus sequence typing (MLST) for the typing of multiple loci. In this technique, housekeeping genes along with other conserved genes are analyzed according to the nucleotide variation for the characterization of bacterial pathogens (Chang *et al.*, 2016).

The MLST was developed as a scalable typing system to determine the diversity and phylogenetic relationships of the isolates based on five housekeeping

genes, and it provide reproducibility, comparability, and transferability between laboratories.

The sequence data obtained for MLST for determining the population structures analyzing the extent of linkage disequilibrium between alleles for phylogenetic relationships between 15 isolates. Gel electrophoresis for each gene shown in **figure(3-6).**

	1	2	3	4	5	6	7	8	9	10
										18-EF-4
B	7-EF-1	15-EF-1	8-EF-5	16-EF-5	9-EF-2	17-EF-2	10-EF-3	18-EF-3	11-EF-4	19-EF-4
C	8-EF-1	16-EF-1	9-EF-5	17-EF-5	10-EF-2	18-EF-2	11-EF-3	19-EF-3	12-EF-4	20-EF-4
D	9-EF-1	17-EF-1	10-EF-5	18-EF-5	11-EF-2	19-EF-2	12-EF-3	20-EF-3	13-EF-4	
E	10-EF-1	18-EF-1	11-EF-5	19-EF-5	12-EF-2	20-EF-2	13-EF-3	6-EF-4	14-EF-4	
F	11-EF-1	19-EF-1	12-EF-5	20-EF-5	13-EF-2	6-EF-3	14-EF-3	7-EF-4	15-EF-4	
G	12-EF-1	20-EF-1	13-EF-5	6-EF-2	14-EF-2	7-EF-3	15-EF-3	8-EF-4	16-EF-4	
H	13-EF-1	6-EF-5	14-EF-5	7-EF-2	15-EF-2	8-EF-3	16-EF-3	9-EF-4	17-EF-4	



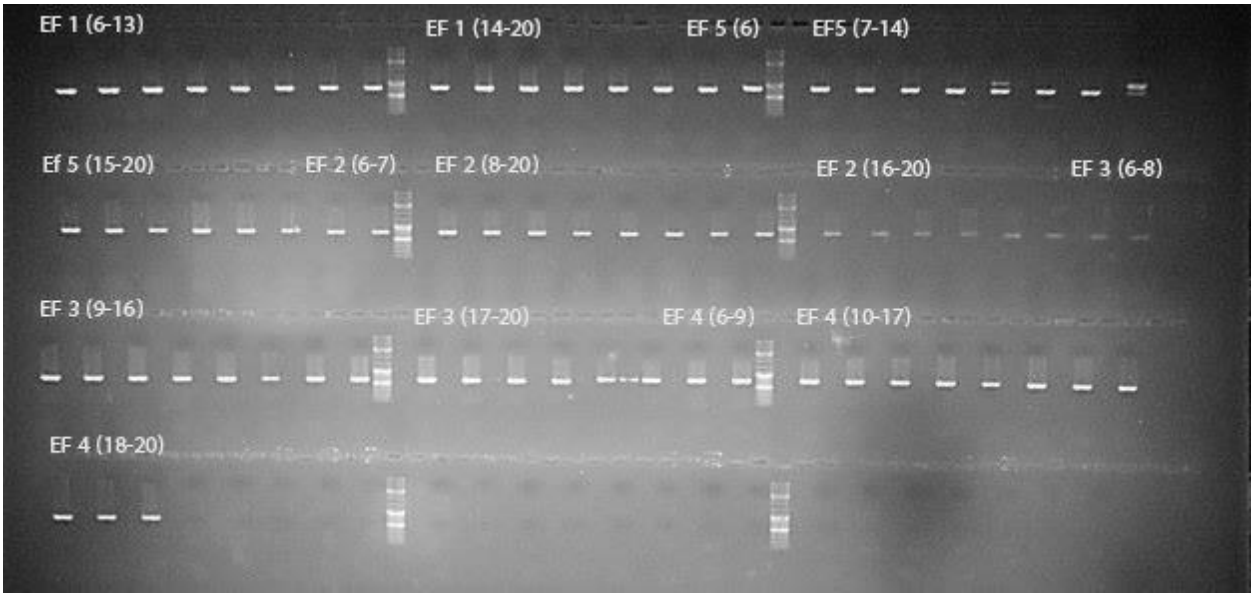


Fig. (3-6) :1% agarose gel electrophoresis for housekeeping genes for 15 *E. faecalis* isolates.

To identify each locus accurately ,it was used sequence method .For all isolate ,the five gene were successfully sequenced and analyzed by MLST ,Polymorphic site ,GC content ,K (rate of nonsynonymous (dN) ,synonymous (dS) substitutions and the nucleotide diversity for each locus (*pepC , clpX , recA , rpoB, & groEL*)were determined as shown in **table (3-4)**

Table (3-4). Nucleotide and allelic diversity of the 5 housekeeping genes evaluated

Locus	Size (bp)	Alleles	Polymorphic sites	GC content (%)	Nucleotide Diversity	*k*	*Codon Bias*
pepC	572	4	5	35	0.00117	0.66667	0.466
clpX	619	4	8	37	0.00300	1.848	0.413
recA	557	2	2	43	0.00048	0.26667	0.605
rpoB	565	7	22	40	0.00999	5.55238	0.336
groEL	479	4	8	40	0.00522	2.47619	0.919

k : Average number of nucleotide differences.

The mean GC content of sequnces of five gene fragments ranged from 35%(*pepC*) to 43%(*recA*) :Trimmed fragment size of the five selected loci ranged from 479 bp (*groEL*)to 619 bp (*clpX*).The nucleotide diversity ranging from 0.00048

to 0.00999 per gene . Moreover , the number of polymorphic site per locus ranged from 2 (*recA*) to 22(*rpoB*) and harbored a total of 45 SNP.

The proportion of nucleotide substitution that changed the amino acid sequence (nonsynonymous base substitution [dn] and the proportion that did note synonymous base substitution [ds]) were calculated the ratio (dn/ds) measure the level of selection in a protein coding gene. The ratio of dn/ds indicates purifying selection if dn/ds <1, positive selection if dn/ds >1. However, the high ratios of nonsynonymous to synonymous substitution indicat a role for diversitying selection these loci ,and the d_N/d_S ratios which indicated negative selection were determined to be less than 1 for the *pepC & recA* genes while were determined to be more than 1 for the *clpX, rpoB &groEL* genes which have indication of positive selection .

MLST protocal had sufficient discriminatory power to type isolates within asingle species , it was analyze sequence diversity of five housekeeping genes from is *E. feacalis* isolates . four loci had low polymorphism (*pepC, clpX, recA, & gerEL* loci) indicated that they had similar sequence at the species level . The *rpoB* had 22 sites suggesting recombination was evident &representing a significant source of genetic diversity of *E. faecalis* , fifteen *E. faecalis* isolates were typed using MLST protocol.In present study, MLST was used to explore the population structure & evolution of 15 *E. feacalis* isolates from different clinical specimens which may provide better information concerning their biological properties. To initiate analysis ,the sequence diversity of the 5 housekeeping genes was calculate . This step was carried out to measure whether these selected loci had sufficient typing discrimination. the number of alleles in these gene loci ranged from 2 to 7 compared with nucleotide sequence diversities reported in *E. feacalis.*

E. feacalis housekeeping genes have similar but low nucleotide diversity values in general, reflacting that these genes are conserved.

According to allelic profile it was found that the presence of allelic variant (SNP, insertion, or deletion) between isolates. In the case *rpoB* was more variant or mutant than other 4 housekeeping genes, contrary to the *recA* which was the least variant. The gene chosen for the present MLST scheme seem to be representatives of the general polymorphism seen in housekeeping genes of *E. feacalis*.

The polymorphic sites found in the present sequence data might therefore be useful in the development of a molecular bacterial typing scheme based on detection of single nucleotide differences.Isolates could be divided into 15 ST using combined data from S loci as shown in **table (3-5).**

Table (3-5): Allelic profiles based on 5 housekeeping genes from all isolates of *Enterococcus faecalis* evaluated

Sequence type	Sample N.	*rpoB*	*recA*	*pepC*	*groEL*	*clpX*
1	20-EF	1	1	1	1	1
2	17-EF	1	1	1	1	2
3	15-EF	3	1	1	1	2
4	10-EF	2	1	2	3	2
5	11-EF	2	1	1	3	2
6	9-EF	6	1	1	3	1
7	12-EF	5	1	1	2	3
8	18-EF	1	1	2	3	4
9	7-EF	4	1	2	3	2
10	16-EF	6	1	2	1	2
11	19-EF	2	2	1	3	3
12	6-EF	5	1	2	3	2
13	8-EF	6	1	1	3	2
14	14-EF	7	1	4	2	2
15	13-EF	7	1	3	4	2

In MLST different sequence at each locus are assigned with specific allelic profile and assigned as a sequence type which is the unambiguous descriptor of the strain. To our knowledge, this is the first study report describing the development and use of MLST of *E. feacalis* to characterize this important human pathogen in Iraq. The present study ST (ST1 to ST15) were detected, these could be unique to

the region, further analysis, possibly sequencing the gene of these strains could help to define the characteristics of these ST.

However the variation between this study and other studies due to the limited number of isolates, or may be due to increased travel or undergoing natural accumulation of sequence variation in housekeeping genes. Moreover, isolates obtained from diverse geographical locations, and during extended periods of time may give more genetic variability.

The DNA sequence of each of the five genes were analyzed by the maximum likehood method to be well suited for determination of phylogenetic relationships among *E. feacalis* isolates the housekeeping genes are assumed to be suitable for a population genetic study.

Phylogenetic relationships among *E. feacalis* isolates were **shown in figure (3-7)**, the DNA sequence were aligned and analyzed for each gene fragments. The phylogeny of these ST, an MLST phylogenetic tree of all the *E. feacalis* strains was inferred maximum like hood approach from concatenated sequence. All *E. feacalis* isolates showed polyphyletic lineage and revealed three distinct clusters, cluster A contain one isolate (13EF), cluster B contain one isolate (14EF) and cluster C contain 13 isolates, and this cluster was divided into subclusters.

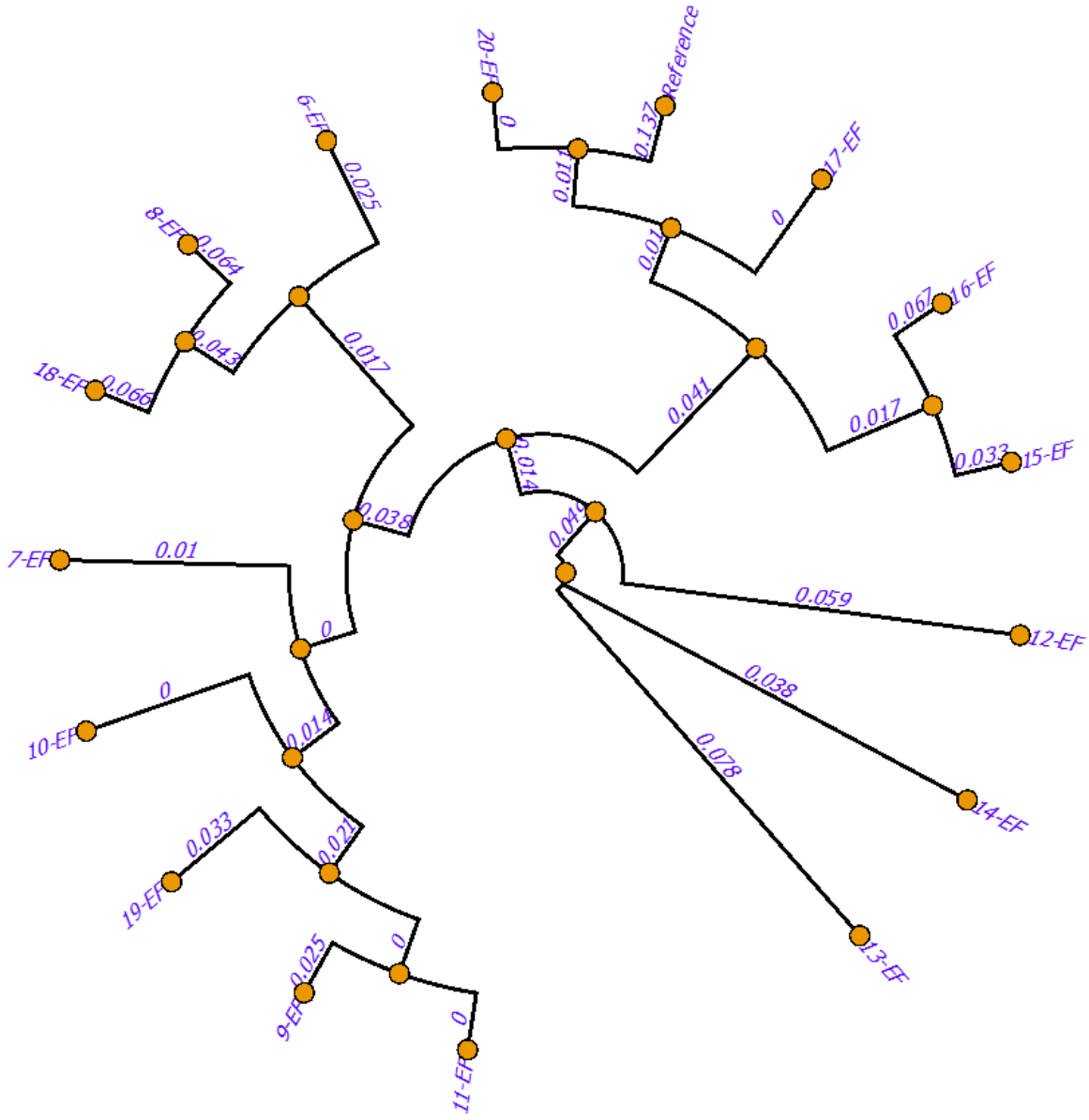


Fig. (3-7): Circular phylogenetic analysis based on concatenated sequences of 5 housekeeping genes from 15 *Enterococcus faecalis* isolates by maximum likelihood method, and comparison with the concatenated Reference sequences (NC_004668).

The phylogenic relationship between bacterial organism, potential problems associated with its ability to resolve the relationship between closely related sp. due to an extremely low rate of natural mutation.

The present finding provided strong evidence that *E. feacalis* strains possess a high level of temporal stability and phylogeographical structuring, supported largely by the phylogeographical signals observed in the phylogenic tree. However, the phylogeny tree for each gene was **shown in appendix (1).**

Splite decomposition analysis was performed on each locus separately and on the concatenated sequences of all ST ,as shown in the split graphs **figure (3-8)**.

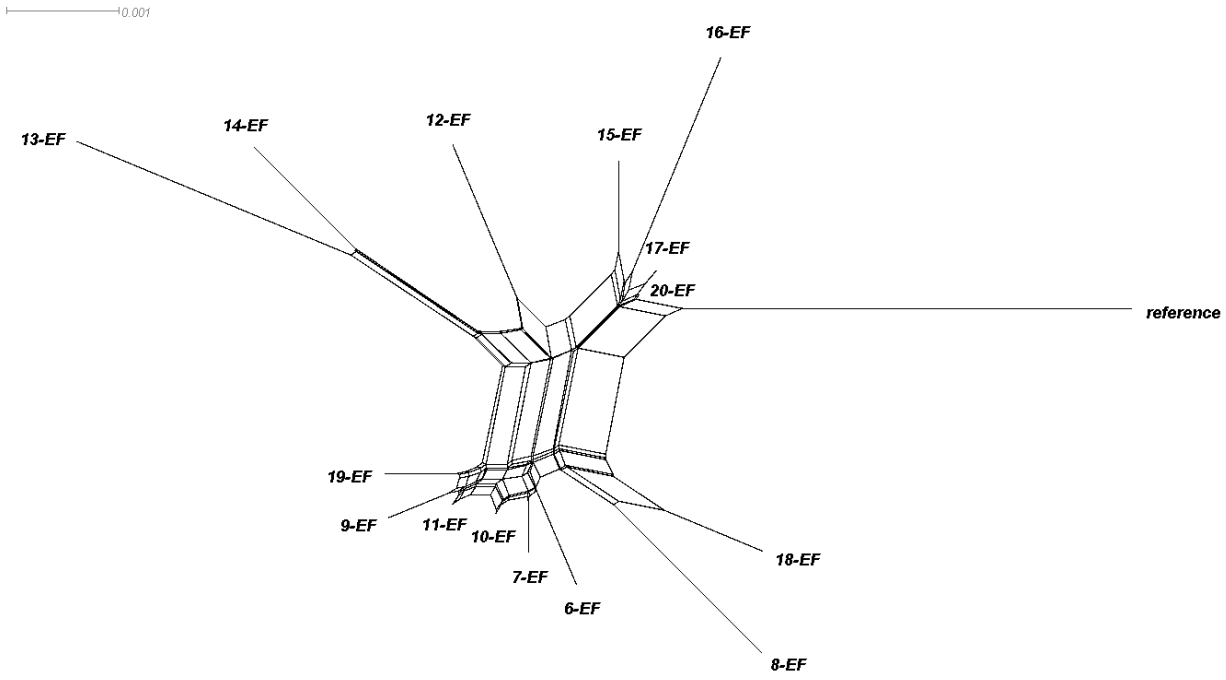


Fig. (3-8): Split-decomposition analysis based on concatenated sequences of 5 housekeeping genes from 15 *Enterococcus faecalis* isolates and comparison with the concatenated Reference sequences (NC_004668) . Note: multiparallelogram formations indicate recombination events.

The split graphs for *clpX* , *groEL & rpoE* revealed network like with parallelogram structures indicating that intergenic recombination had occurred during the evolutionaty history of these genes . However, the split graphs of *pepC &recA* are tree like structures suggesting that the descent of these genes was clonal and absence of recombination. The split decomposition analysis of combined five

MLST Loci display network like structure with rays of different length as shown in **figure (3-9)**.

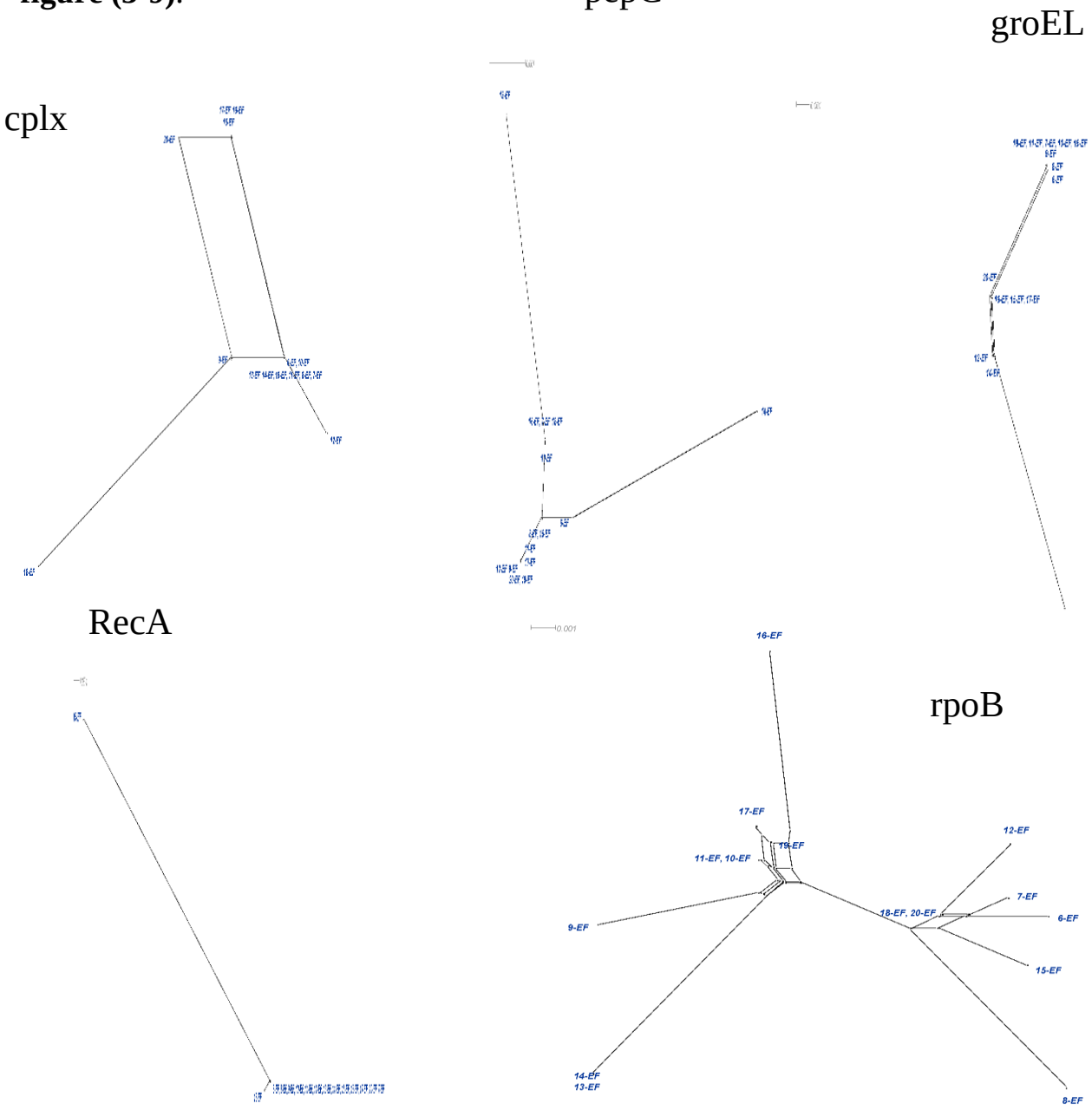


Fig. (3-9): Split-decomposition analysis based on *cplx* , *groEL*, *pepC*, *RecA*, *rpoB* gene sequences of 5 housekeeping genes from 15 *Enterococcus faecalis* isolates. Note: multiparallelogram formations indicate recombination events.

The 15 STs representing all isolates divided into subpopulation was completely disconnected. Split decomposition analysis based on the allelic profiles of isolates have provided evidence of recombination that play a role in generating genotyping diversity among isolates.

In this study, tree like structures or parallelogram. Shaped structures were commonly found in the split graphs for all the five housekeeping genes evaluated, illustrating that recombination had occurred in these MLST loci.

eBURST is an algorithm that can identifies groups of closely associated sequence types from MLST data. It was used to analyze the possible similarity, variability and evolutionary relationships among different ST types of *E. faecalis* in the present study. The genetic backgrounds were found to be diverse among the STs identified in the present study. As shown in **figure (3-10)** 2cc(cc1&cc2) were observed for the 15 ST. Among the 2 cc, cc1 was the largest and comprised 8 link ST, namely ST1, ST3, ST2, ST5, ST7, ST11, ST13 & ST6. These included four isolates from urine, two isolate from vagina &two isolates from wound. Clonal complex 2, representing 7 ST, which includes two isolate from urine ,two isolates from vagina &three isolates from wound .Therefore ,it is unsurprising that the relationship between the isolates from clinical different source was closely related. Often, only some isolates of the same source or location were clustered to gather, whereas the rest were dispersed across other clusters.

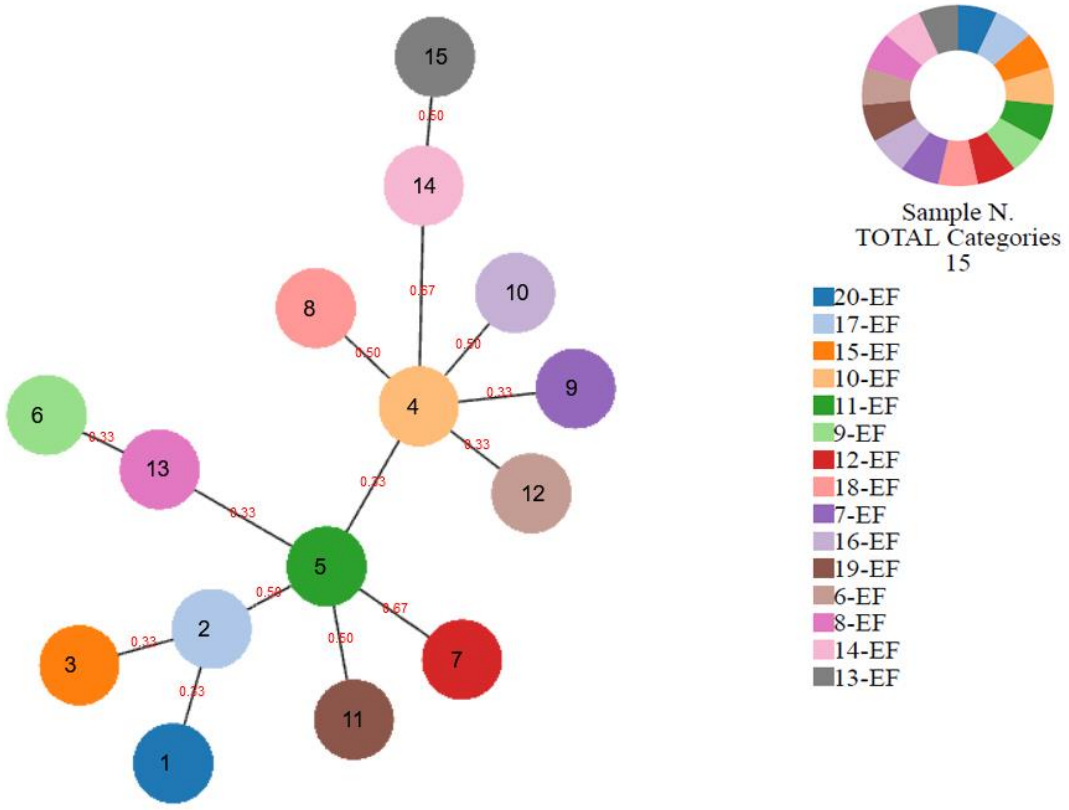


Fig (3-10): Comparative eBURST analysis showing the clonal assignment and the relative distances of the identified STs

The sequence of fragments of five housekeeping genes from 15 of *E. faecalis* isolates provide data that can be used to address aspects of the population & evolutionary biology of the species.

E. faecalis frequent horizontal DNA exchange has eliminated the phylogenetic signal in each housekeeping gene. We determined the degree of allelic variations in five housekeeping genes of *E. faecalis* by using a sample of 15 isolates originated from different clinical specimens. The degree of isolates differentiation by MLST appear adequate for use in epidemiological investigation, as the number of different types obtained by MLST. Single locus phylogenetic tree were noncongrunent , suggesting that recombination plays a role in the generation of diversity of *E. faecalis* population .MLST was performed for only 15 isolates to

determine the STs due to its high cost &labor intensive. The high genetic variability amongst enterococci isolates in this study provides some information on the local dissemination and genetic relatedness.

The MLST was developed as a scalable typing system to determine the diversity and phylogenetic relationships of the isolates based on five housekeeping genes, and it provide reproducibility, comparability, and transferability between laboratories.

Most previous studies showed that MLST was useful to accurately identify bacterial lineages, but few studies have considered the relationships between isolates &their source. In this study, we used MLST to type *E. feacalis* isolated from different clinical specimens and look for relatedness to isolates that were pathogens. These representative isolates were unique in their diversity of sources and provide some necessary information required to understand genetic diversity persistence & movement in this species.

Results of this study indicates that the majority of *E. feacalis* studied may have descended from ancestor that exist many years ago. However, genetic variation in pathogen population is a major barrier to disease control.

MLST as a tool for epidemiological studies to investigate the evolutionary pathogen and clonal lineages of bacteria. MLST differentiated strains into sequence of 5 housekeeping genes with appropriate level discrimination using allelic differences.

The study was showed the genetic relationships between the *E. feacalis* clones. The presence of dominant clone in the samples of hospital showed the presence of shared infection source among the patients.

The genetic diversity among these strains may be related to gene deletion, insertion, duplication, within-patient mutations, or high rate of horizontal gene transfer mechanism.

Although the number of isolates studied was not large, this work has shown that the specimens collected are highly divers. This is due either to a dynamic evolution of the local strain of the organism, or to the continuous introduction of new isolates from abroad. This study provide valuable information that is important for the understanding of the poor adaptation of *E. feacalis* with uncommon STs, which may otherwise be capable of disseminating globally.

The data of this study have public-health implication, where the high diversity and emergence of a new clone of *E. feacalis* in Babylon province calls attention for the epidemic and the recognition of the strains with new clones that could protrude in the future, that in turn very important for understanding the evolution.

The high genetic variability amongst enterococci isolates in this study provide some information on the local dissemination & genetic relatedness.

MLST is based on allelic variation in housekeeping genes, and while it monitors change over just a small portion of the genome, its highly discrimination and provides insight into genetic structure. Also, it reveals highly detailed information on genetic changes in specific housekeeping genes, and thus provides direct insight into evolutionary changes of the core genome. In addition, MLST proved useful for detection of novel and previously known strain and for inferring relatedness among isolates.

Generally, molecular typing methods are intended to tackle two different levels of epidemiological problems, which reflect different insights toward solving a local or global epidemiology in different timeframes. In one hand localized outbreak of disease in a short period of time should be assessed and on the other,

relation between strains causing a disease in one geographic area with those observed around the world during a longer period would be investigated. These two different conceptual views demand different appropriate scheme of molecular typing, so that isolates recorded in same molecular type are likely to be descended from a younger ancestor and those belonging to more distant ancestors are expected to differ in type unless a relative higher clonal population would be under study.

High diversity in *recA* alleles and evidence for frequent recombination at this locus has been observed in previous studies (Han *et al*., 2014; Chen *et al*.,2015). While study by Sun *et al*., (2015), Were found that the housekeeping loci are all under negative selection.

This result was similar to that of previous studies. Ruiz-Garbajosa *et al*., (2006) provided evidence that recombination was an important mechanism driving genetic variation in the *E. faecalis* isolates they evaluated.

MLST scheme provides an excellent tool for investigating local and short-term epidemiology as well as global epidemiology, population structure, and genetic evolution of *E. faecalis* (Ruiz-Garbajosa *et al* .,2006).

It is true that the incorporation of virulence genes, as described in the previous *E. faecalis* MLST schemes (Nallapareddy *et al*.,2002; Nallapareddy *et al*.,2005), may improve epidemiological resolution. Nevertheless, selection on these genes may also obscure patterns of evolutionary descent in phylogenetic studies. Furthermore, adding rapidly evolving genes, like virulence genes, identifying microvariation may lead to a scheme that is too discriminatory for long-term and global epidemiology, since it may reduce the capability of grouping isolates with common features (e.g., hospital-adapted clones) from different time periods and continents in common globally distributed lineages. *E. faecalis* obtained from

different epidemiological sources (hospitalized patients and community) frequently shared identical STs and grouped together in common complexes. Despite this alleged random dispersion of human clinical and surveillance isolates, two of the major complexes (CC2 and CC9) contained almost exclusively hospital-derived isolates, suggesting they were well adapted to persist in the hospital environment (Nallapareddy *et al*.,2005).

The data resulting from the application of MLST to explore the population structure of *E. faecalis* reveal an epidemic structure where recombination plays an important role and two major hospital-adapted CCs have emerged. However, more studies are needed to improve our understanding of the population biology and the mechanisms involved in horizontal genetic transfer and recombination in *E. faecalis*. With this MLST scheme, comparison of population structures of different enterococcal species is becoming a reachable objective that should provide new insight into the evolutionary history of this important group of bacteria (Ruiz-Garbajosa *et al* .,2006).

eBURST is an algorithm that can be used to subdivide MLST data into nonoverlapping groups of STs with a user-defined level of similarity in their allelic profiles (Feil *et al*.,2004). The most stringent definition of an eBURST group, where all STs assigned to the same group must share alleles at least five of the seven MLST loci with at least one other ST in the group, identifies clusters of closely related genotypes that are considered to be descended from the same founder and that are defined as clonal complexes (Feil *et al*.,2004).

The total number of STs within each clonal complex identified by eBURST was rather low and probably reflects sampling strategy. In general, eBURST may identify few clonal complexes, and few large clonal complexes, in populations where sampling has largely been designed to uncover the genetic diversity within the species (Spratt *et al*.,2001).

eBURST analysis of MLST datasets of highly recombinogenic species results in a single large straggly eBURST group, which results from the incorrect linking of unrelated groups of strains (Turner *et al*.,2007).

In particular, the Multilocus Sequence Typing (MLST) is an ordinary typing method that is based on the characterizing bacterial species via sequencing of internal fragments of multiple housekeeping genes. Usually, seven housekeeping gene evaluate by the MLST method via the internet (Kalia *et al*.,2001;Willems *et al*.,2001). In this process, each sequence of internal fragments compares with the other alleles that they were already characterized. Then, each sequence classified at one of those seven housekeeping genes category. Ultimately, by the combination of obtained data, the allelic profile will be construct and each distinct profile consider as absolute sequence type.

In a study by (Ruiz-Garbajosa *et al.*,2006) in 110 different *E. faecalis*, 55 STs were obtained that showed the diversity of samples and low possibility of common accient in *E. faecalis*. Other study by Zalipour and collegues (2002) in Isfahan, among 53 *E. faecalis* isolates, 8 different STs obtained that ST 6 and 422 were dominant. Nallapareddy and *et al.*,(2002) were demonstrated among 22 different *E. faecalis*, 13 STs were obtained that showed the diversity of isolates.

Diverse STs of *E. faecalis*, including strains associated with common nosocomial infections are circulating in the healthcare facility of Saudi Arabia and carried multi-drug resistance, which has important implications for infection control (Muhammad *et al*.,2019).

Different allelic profile in our isolates showed a decrease of possiblity of samples to be from a clonal lineage. So, elaborating the relationship among the *E. faecalis* isolates from different hosts is becoming increasingly important.The ability for *E. faecalis* to jump into different host types is a major threat to public health.

The high genetic variability amongst enterococci isolates provides some information on the local dissemination and genetic relatedness, as well as the antibiotic patterns of our enterococcal isolates (Poh LengWeng *et al*.,2013).

3.8 Clustered regularly interspaced short palindromic repeats (CRISPRs):

In this study , we determined the occurrence of CRISPR loci in *E. faecalis* isolated from different infectious source (urine ,vagina &wound) . The distribution & the frequency of three different CRISPR- cas elements in *Enterococcus faecalis* isolates is presented in **table (3-6) & figures (3-12)(3-13)** .

Table (3-6): Identification of Crisper I, Crisper II and Crisper III of *E. faecalis* in all studied samples

Results	Crisper I N (%)	Crisper II N (%)	Crisper III N (%)	P value
Positive	3 (20)	8 (53.3)	5 (33.3)	<0.0001*
Negative	12 (80)	7 (46.7)	10 (66.7)	
Total	15	15	15	

* represent a significant difference at p<0.05.

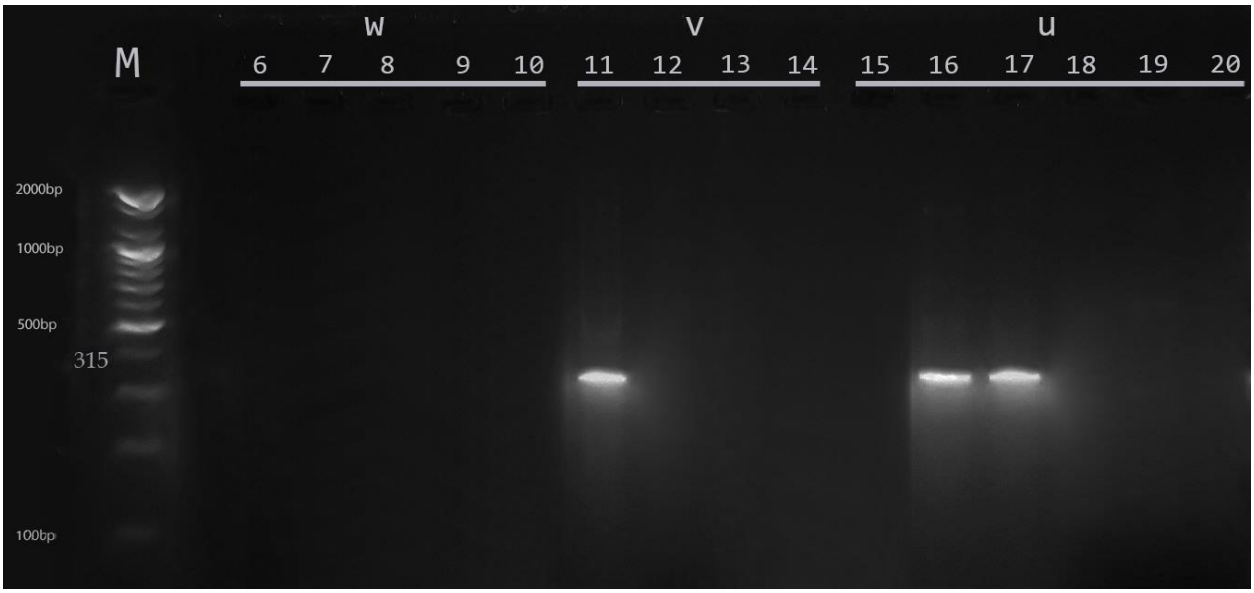


Fig. (3-12) :Identification of CRISPR1 – Cas genes of *E. faecalis* in patients with different sample sources.

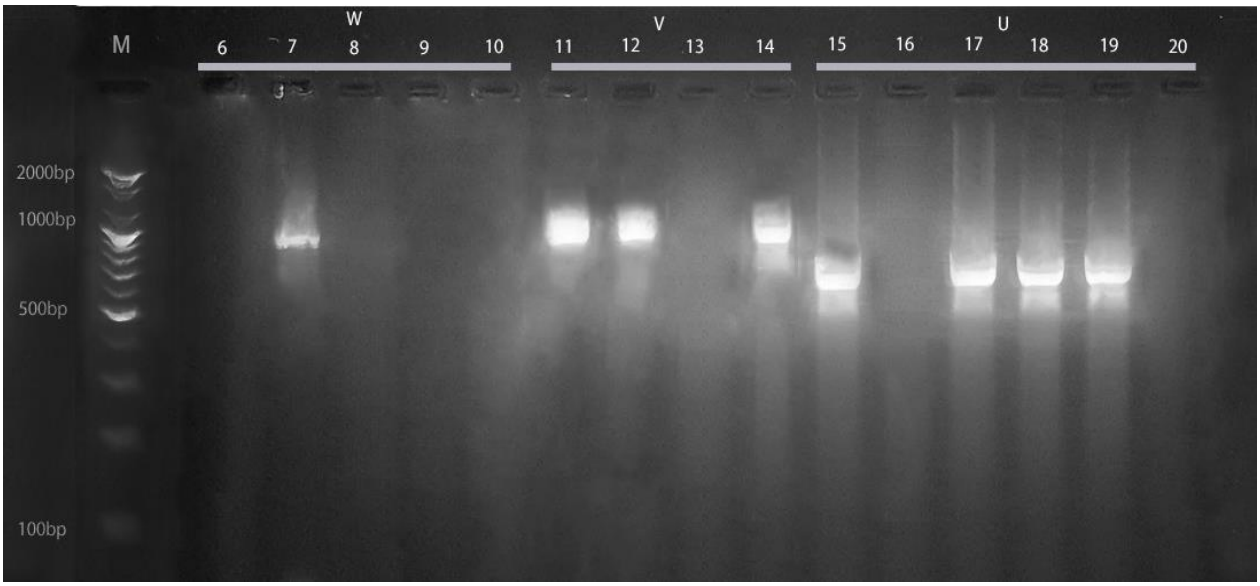


Fig. (3-13) :Identification of CRISPR2 – Cas genes of *E. faecalis* in patients with different sample sources.

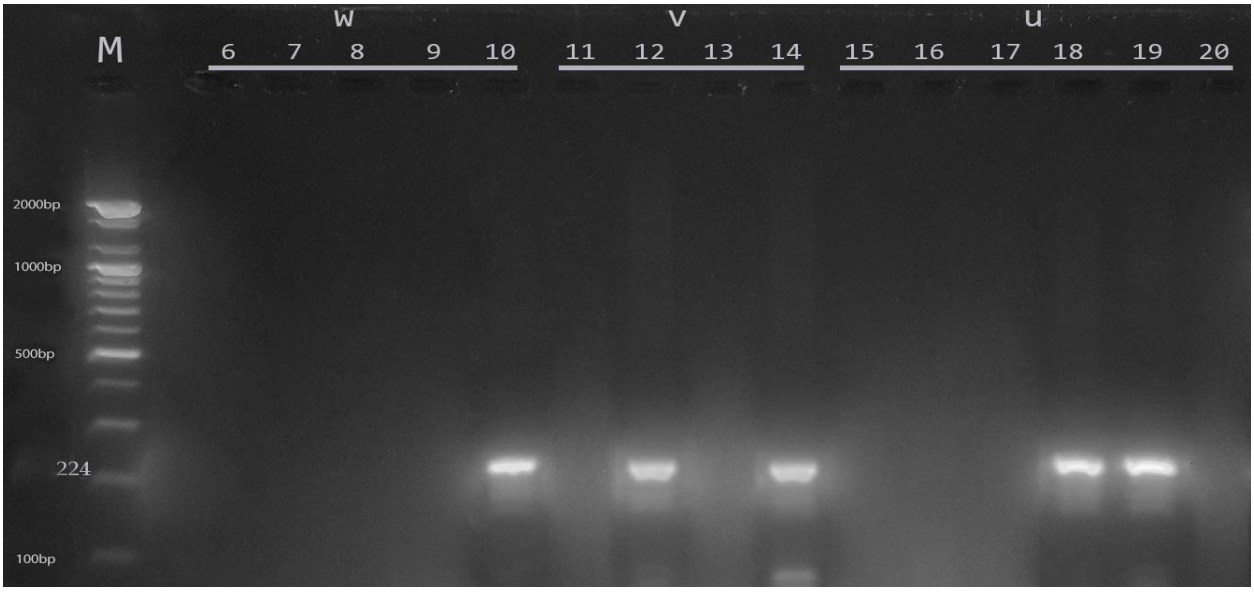


Fig. (3-14) :Identification of CRISPR3 – Cas genes of *E. faecalis* in patients with different sample sources.

Over all , CRISPR2 was identified in 8(53.3%) of the isolates followed by CRISPR3- Cas and CRISPR1 – Cas (33.3 & 20% respectively). The current study was demonstrated that the presence of CRISPR loci was variable among *E. faecalis* isolates . The isolates were more likely to harbor orphan CRISPR2 than CRISPR1 – cas & CRISPR3 – Cas .

Difference in the distribution and the frequency of CRISPR (CRISPR 1 – Cas, CRISPR2, & CRISPR3 – Cas) in different clinical isolates of *E. faecalis* were observed in **table (3-7)** .

Table (3-7): Identification of Crisper 1cas, Crisper 2 and Crisper 3 Cas genes of *E. faecalis* in patients with different sample sources.

Sample Sources	Crisper I N (%)	Crisper II N (%)	Crisper III N (%)
Wound	0 (0)	1 (12.5)	1 (20)
Vagina	1 (33.3)	3 (37.5)	2 (40)
Urine	2 (66.7)	4 (50)	2 (40)
P value	0.001*	<0.0001*	0.018*
Total	3/15	8/15	5/15

* represent a significant difference at p<0.05.

The presence of CRISPR loci among the UTI *E. faecalis* (8) isolates was higher than for vagina (6) isolates & wound (2) isolates which were multidrug resistance isolates. The reason of higher presence of CRISPR loci among UTI isolates is not clear but could confer antibiotic resistance to them.

Alduhaidhawi *et al*.,(2022) were revealed the incidence of 50.0%, 78.0%, and 36.0% for CRISPR1, CRISPR2, and CRISPR3 in *E. faecalis* isolates, respectively. In a previous report by Gholizadeh *et al*.,(2020) from Iran, the attendance of CRISPR1-cas, orphan CRISPR2, and CRISPR3-cas was seen in 13%, 55.3%, and 17.4% of clinical *E. faecalis*, respectively. Also, in another study from Iran, the prevalence rates of 53.4%, 17.7%, and 10.4% were reported for CRISPR2, CRISPR3, and CRISPR1 in *E. faecalis* isolates from urinary tract infections (UTI) and dental-root canal (DRC) samples (Gholizadeh *et al*.,2021). These discrepancies in our & other results of various studies can be due to the sample size, the source of the sample collection, and the technique used in the screening of CRISPR-Cas systems.

Palmer *et al*., (2010); Hullahalli *et al*., (2017) suggested that CRISPR2 is functional for sequence interference and is functionally linked to CRISPR1-Cas or CRISPR3-Cas. Several studies have reported that CRISPR loci play an inverse role

in some virulence factors and acquisition of antibiotic resistance (Palmer & Gilmore.2010; Lindenstrauß *et al*.,2011; Burley & Sedgley,2012), such as, reporting that CRISPR loci were inversely associated with antibiotic resistance and some virulence factors in *E. faecalis* strains. demonstrated that CRISPR-cas could prevent the acquisition of antibiotic resistance genes in *E. faecalis* and other bacteria.

orphan CRISPR2 is found in all *E. faecalis* strains, but the presence of CRISPR1-Cas and CRISPR3-Cas is varied among strains. In addition, (Palmer and Gilmore,2010) found that CRISPR1-Cas and CRISPR2 are functionally linked to each other.

The lack of required functional genes in strains with the CRISPR2 orphan locus, together with the abundance of antibiotic resistance genes in these strains, indicates that CRISPR2 alone does not confer immunity in *E. faecalis* hosts. Ultimately, a functional analysis of the CRISPR1-cas and CRISPR2 loci will be required to confirm this hypothesis (Kelli and Michael**,** 2010).

Mojica et al., (2005) for instance, have suggested that the pathogenicity of bacteria is largely controlled by conjugative plasmids and bacteriophages on an evolutionary timescale. As well, those CRISPR spacers that target these mobile elements might affect bacterial pathogenicity and virulence traits.

An application for CRISPR-mediated toxicity is in the selective depletion of hospital-adapted strains from heterogeneous populations. CRISPR targeting to distinguish very closely related strains was first demonstrated in E. coli (Gomaa et al., 2014).

The CRISPR system is a diverse defence mechanism towards invading nucleic acids. CRISPR motives are typically located near CRISPR-associated (cas) genes (Sorek *et al*.,2008; Koonin & Makarova,2009).

Distribution of CRISPR-Cas systems differs among isolates from various settings as well as particular strains and clonal complexes of *E.faecalis* (Iwona *et al*.,2015).

Microorganisms are vulnerable to invasion by mobile genetic elements such as viruses, plasmids, and transposons. CRISPR-Cas systems are highly adaptive immune systems present in most archaea and many bacteria that provide intracellular protection against these invading genetic elements (Koonin & Makarova,2009;Makarova *et al*.,2011).

The correlation between the presence of CRISPR – cas & phenotypic antibiotic susceptibility was also studied , CRISPR – cas distribution varies among antibiotic sensitive & antibiotic resistance isolates were shown in **table (3-8)** .

Table (3-8) :Association between Antibiotic sensitivity and the occurrence of CRISPR-I, II, III in *E. faecalis*

Antibiotic		**Crisper I**		**P**	**Crisper II**		**P**	**Crisper III**		**P**
		Present	**Absent**	**value**	Present	Absent	**value**	Present	Absent	**value**
E	R	3	9	0.333	6	6	0.605	3	9	0.171
	S	0	3		2	1		2	1	
K	R	3	12	ns	8	7	ns	5	10	ns
	S	0	0		0	0		0	0	
PRL	R	0	1	0.605	1	0	0.333	0	1	0.464
	S	3	11		7	7		5	9	
TEC	R	0	0	ns	0	0	ns	0	0	ns
	S	3	12		8	7		5	10	
LEV	R	0	2	0.448	2	0	0.155	0	2	0.283
	S	3	10		6	7		5	8	
CIP	R	3	12	ns	8	7	ns	5	10	ns
	S	0	0		0	0		0	0	
VAN	R	3	8	0.243	7	4	0.185	2	9	0.039*
	S	0	4		1	3		3	1	
DA	R	3	12	ns	8	7	ns	5	10	ns
	S	0	0		0	0		0	0	
IMI	R	3	11	0.605	8	6	0.268	4	10	0.143
	S	0	1		0	1		1	0	
REF	R	2	11	0.255	7	6	0.919	4	9	0.591
	S	1	1		1	1		1	1	

NOR	R	3	10	0.448	7	6	0.919	4	9	0.591
	S	0	2		1	1		1	1	

* represent a significant difference at p<0.05. ns: no statistics are computed.

The CRISPR – cas positive *E. faecalis* showed lower resistance rates against almost antibiotic in comparision with CRISPR – cas negative *E. faecalis* isolates. Overall, CRISPR2 was predominant in almost antibiotic susceptible isolates . The presence of CRISPR – cas is associated with the absence of some antibiotic resistance acquired by horizontal gene transfer.

The relationship between CRISPR loci and antibiotic resistance phenotypes and genotypes may provide new insights into combating infections caused by resistant pathogens.

E. faecalis strains with CRISPR-cas possess significantly fewer acquired antibiotic resistance genes than those lacking CRISPR-cas (Kelli and Michael**,** 2010).

The lack of CRISPR-Cas genes was associated with more antibiotic resistance rates and multidrug resistance in *E. faecalis* (Alduhaidhawi *et al*.,2022).

Notably, the presence of CRISPR-Cas elements was lower in MDR isolates compared to non MDR enterococci isolates Gholizadeh *et al*.,(2021) who showed that CRISPR-cas loci are negatively correlated with antibiotic resistance, as well as carrying antibiotic-resistant genes in *E. faecalis* isolates from UTI and DRC samples. Palmer and Gilmore, (2010); Burley and Sedgley,(2012) ; Dos Santos et al.,(2020) who were showed that MDR properties among enterococci isolates were associated with a lack of CRISPR-Cas systems. It is believed that CRISPR-Cas acts as a barrier to the acquisition of antibiotic resistance genes because most of these

genes are commonly spread by plasmids in enterococci isolates (Gholizadeh *et al*.,2021).

significant association between the absence of CRISPR and the presence of antibiotic resistance traits in clinical isolates, versus the presence of CRISPR elements in commensal strains was demonstrated (Palmer and Gilmore,2010). While CRISPR elements are highly diverse and can only be detected in genome sequencing, cas genes exhibit homologies and may serve as PCR-detectable markers pinpointing to active CRISPR elements.

Details are rapidly emerging about the acquisition, mechanisms, and dynamic evolution of the various CRISPR-Cas immune systems and their associated genes (Makarova *et al*.,2011; Terns,2011; Takeuchi *et al*.,2012). From a clinical perspective the absence of a CRISPR-Cas immunity system might facilitate cell survival under certain conditions, eg, by allowing uptake of antibiotic resistance genes in an antibiotic environment, but could also render the cell more vulnerable to attack by other selfish genetic elements (eg, phages). Conversely, possession of a functional CRISPR-Cas system might facilitate survival by way of stabilizing the genome, while allowing the cell to acquire information about the external environment via foreign DNA, integrate this information into the genome, and subsequently pass it on to progeny (Takeuchi *et al*.,2012).

CRISPR-Cas system is one of the factors limiting the development and evolution of bacterial antibiotic resistance. (Gholizadeh *et al*.,2020) CRISPR-Cas is considered as a natural barrier to horizontal gene transfer and transmission of antibiotic resistance (Wheatley *et al*.,2021).

Orphan CRISPR2 is thought to be inactive due to the lack of associated Cas proteins. A study revealed most multidrug-resistant *Enterococcus faecalis* isolates

lack functional CRISPR-Cas and possess only the orphan CRISPR2 (Price *et al*.,2016)

In a study by Price *et al*.,(2016) demonstrated that the orphan CRISPR2 locus requires the presence of CRISPR1-Cas from *Enterococcus faecalis* for genomic defense against mobile genetic element MGE.

Antibiotic resistance genes are mainly transmitted through HGT (McInnes *et al*.,2020). The acquired immune system CRISPR-Cas is considered as a barrier to HGT (Zheng *et al*.,2020).

Several studies have shown that the presence of the CRISPR-Cas system is inversely associated with the incidence of bacterial resistance (Pursey *et al*.,2022).

Shuan *et al*., (2022) the study revealed that the CRISPR-Cas system of *Enterococcus faecalis* has a higher carriage rate concluded that CRISPR-Cas system may hinder the transmission of antibiotic resistance genes, which provides a reference for the prevention and control of enterococcal nosocomial infection.

Conclusions and Recommendation

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* were shown and have different behavior of resistance against to one or more of antibiotic.

3. The RAPD method was cheap, affordable & powerful means for microbiologist to find out relatedness of *E. faecalis* isolated from different clinical specimensn. However , this genotype gave a significant assumption for evolution ancestral gene

4. MLST has emerged as an important tool to study the long-term epidemiology and the population structure and patterns of evolutionary descent. The high genetic variability amongst enterococci isolates in this study provides some information on the local dissemination and genetic relatedness, as well as the antibiotic patterns of our enterococcal isolates.

5. Notwithstanding high discriminatory power, nucleotide changes accumulate in housekeeping genes in long period of time. That is why the allelic profile of isolates persists unchanged over a longer timeframe, which make MLST a desirable tool for global epidemiology.

6. Molecular tool such as CRISPR –Cas may be promising alternative in control of hospital spread . Isolates that do not have this system are more susceptible to acquiring resistance.

7. It was found that *E. faecalis* carry different types of CRISPR-Cas system , the highest type was CRISPR 2 flowed by CRISPR 3 & CRISPR 1.

8. The presence of CRISPR loci among the UTI *E. faecalis* isolates was higher than for vagina & wound isolates which were multidrug resistance isolates.

Recommendation:

1. Further global investigations covering more isolates and methods like whole genome sequencing would be advisable.
2. Using the laboratory animals for *in vivo* study is needed to help clarify the role of bacterial mutations and genomic rearrangements in *E. faecalis* infection.
3. Re-evaluation of antibiotics currently used to treat *E. faecalis*, especially after the bacteria have developed resistance to most of them.
4. Using Real-time PCR to detect pathogen as the main causative agent depending on the copy number
5. Further studies can determine the exact role of CRISPR-*cas* with virulence factor of *E. faecalis* .
6. Detection of mutation ratein *E. faecalis* through using DNA microarray .

REFERENCES:

Abat, C., Chaudet, H., Rolain, J. M., Colson, P., & Raoult, D. (2016). Traditional and syndromic surveillance of infectious diseases and pathogens. *International Journal of Infectious Diseases*, *48*, 22-28.

Abitzsch S, Stepan H, Sack U, Faber R. (2001) Detection of cervical immunoglobulin a in normal pregnancy. Journal of Perinatal Medicine. 2001; 29: 36–41.

Afriyie D. K., Adu L. B., Dzradosi M., Amponsah S. K., Ohene-Manu P., Manu-Ofei F. (2018). Comparative *in vitro* activity of ciprofloxacin and levofloxacin against isolated uropathogens in Ghana: a pilot study. *Pan Afr. Med. J.* 30, 194.10.11604/pamj.2018.30.194.15457.

Agudelo Higuita NI, Huycke MM. (2014).Enterococcal disease, epidemiology, and implications for treatment. In: Gilmore MS, Clewell DB, Ike Y, Shankar N, eds. *Enterococci: From Commensals to Leading Causes of Drug Resistant Infection*. Massachusetts Eye and Ear Infirmary.

Ahmad, A., Dada, A. C., & Usup, G. (2014). Application of multilocus sequence analysis for molecular characterization of enterococci with virulence factors recovered from a tropical recreational beach. *Southeast Asian Journal of Tropical Medicine and Public Health*, *45*(3), 700.

References

Aiyegoro, O.A., Igbinosa, O. O.,Ogunmwonyi, I. N., Odjadjare, E.E.,Igbinosa, O.E. and Okoh, A.I. (2007). Incidence of urinary tract infection among children and adolescent in lle-lfe, Nigeria. Afr. J. Microbiol. Res.68:013-019.

Al-Badah AS, Ibrahim ASS, Al-Salamah AA, Ibrahim SSS. (2015). Clonal diversity and antimicrobial resistance of Enterococcus faecalis isolated from endodontic infections. *Electronic journal of Biotechnology*, *18*(3), 175-180

Al-Dahmoshi, H. O., Rabeea, H. W., Aridhee, A. S. A., Al-Khafaji, N.S., Al-Allak, M. H., Lazm, A. M., & Jebur, M. S. (2019). Phenotypic Investigation of Vancomycin, Teicoplanin and Linezolid Resistance Among *Enterococcus* spp. Isolated from Children Diarrhea. J Pure Appl Microbiol, 13(1), 531-6.

Alduhaidhawi, A. H. M., AlHuchaimi, S. N., Al-Mayah, T. A., Al-Ouqaili, M. T., Alkafaas, S. S., Muthupandian, S., & Saki, M. (2022). Prevalence of CRISPR-cas systems and their possible association with antibiotic resistance in Enterococcus faecalis and Enterococcus faecium collected from hospital wastewater. *Infection and Drug Resistance*, 1143-1154.

Alhajjar N., Chatterjee A., Spencer B.L., Burcham L.R., Willett J.L.E., Dunny G.M., Duerkop B.A., Doran K.S.(2020). Genome-Wide Mutagenesis Identifies Factors Involved in Enterococcus

faecalis Vaginal Adherence and Persistence. Infect. Immun. 2020;88:e00270-20. doi: 10.1128/IAI.00270-20.

Al-Halaby, A. H., Al-Hashimy, A. B., Al-Kadmy, I., & Al_Ajeeli, E. (2017). Molecular Detection of Enterococcus Surface Protein (Esp) Gene in E. faecalis and E. faecium and their Role in Biofilm Formation. *Iraqi journal of biotechnology*, *16*(1).

Almeida JA., et al., (2020). Antibiotic resistance in clinical isolates of Escherichia coli non-producer of Extended Spectrum Betalactamases (ESBL), obtained from urine cultures in patients with urinary tract infection., medRxiv 2020.08.15.20173146; doi:https://doi.org/10.1101/2020.08.15.20173146

Almeida JA., et al.,(2020).Quinolone resistance in different uropathogens isolated from urine cultures in patients with urinary tract infection., Biorxiv., doi: https://doi.org/10.1101/2020.09.07.286674

Al-Sa'ady, A. T. (2019). Detection of vancomycin resistance in multidrug-resistant *Enterococcus faecalis* isolated from burn infections. Drug Invention Today, 11(11), 2984-2989.

AL-saadi, S. A. (2013). Molecular Characterization of Some Virulence Factors of Clinical Isolates of *Enterococcus faecalis*. MSc Iraq.

Al-Temimay, I. A., & Aswad, E. M. (2018). Identification of Cryptococcus neoformans Isolates by PCRITS regions. *Iraqi Journal of Science*, *59*(4A), 1792-1805.

Alwan NAS.(2018). Tumor characteristics of female breast cancer: pathological review of mastectomy specimens belonging to Iraqi patients. World J Breast Cancer Res..

Anders C, Niewoehner O, Duerst A, Jinek M. 2014. Structural basis of PAM-dependent target DNA recognition by the Cas9 endonuclease. *Nature* 513:569–573. doi: 10.1038/nature13579, PMID: 25079318

Anderson A C, Jonas D, Huber I, et al.,(2015) *Enterococcus faecalis* from food, clinical specimens, and oral sites: prevalence of virulence factors in association with biofilm formation[J]. Frontiers in Microbiology, 6: 1534.

Anderson KL, Whitlock JE, Harwood VJ. (2005).Persistence and differential survival of fecal indicator bacteria in subtropical waters and sediments. *Appl Environ Microbiol*; 71: 3041-8.

Arias, C. A., Contreras, G. A., & Murray, B. E. (2010). Management of multidrug-resistant enterococcal infections. *Clinical microbiology and infection*, *16*(6), 555-562.

Arias, C.A.; Murray, B.E.(2012).The rise of the *Enterococcus*: Beyond vancomycin resistance. *Nat. Rev. Microbiol., 10*, 266²278.

References

Artero E, Nuñez AC, Bravo MG, Calvo OC, Garcia MB, Lledias JP, et al.,(2019). Infección urinaria en el anciano. Revista Clínica Española. Elsevier España, S.L.U. and Sociedad Española de Medicina Interna (SEMI); Feb 13;:1–5.

Atlas, R., & Snyder, J. (2015). Reagents, stains, and media: bacteriology. In Manual of Clinical Microbiology, Eleventh Edition (pp. 316-349). American Society of Microbiology.

Baldassarri, L., L. Bertuccini, R. Creti, P. Filippini, M. G. Ammendolia, S. Koch, J. Huebner, and G. Orefici. 2005.'Glycosaminoglycans mediate invasion and survival of Enterococcus faecalis into macrophages', J Infect Dis, 191:1253–62.

Banerjee, T. (2013). Random amplified polymorphic DNA (RAPD) typing of multidrug resistant Enterococcus faecium urinary isolates from a tertiary care centre, Northern India. *Journal of Clinical and Diagnostic Research: JCDR*, *7*(12), 2721.

Barnes AMT, Dale JL, Chen Y, Manias DA, Greenwood Quaintance KE, Karau MK, Kashyap PC, Patel R, Wells CL, Dunny GM.2017. *Enterococcus faecalis* readily colonizes the entire gastrointestinal tract and forms biofilms in a germ-free mouse model.Virulence 8:282–296.

Baron, E. J., Peterson, L. R. and Finegoldens, S. M. (1994). Bailey and Scott's diagnostic microbiology. 9th Ed. Mosology. Co. USA.

Barrangou R, van Pijkeren J-P. 2016. Exploiting CRISPR–Cas immune systems for 669 genome editing in bacteria. Curr Opin Biotechnol 37:61–68

Bertuccini, L., M. G. Ammendolia, F. Superti, and L. Baldassarri. 2002. 'Invasion of HeLa cells by Enterococcus faecalis clinical isolates', Medical microbiology and immunology, 191: 25–31.

Bi, R., Qin, T., Fan, W., Ma, P., & Gu, B. (2018). The emerging problem of linezolid-resistant enterococci. Journal of global antimicrobial resistance, 13, 11-19.

Billington, E. O., Phang, S. H., Gregson, D. B., Pitout, J. D. D., Ross, T., Church, D. L., ... & Parkins, M. D. (2014). Incidence, risk factors, and outcomes for Enterococcus spp. blood stream infections: a population-based study. *International Journal of Infectious Diseases*, *26*, 76-82.

Bitsori, M., Maraki, S., Raissaki, M., Bakantaki, A. & Galanakis, (2005).E. Communityacquired enterococcal urinary tract infections. *Pediatr Nephrol* 20, 1583-1586.

Boeree MJ, Diacon AH, Dawson R, et al. (2015) A dose-ranging trial to optimize the dose of rifampin in the treatment of tuberculosis. Am J Respir Crit Care Med; 191:1058.

Bolocan AS, Upadrasta A, de Almeida Bettio PH, Clooney AG, Draper LA, Ross RP, *et al*.,(2019) .Evaluation of Phage Therapy

in the Context of *E. faecalis* and its Associated Diseases. Viruses.; 11: 366.

Bondi, M., Laukova, A., de Niederhausern, S., Messi, P., Papadopoulou, C., and Economou, V. (2020). Controversial aspects displayed by enterococci: probiotics or pathogens? *Biomed. Res. Int.* 2020:9816185.

Bortolaia, V., Espinosa-Gongora, C., & Guardabassi, L. (2016).Human health risks associated with antimicrobial-resistant enterococci and *Staphylococcus aureus* on poultry meat. Clinical Microbiology and Infection, 22(2), 130-140.

Bourgogne A, Garsin DA, Qin X, Singh KV, Sillanpaa J, Yerrapragada S, Ding Y, Dugan-Rocha S, Buhay C, Shen H, Chen G, Williams G, Muzny D, Maadani A, Fox KA, Gioia J, Chen L, Shang Y, Arias CA, Nallapareddy SR, et al. 2008. Large scale variation in *Enterococcus faecalis* illustrated by the genome analysis of strain OG1RF. *Genome Biology* 9:R110. doi: 10.1186/gb-2008-9-7-r110, PMID: 18611278

Bruno Ribeiro-Gonçalves, Alexandre P. Francisco, Cátia Vaz, Mário Ramirez, João André Carriço, PHYLOViZ Online (2016): web-based tool for visualization, phylogenetic inference, analysis and sharing of minimum spanning trees, *Nucleic Acids Research*, Volume 44, Issue W1, 8 July 2016, Pages W246–W251.

Brunton LL, Hilal-Dandan R, Knollmann BC. eds (2018). Goodman & Gilman's: The Pharmacological Basis of Therapeutics (13th ed.). McGraw-Hill Education. [ISBN:978-1-25-958473-2]

Buetti, N., Atkinson, A., Marschall, J., & Kronenberg, A. (2017). Incidence of bloodstream infections: a nationwide surveillance of acute care hospitals in Switzerland 2008–2014. *BMJ open*, *7*(3), e013665.

Burley KM, Sedgley CM.(2012). CRISPR-Cas, a prokaryotic adaptive immune system, in endodontic, oral, and multidrug-resistant hospital-acquired enterococcus faecalis. J Endod. 2012;38:1511–1515.

Caballero-Granado FJ, Cisneros JM, Luque R, Torres-Tortosa M, Gamboa F, Ez FD, Villanueva JL, Pé Rez-Cano R, Pasquau J, Merino D. 1998 Comparative Study of Bacteremias Caused by Enterococcus spp. with and without High-Level Resistance to Gentamicin. *Journal of Clinical Microbiology*, *36*(2), 520-525.

Cai T, Mazzoli S, Mondaini N, Meacci F, Nesi G, D'Elia C, et al. (2012).The Role of Asymptomatic Bacteriuria in Young Women With Recurrent Urinary Tract Infections: To Treat or Not to Treat? Clinical Infectious Diseases. Aug 21;55(6):771–7.

Calderaro, A., Maugeri, A., Magazù, S., Laganà, G., Navarra, M., & Barreca, D. (2021). Molecular basis of interactions between the antibiotic nitrofurantoin and human serum albumin: A

mechanism for the rapid drug blood transportation. *International Journal of Molecular Sciences*, *22*(16), 8740.

Campbell EA, Korzheva N, Mustaev A, et al. (2001) Structural mechanism for rifampicin inhibition of bacterial rna polymerase. Cell 2001; 104:901.

Campoccia, D., F. Testoni, S. Ravaioli, I. Cangini, A. Maso, P. Speziale, L. Montanaro, L. Visai, and C. R. Arciola. 2016.'Orthopedic implant infections: Incompetence of Staphylococcus epidermidis, Staphylococcus lugdunensis, and Enterococcus faecalis to invade osteoblasts', Journal of biomedical materials research. Part A, 104: 788–801.

Chanderraj R, Millar JA, Patel TS, Read AF, Washer L, Kaye KS, Woods RJ. (2019).Vancomycin-Resistant *Enterococcus* Acquisition in a Tertiary Care Hospital: Testing the Roles of Antibiotic Use, Proton Pump Inhibitor Use, and Colonization Pressure. Open Forum Infect Dis. 2019 Apr;6(4):ofz139.

Chang, Y. C. *et al.* (2016) 'Distribution and factors associated with Salmonella enterica genotypes in a diverse population of humans and animals in Qatar using multi-locus sequence typing (MLST)', *Journal of Infection and Public Health*, 9(3), pp. 315–323. doi: 10.1016/j.jiph.2015.10.013.

Chen, A. Y., & Zervos, M. J. (2009). Enterococcus: antimicrobial resistance in enterococci epidemiology, treatment, and control.

In *Antimicrobial Drug Resistance: Clinical and Epidemiological Aspects* (pp. 715-733). Totowa, NJ: Humana Press.

Chen, X., Song, Y. Q., Xu, H. Y., Menghe, B. L. G., Zhang, H. P., & Sun, Z. H. (2015). Genetic relationships among Enterococcus faecalis isolates from different sources as revealed by multilocus sequence typing. *Journal of Dairy Science*, *98*(8), 5183-5193.

Ch'ng J-H, Chong KKL, Lam LN et al.(2019) Biofilm-associated infection by enterococci. *Nat Rev Microbiol*. 17:82–94.

Cho S, Shin J, Cho BK.(2018) Applications of CRISPR/Cas system to bacterial metabolic engineering. Int J Mol Sci.19(4):1089. doi:10.3390/ijms19041089.

Choi J, Rieke EL, Moorman TB, Soupir ML, Allen HK, Smith SD, et al., (2018) Practical implications of erythromycin resistance gene diversity on surveillance and monitoring of resistance. *FEMS Microbiol Ecol.* Apr;94((94)):1–11.

Chong, K. K. L., W. H. Tay, B. Janela, A. M. H. Yong, T. H. Liew, L. Madden, D. Keogh, T. M. S. Barkham, F. Ginhoux, D. L. Becker, and K. A. Kline. 2017. 'Enterococcus faecalis Modulates Immune Activation and Slows Healing During Wound Infection', J Infect Dis, 216: 1644–54.

CLSI, Clinical and Laboratory Standards Institute. (2019). Performance standards for antimicrobial susceptibility testing. Approved standard M100-S17 .

Collee, J.G.; Fraser, A.G.; Marmion, B.P. and Simmons, A. (1996): Mackie and McCartney Practical Medical Microbiology, 14th ed. Churchill Livingstone, New York.

Colodner, R., Eliasbery, T., Chazan, B. & Raz, R.(2006) Clinical significance of bacteriuria with low colony counts of Enterococcus species. *Eur J Clin Microbiol Infect Dis* 25, 238-241.

Combarros-Fuertes, P., Fresno, J. M., Estevinho, M. M., Sousa-Pimenta, M., Tornadijo, M. E., & Estevinho, L. M. (2020).Honey: Another Alternative in the Fight against Antibiotic- Resistant Bacteria?. Antibiotics, 9(11), 774.

Costa Y, Galimand M, Leclercq R, Duval J, Courvalin P.(1993). Characterization of the chromosomal aac(6')-Ii gene specific for Enterococcus faecium. Antimicrob Agents Chemother. Sep;37(9):1896-903.

Da Costa, P. M., Loureiro, L., & Matos, A. J. (2013). Transfer of multidrug-resistant bacteria between intermingled ecological niches: the interface between humans, animals and the environment. International journal of environmental research and public health, 10(1), 278-294.

Dan Brennan, MD on May 11, 2021.What Are Enterococcal Infections.

de Almeida, Carolina Vieira; Taddei, Antonio; Amedei, Amedeo (2018-01-01). "The controversial role of Enterococcus faecalis in colorectal cancer" (https://www.ncbi.nlm.nih.gov/pmc/articles/PMC6044 108). Therapeutic Advances in Gastroenterology. SAGE Publications. 11: 175628481878360.

de Lastours, V. *et al.* (2017) Ecological impact of ciprofloxacin on commensal enterococci in healthy volunteers. *J Antimicrob Chemother* 71, 1574-1580. diversification of bacterial clones.

De Seta F, Campisciano G, Zanotta N, Ricci G, Comar M. (2019) The Vaginal Community State Types Microbiome-Immune Network as Key Factor for Bacterial Vaginosis and Aerobic Vaginitis. Frontiers in Microbiology. 10: 2451.

Dicks, L. M., Todorov, S. D., & Franco, B. D. G. M. (2011). Current status of antibiotic resistance in lactic acid bacteria. Antibiotic resistance: causes and risk factors, mechanisms and alternatives. Pharmacology-research, safety testing and regulation. Nova Publisher, New York, 379-425.

Domig KJ, Mayer HK, Kneifel W. (2003) Methods used for the isolation, enumeration, characterisation and identification of Enterococcus spp. 2. Pheno- and genotypic criteria.

Donders GGG, Bellen G, Grinceviciene S, Ruban K, Vieira-Baptista P.(2017). Aerobic vaginitis: no longer a stranger. Research in Microbiology. 168: 845–858.

Donders GGG, Vereecken A, Bosmans E, Dekeersmaecker A, Salembier G, Spitz B.(2002) .Definition of a type of abnormal vaginal flora that is distinct from bacterial vaginosis: aerobic vaginitis. British Journal of Obstetrics and Gynaecology.; 109: 34–43.

Dos Santos BA, de Oliveira JD, Parmanhani-da-silva BM, Ribeiro RL, Teixeira LM, Neves FP.(2020) . CRISPR elements and their association with antimicrobial resistance and virulence genes among vancomycin-resistant and vancomycin-susceptible enterococci recovered from human and food sources. *Infect Genet Evol*. 80:104183. doi:10.1016/j.meegid..104183.

Dowd, S. E., R. D. Wolcott, Y. Sun, T. McKeehan, E. Smith, and D. Rhoads. 2008. 'Polymicrobial nature of chronic diabetic foot ulcer biofilm infections determined using bacterial tag encoded FLX amplicon pyrosequencing (bTEFAP)', PLoS ONE, 3: e3326.

Dunny GM, Hancock LE, Shankar N. (2014). Enterococcal biofilm structure and role in colonization and disease. In: Enterococci: From Commensals to Leading Causes of Drug Resistant

Infection Gilmore MS, Clewell DB, Ike Y, Shankar N (Eds).Massachusetts Eye and Ear Infirmary, MA, USA.

el Amin NA, Jalal S, Wretlind B. 1999. Alterations in GyrA and ParC associated with fluoroquinolone resistance in Enterococcus faecium. Antimicrob Agents Chemother 43:947–949.

Emaneini, M., Khoramian, B., Jabalameli, F., Beigverdi, R., Asadollahi, K., Taherikalani, M., & Lari, A. R. (2016). Prevalence of high-level gentamicin-resistant Enterococcus faecalis and Enterococcus faecium in an Iranian hospital. *Journal of preventive medicine and hygiene*, *57*(4), E197.

Escolà-Vergé L, Fernández-Hidalgo N, Rodríguez-Pardo D et al., (2019). Teicoplanin for treating enterococcal infective endocarditis: a retrospective observational study from a referral centre in Spain. *Int J Antimicrob Agents*; 53: 165–70.https://doi.org/10.1016/j.ijantimicag.2018.10.003

Esmail M.A.M., Abdulghany H.M., Khairy R.M. (2019). Prevalence of Multidrug-Resistant Enterococcus faecalis in Hospital-Acquired Surgical Wound Infections and Bacteremia: Concomitant Analysis of Antimicrobial Resistance Genes. Infect. Dis.;12:1178633719882929.

Falagas M.E., Karageorgopoulos D.E.(2008) Pandrug Resistance (PDR), Extensive Drug Resistance (XDR), and Multidrug

Resistance (MDR) among Gram-Negative Bacilli: Need for International Harmonization in Terminology. Clin. Infect. Dis. 2008;46:1121–1122.

Fan A, Yue Y, Geng N, Zhang H, Wang Y, Xue F. 2013.Aerobic vaginitis and mixed infections: comparison of clinical and laboratory findings. Archives of Gynecology and Obstetrics. 287: 329–335.

Feil, E. J., Li, B. C., Aanensen, D. M., Hanage, W. P., & Spratt, B. G. (2004). eBURST: inferring patterns of evolutionary descent among clusters of related bacterial genotypes from multilocus sequence typing data. *Journal of bacteriology*, *186*(5), 1518-1530.

Fernández-Hidalgo N, Escolà-Vergé L. (2019). Enterococcus faecalis Bacteremia: Consider an Echocardiography, But Consult an Infectious Diseases Specialist. J Am Coll Cardiol. Jul 16;74(2):202-204.

Fiore, E., Van Tyne, D., & Gilmore, M. S. (2019). Pathogenicity of Enterococci. Gram-Positive Pathogens, 378-397.

Fisher, K. & Phillips, C. (2009) The ecology, epidemiology and virulence of Enterococcus. *Microbiol* 155, 1749-1757.

Flores-Mireles, A. L., Walker, J. M., Caparon, M. & Hultgren, S. J. (2015). Urinary tract infections: epidemiology, mechanisms of infection and treatment optios. *Nat Rev Microbiol* 13, 269-284.

Fonfara I, Le Rhun A, Chylinski K, Makarova KS, Le´ crivain AL, Bzdrenga J, Koonin EV, Charpentier E. 2014. Phylogeny of Cas9 determines functional exchangeability of dual-RNA and Cas9 among orthologous type II CRISPR-Cas systems. *Nucleic Acids Research* 42:2577–2590. doi: 10.1093/nar/gkt1074, PMID: 24270795

Forbes, B. A., Sahm, D. F., & Weissfeld, A. S. (2007). Diagnostic microbiology. St Louis: Mosby.

Gadeberg, O. V., Orskov, I., & Rhodes, J. M. (1983). Cytotoxic effect of an alpha-hemolytic Escherichia coli strain on human blood monocytes and granulocytes in vitro. Infection and immunity, 41(1), 358-364.

Gagetti, P., Bonofiglio, L., Gabarrot, G. G., Kaufman, S., Mollerach, M., Vigliarolo, L. & Lopardo, H. A. (2019). Resistance to β-lactams in enterococci. Revista Argentina de microbiologia, 51(2), 179-183.

Galimand, M., Schmitt, E., Panvert, M., Desmolaize, B., Douthwaite, S., Mechulam, Y., and Courvalin, P. (2011). Intrinsic resistance to aminoglycosides in *Enterococcus faecium* is conferred by the 16S rRNA m5C1404-specific methyltransferase EfmM, RNA, 17(2), 251-62.

García-Solache M, Rice LB. (2019) The *Enterococcus*: a model of adaptability to its environment. *Clin Microbiol Rev*.32(2):e00058–18.doi:10.1128/CMR.00058-18.

García-Solache M, Rice LB. (2019) The Enterococcus: a Model of Adaptability to Its Environment. Clin Microbiol Rev. Mar 20;32(2).

Gardiner BJ, Stewardson AJ, Abbott IJ, Peleg AY. 2019 Nitrofurantoin and fosfomycin for resistant urinary tract infections:old drugs for emerging problems. Aust Prescr. Feb;42(1):14-19.

Gardy, Jennifer L., and Nicholas J. Loman. 2018. "Towards a Genomics-Informed, Real-Time, Global Pathogen Surveillance System." *Nature Reviews. Genetics* 19 (1):9–20.

Garsin, D. A., Frank, K. L., Silanpää, J., Ausubel, F. M., Hartke, A., Shankar, N., & Murray, B. E. (2014). Pathogenesis and models of enterococcal infection. *Enterococci: from commensals to leading causes of drug resistant infection [internet]*.

Gawryszewska I, Z˙ abicka D, Bojarska K, Malinowska K,Hryniewicz W, Sadowy E. 2016. Invasive enterococcal infections in Poland: The current epidemiological situation. Eur J Clin Microbiol Infect Dis 35:847–856.

Gholizadeh P, Aghazadeh M, Ghotaslou R, et al., (2020).CRISPR-cas system in the acquisition of virulence genes in dental-root canal

and hospital-acquired isolates of *Enterococcus faecalis*. *Virulence.*11(1):12571267.doi:10.1080/21505594.2020.180939

Gholizadeh P, Aghazadeh M, Ghotaslou R, et al., (2021) Role of CRISPR-Cas system on antibiotic resistance patterns of *Enterococcus faecalis*. *Ann Clin Microbiol Antimicrob*.20(1):49. doi:10.1186/s12941-021-00455-6

Gholizadeh P, Köse Ş, Dao S, et al., (2020). How CRISPR-Cas system could be used to combat antimicrobial resistance. *Infect Drug Resist*.;13:1111–1121. doi:10.2147/IDR.S247271

Gholizadeh, P., Aghazadeh, M., Ghotaslou, R., Ahangarzadeh Rezaee, M., Pirzadeh, T., Köse, Ş., Ganbarov, K., Yousefi, M., & Kafil, H. S. (2020). CRISPR-*cas* system in the acquisition of virulence genes in dental-root canal and hospital-acquired isolates of *Enterococcus faecalis*. *Virulence*, *11*(1),1257–1267.

Giedraitiene A, Pereckaite L, Bredelyte-Gruodiene E, Virgailis M, Ciapiene I, Tatarunas V. 2022 . CTX-M-producing *Escherichia coli* strains: resistance to temocillin, fosfomycin, nitrofurantoin and biofilm formation. Future Microbiol. Jul;17:789-802.

Giedraitienė, A., Vitkauskienė, A., Naginienė, R., & Pavilonis, A. (2011). Antibiotic resistance mechanisms of clinically important bacteria. Medicina, 47(3), 19.

Gilmore MS, Clewell DB, Shankar N, Ness IF, Diep DB, Ike Y. 2014. Enterococcal Bacteriocins and Antimicrobial Proteins that

Contribute to Niche Control. Boston: Massachusetts Eye and Ear Infirmary.

Gilmore MS, Clewell DB. 2002. The Enterococci: pathogenesis, molecular biology, and antibiotic resistance, Vol. 10, Washington, DC: ASM press, 439.

Giulieri, S. G., Tong, S. Y., & Williamson, D. A. (2020). Using genomics to understand meticillin-and vancomycin-resistant Staphylococcus aureus infections. Microbial genomics, 6(1): 32-40.

Gjodsbol, K., J. J. Christensen, T. Karlsmark, B. Jorgensen, B. M. Klein, and K. A. Krogfelt. 2006. 'Multiple bacterial species reside in chronic wounds: a longitudinal study', International wound journal , 3: 225–31.

Goh HMS, Yong MHA, Chong KKL, Kline KA. 2017. Model systems for the study of Enterococcal colonization and infection.Virulence. Nov 17;8(8):1525-1562.

Golob, M., Pate, M., Kušar, D., Dermota, U., Avberšek, J., Papić, B., & Zdovc, I. (2019). Antimicrobial resistance and virulence genes in Enterococcus faecium and Enterococcus faecalis from humans and retail red meat. *BioMed research international*.

Gomaa AA, Klumpe HE, Luo ML, Selle K, Barrangou R, Beisel CL. 2014. Programmable removal of bacterial strains by use of

genome-targeting CRISPR-Cas systems. *mBio* 5:e00928-13. doi: 10.1128/mBio.00928-13, PMID: 24473129.

Guo M, Chen H, Dong S, Zhang Z, Luo H. 2022. CRISPR-Cas gene editing technology and its application prospect in medicinal plants. Chin Med.17(1):33. doi:10.1186/s13020-022-00584-w.

Gupta V., Singla N., Behl P., Sahoo T., Chander J. 2015. Antimicrobial susceptibility pattern of vancomycin resistant enterococci to newer antimicrobial agents. Indian J. Med. Res.141:483–486. doi: 10.4103/0971-5916.159309.

Gupta V., Singla N., Behl P., Sahoo T., Chander J. 2015.Antimicrobial susceptibility pattern of vancomycin resistant enterococci to newer antimicrobial agents. Indian J. Med. Res.141:483–486. doi: 10.4103/0971-5916.159309.

Guzman Prieto A M, Van Schaik W, RogersM R, et al., (2016) Global emergence and dissemination of enterococci as nosocomial pathogens: attack of the clones?[J]. Frontiers in Microbiology,7: 788. DOI:10.3389/fmicb.2016.00788.

Gzyl A, Augustynowicz E. 1998.Technical aspects of random amplified polymorphic DNA (rapd) technique in genotyping of bacterial strains. *Acta Microbiol Polonica*; 48: 243-59.

Haghi, F., Lohrasbi, V., & Zeighami, H. (2019). High incidence of virulence determinants, aminoglycoside and vancomycin

resistance in enterococci isolated from hospitalized patients in Northwest Iran. BMC infectious diseases, 19(1), 1-10.

Haider MZ, Shabbir MAB, Yaqub T, et al., (2022). CRISPR-Cas system: an adaptive immune system's association with antibiotic resistance in Salmonella enterica serovar enteritidis. Biomed Res Int.2022:9080396.

Hammerum A M. Enterococci of animal origin and their significance for public health[J]. Clinical Microbiology and Infection, 2012, 18(7): 619-625. DOI:10.1111/j.1469-0691.2012.03829.x.

Han C, Wu W, Fan A, Wang Y, Zhang H, Chu Z, *et al*., (2015).Diagnostic and therapeutic advancements for aerobic vaginitis. Archives of Gynecology and Obstetrics. 291: 251–257.

Han et al., 2014 D. Han, H. Tang, J. Lu, G. Wang, L. Zhou, L. Min, C. Han Population structure of clinical Vibrio parahaemolyticus from 17 coastal countries,determined through multilocus sequence analysis PLoS ONE, 9 (2014), p. e107371

Hasan, A. R. S., Al-Duliami, A. A., Al-Ajeely, K. S., & Al-Azawi, Z. H. (2011). Antimicrobial Susceptibility Patterns of Enterococcal Isolates and its Relevance With Biofilms Formation And B-Lactamase Production. Diyala Journal of Medicine, 1(1), 21-26.

Hashem, Y. A., Abdelrahman, K. A., & Aziz, R. K. (2021). Phenotype–Genotype Correlations and Distribution of Key

Virulence Factors in *Enterococcus faecalis* Isolated from Patients with Urinary Tract Infections. Infection and Drug Resistance, 14, 1713.

Hassan MF, Rund NMA, El-Tohamy O, Moussa M, Ali YZ, Moussa N, *et al*. 2020 Does Aerobic Vaginitis have Adverse Pregnancy Outcomes? Prospective Observational Study. Infectious Diseases in Obstetrics and Gynecology. 2020: 5842150.

Healy, M., J. Huong, T. Bittner, M. Lising, S. Frye, S. Raza, R. Schrock, J. Manry, A. Renwick, R.Nieto, C. Woods, J.Versalovic, and J.R. Lupski. 2005. Microbial DNA typing by automated repetitive sequence based PCR. J.Clin. Microbiol. 43:199-207.

Hegstad K, Mikalsen T, Coque TM, Werner G, Sundsfjord A. 2010. Mobile genetic elements and their contribution to the emergence of antimicrobial resistant *Enterococcus faecalis* and *Enterococcus faecium*. Clin Microbiol Infect 16:541–554.

Heitkamp, R. A., P. Li, K. Mende, S. T. Demons, D. R. Tribble, and S. D. Tyner. 2018. 'Association of Enterococcus spp. With Severe Combat Extremity Injury, Intensive Care, and Polymicrobial Wound Infection', Surg Infect (Larchmt) , 19: 95–103.

Hidron, Alicia I.; Edwards, Jonathan R.; Patel, Jean; Horan, Teresa C.; Sievert, Dawn M.; Pollock, Daniel A.; Fridkin, Scott K.;

National Healthcare Safety Network Team; Participating National Healthcare Safety Network Facilities (November 2008). "Antimicrobial-Resistant Pathogens Associated WithHealthcare-Associated Infections: Annual Summary of Data Reported to the National HealthcareSafety Network at the Centers for Disease Control and Prevention, 2006–2007". Infection Control &Hospital Epidemiology. 29 (11): 996–1011.

Higuita, N. I. A., & Huycke, M. M. (2014). Enterococcal disease, epidemiology, and implications for treatment. In Enterococci: From commensals to leading causes of drug resistant infection. Massachusetts Eye and Ear Infirmary.

Hollenbeck, B.L. and L.B. Rice, 2012 .Intrinsic and acquired resistance mechanisms in enterococcus. Virulence, 3(5): p. 421-569.

Holmberg A, Morgelin M, Rasmussen M. 2012. Effectiveness of ciprofloxacin or linezolid in combination with rifampicin against *Enterococcus faecalis* in biofilms. J Antimicrob Chemother, 67:433–9.

Homan et al., 2002 W.L. Homan, D. Tribe, S. Poznanski, M. Li, G. Hogg, E. Spalburg, J.D. Van Embden, R.J. Willems Multilocus sequence typing scheme for Enterococcus faecium J. Clin. Microbiol., 40 (2002), pp. 1963-1971.

Horsley, H. *et al.,* (2013). *Enterococcus faecalis* subverts and invades the host urothelium in patients with chronic urinary tract infection. *PLoS One* 8, e83637.

Horsley, H., D. Dharmasena, J. Malone-Lee, and J. L. Rohn. 2018. 'A urine-dependent human urothelial organoid offers a potential alternative to rodent models of infection', Sci Rep, 8: 1238.

Hullahalli K, Rodrigues M, Palmer KL. Exploiting CRISPR-Cas to manipulate Enterococcus faecalis populations. eLife. 2017;6:e26664.

Hullahalli K, Rodrigues M, Schmidt BD, Li X, Bhardwaj P, Palmer KL. 2015. Comparative analysis of the Orphan CRISPR2 Locus in 242 *Enterococcus faecalis* Strains. *PLoS One* 10:e0138890. doi: 10.1371/journal.pone.0138890, PMID: 26398194

Humphries R. M., Hindler J. A., Shaffer K., Campeau S. A. (2019). Evaluation of ciprofloxacin and levofloxacin disk diffusion and etest using the 2019EnterobacteriaceaeCLSI breakpoints. *J. Clin. Microbiol.*57 (3). 10.1128/jcm.01797-18.

Huycke, M. M., Spiegel, C. A., & Gilmore, M. S. (1991). Bacteremia caused by hemolytic, high-level gentamicin-resistant Enterococcus faecalis. *Antimicrobial agents and chemotherapy*, *35*(8), 1626-1634.

Huys G., et al., 2004 Prevalence and Molecular Characterization of Tetracycline Resistance in Enterococcus Isolates from Food., Appl. Environ. Microbiol ,70(3),1555-1562.

Iacumin, L., Ginaldi, F., Manzano, M., Anastasi, V., Reale, A., Zotta, T., ... & Comi, G. (2015). High resolution melting analysis (HRM) as a new tool for the identification of species belonging to the Lactobacillus casei group and comparison with species-specific PCRs and multiplex PCR. *Food Microbiology*, *46*, 357-367.

Ibtisam H. Al-Azawi1, Mohammed H. Abbas, (2020). Determination *Enterococcus faecalis* in Asymptomatic Urinary Tract Infection Associated with Diabetes Type 2 Patients in Suwayrah General Hospital-Iraq.*Medico-Legal Update*, *20*(1).

Inabo, H.I. and Obanibi, H.B.T. (2006). Antimicrobial susceptibility of some urinary tract clinical isolates to commonly used antibiotics. Africa. J. Biotech. 5 (5): 487-489.

Iwona Gawryszewska, Waleria Hryniewicz, Ewa Sadowy Natinal Medicines Institute, ul. 2015. Chelmska30/34, 00-725 Warsaw, Poland, Distribution of CRISPR-Cas systems vs. virulence and resistance genes among *Enterococcusfaecalis*from various settings.

Jabbari Shiadeh SM, Pormohammad A, Hashemi A, Lak P. 2019. Global prevalence of antibiotic resistance in blood-

isolated Enterococcus faecalis and Enterococcus faecium: a systematic review and meta-analysis. *Infection and Drug Resistance*.12:2713-2725. doi:10.2147/IDR.S20608.

Jahic M. 2007. Clinical, microbiological and cytological characteristics of enterococcal vaginitis [Doctoral thesis]. Tuzla, B&H: Medical University Tuzla.

Javed, A., & Manzoor, S. (2020). Comparative analysis of bacterial vaginosis microbiota among pregnant and non-pregnant females and isolation of phages against *Enterococcus faecalis*, *Enterococcus faecium*, and *Shigella flexneri* strains. Microbial Pathogenesis, 149, 104588.

Jett BD, Huycke MM, Gilmore MS. 1994.Virulence of enterococci. Clinical Microbiology Reviews.7: 462–478.

Jiang Y, Chen B, Duan C, Sun B, Yang J, Yang S. 2015. Multigene editing in the 654 Escherichia coli genome via the CRISPR-Cas9 system. Appl Environ Microbiol 81:2506–655 14.

Jinek M, Jiang F, Taylor DW, Sternberg SH, Kaya E, Ma E, Anders C, Hauer M, Zhou K, Lin S, Kaplan M, Iavarone AT, Charpentier E, Nogales E, Doudna JA. 2014. Structures of Cas9 endonucleases reveal RNA-mediated conformational activation. *Science* 343:1247997. doi: 10.1126/science.1247997, PMID: 24505130

John, G., Kumar, K. P., Gopal, S. S., Kumari, S., & Reddy, B. K. (2015*). Enterococcus faecalis*, a nightmare to endodontist: A systematic review. African journal of microbiology research, 9(13), 898-908.

Johnson CN, Sheriff EK, Duerkop BA & Chatterjee A (2021) Let Me Upgrade You: Impact of Mobile Genetic Elements on Enterococcal Adaptation and Evolution. J Bacteriol 203: e0017721.

Jolley, K. A., Bray, J. E., & Maiden, M. C. (2018). Open-access bacterial population genomics: BIGSdb software, the PubMLST. org website and their applications. *Wellcome open research*, *3*.

Jugdave, A. (2007). *Molecular characterization of selected enterococcus strains (previously streptococcus) using genotyping techniques* (Doctoral dissertation).

Kadhem, H. S., & Flayyih, M. T. (2014). Isolation and Identification of Vancomycin-Resistant *Enterococcus Faecalis*. Iraqi *Journal of Science, 55(4B), 1811-1816.*

Kafil, H.S. and M. Asgharzadeh (2014). Vancomycin-resistant *Enterococcus faecium* and *Enterococcus faecalis* isolated from education hospital of Iran. Maedica, 9(4): 323

Kajihara T, Nakamura S, Iwanaga N, Oshima K, Takazono T, Miyazaki T, Izumikawa K. et al. (2015). Clinical characteristics

and risk factors of enterococcal infections in Nagasaki, Japan: a retrospective study. BMC Infect Dis.15:426. doi: 10.1186/s12879-015-1175-6.

Kalia, A., Enright, M. C., Spratt, B. G., & Bessen, D. E. (2001). Directional gene movement from human-pathogenic to commensal-like streptococci. *Infection and immunity*, *69*(8), 4858-4869.

Kamruzzaman M, Iredell J. 2020. CRISPR-Cas system in antibiotic resistance plasmids in *Klebsiella pneumoniae*. *Front Microbiol*.10:2934.doi:10.3389/fmicb.2019.02934

Kandela, N. J. (2012). A genetic study on enterocin–production from *Enterococcus faecalis* isolated from different clinical sources. Iraqi Journal of Biotechnology, 11(2), 503-518.

Kang ZZ, Lei CW, Kong LH, Wang YL, Ye XL, Ma BH, at al., (2019). Detection of transferable oxazolidinone resistance determinants in enterococcus faecalis and enterococcus faecium of swine origin in Sichuan Province, China. J Glob Antimicrob Resist.;25: doi: 10.1016/j.jgar.2019.05.021.

Kao PHN, Kline KA. Dr. Jekyll and Mr. Hide (2019): How Enterococcus faecalis Subverts the Host Immune Response to Cause Infection. Journal of Molecular Biology. Academic Press; Jul 26;431(16):2932–45.

Kau, A. L. *et al.,* (2005). *Enterococcus faecalis* tropism for the kidneys in the urinary tract of C57BL/6J mice. *Infect Immun* 73, 2461-2468.

Kearns, A. M., Graham, C., Burdess, D., Heatherington, J., & Freeman, R. (2002). Rapid real-time PCR for determination of penicillin susceptibility in pneumococcal meningitis, including culture-negative cases. *Journal of clinical microbiology*, *40*(2), 682-684..

Khalid, H. M. (2016). Molecular Detection of Virulence Factors of Enterococcus Faecalis Isolated From Urine Samples in Duhok City, Kurdistan Region/Iraq. *Science Journal of University of Zakho*, *4*(1), 63-72..

Khazim, K., Azulay, E. E., Kristal, B., & Cohen, I. (2018). Interleukin1 gene polymorphism and susceptibility to disease. Immunological reviews, 281(1), 40-56.

Kirsty Skinner , Jonathan A.T. Sandoe , Ranjith Rajendran , Gordon Ramage , Sue Lang. (2017).Efficacy of rifampicin combination therapy for the treatment of enterococcal infections assessed in vivo using a *Galleria mellonella* infection model. *International journal of antimicrobial agents*, *49*(4), 507-511.

Kline, K. A., & Lewis, A. L. (2017). Gram-positive uropathogens, polymicrobial urinary tract infection, and the emerging

microbiota of the urinary tract. Urinary Tract Infections: Molecular Pathogenesis and Clinical Management, 459-502.

Koneman, E. W., Allen, S. D., Junda, W. M. and Schreckenberger, P. C. (1997). Color atlas and textbook of diagnostic microbiology. Lippincott Raven Publishers. Philadelphia : 577- 620.

Koonin EV, Makarova KS. 2009. CRISPR-Cas: an adaptive immunity system in prokaryotes. F1000 Biol Rep1:95.

Kotov, S. V., Pulbere, S. A., Alesina, N. V., Boyarkin, V. S., Guspanov, R. I., Belomytsev, S. V., & Kotova, D. P. (2021). The problem of antibiotic resistance in patients with urinary tract infection. Urologiia (Moscow, Russia: 1999), (1), 5-12.

Krawczyk, B., Samet, A., Leibner, J., Sledzinska, A., & Kur, J. (2006). Evaluation of a PCR melting profile technique for bacterial strain differentiation. *Journal of Clinical Microbiology*, *44*(7), 2327-2332.

Kristich CJ, Rice LB, Arias CA (2014) Enterococcal Infection-Treatment and Antibiotic Resistance. In:Gilmore MS, Clewell DB, Ike Y, Shankar N, editors. Enterococci: From Commensals to Leading Causesof Drug Resistant Infection. Boston. [Internet].

Kuch A, Willems RJ, Werner G, Coque TM, Hammerum AM, et al. (2012) Insight into antimicrobial susceptibility and population

structure of contemporary human *Enterococcus faecalis* isolates from Europe. J Antimicrob Chemother 67: 551-558.

Kumar L., Cox C.R., Sarkar S.K. 2019.Matrix metalloprotease-1 inhibits and disrupts Enterococcus faecalis biofilms. PLoS ONE.14:e0210218.doi:10.1371/journal.pone.021021.

Kutlu, O., & ARABACI, Ç. (2019). Antibiotic susceptibility pattern of Enterococcus isolates in a five year period at a tertiary care hospital. *Journal of Surgery and Medicine*, *3*(9), 644-647.

Lamichhane P, Joshi D, Subedi Y. 2013. Study on types of vaginitis and association between bacterial vaginosis and urinary tract infection in pregnant women. Internal Journal of Biomedical and Advace Research.15: 304–307.

Lancefield RC. 1933 A Serological Differentiation of Human and Other Groups of Hemolytic Streptococci.

Leavis HL, Bonten MJ, Willems RJ. 2006. Identification of high-risk enterococcal clonal complexes: global dispersion and antibiotic resistance.Curr Opin Microbiol 9:454–460.

Lebreton, F., Manson, A. L., Saavedra, J. T., Straub, T. J., Earl, A. M., and Gilmore, M. S. (2017). Tracing the Enterococci from Paleozoic origins to the hospital.Cell 169, 849. doi: 10.1016/j.cell.2017.04.027

Lebreton, F., Willems, R. J., & Gilmore, M. S. (2014). Enterococcus diversity, origins in nature, and gut colonization. *Enterococci:*

from commensals to leading causes of drug resistant infection [Internet].

Lee T, Pang S, Abraham S, Coombs GW. 2018.Antimicrobial-resistant CC17 Enterococcus faecium: The past, the present and the future. J Glob Antimicrob Resist.16:36-47.

Li, J., Yang, L., Huang, X., Wen, Y., Zhao, Q., Huang, X., ... & Han, X. (2021). Molecular characterization of antimicrobial resistance and virulence factors of *Enterococcus faecalis* from ducks at slaughterhouses. Poultry Science, 101646.

Lin E, Bhusal Y, Horwitz D, Shelburne SA, Trautner BW. 2012. Overtreatment of enterococcal bacteriuria. *JAMA InternalMedicine.* 172(1):33.doi:10.1001/archinternmed.2011.565.

Lindenstrauß AG, Pavlovic M, Bringmann A, et al., (2011). Comparison of genotypic and phenotypic cluster analyses of virulence determinants and possible role of CRISPR elements towards their incidence in Enterococcus faecalis and Enterococcus faecium. Syst Appl Microbiol.34:553–560.

López-Salas, P., Llaca-Díaz, J., Morfin-Otero, R., Tinoco, J. C., Rodriguez-Noriega, E., Salcido-Gutierres, L., ... & Garza-González, E. (2013). Virulence and antibiotic resistance of *Enterococcus faecalis* clinical isolates recovered from three

states of mexico. Detection of linezolid resistance. Archives of medical research, 44(6), 422-428.

Lüddeke, F., Heß, S., Gallert, C., Winter, J., Güde, H., & Löffler, H. (2015). Removal of total and antibiotic resistant bacteria in advanced wastewater treatment by ozonation in combination with different filtering techniques. Water research, 69, 243-251.

MacFadden, J. F., (2000). Biochemical tests for identification of medical bacteria. 3rd ed. ed. Baltimore Md: Lippincott Williams and Wilkins.

Maha, A. N.Al_Hassani1 and Lamees A. Abdul-Lateef (2022). Molecular detection of some virulence genes of Enterococcus faecalis isolated from different clinical samples in Babylon province/ Iraq.

Maiden MC *et al.* 1998. Multilocus sequence typing: a portable approach to the identification of clones within populations of pathogenic microorganisms. *Proc Natl Acad Sci USA*, **95:**3140-3145.

Maiden MC *et al.* 2013. MLST revisited: the gene-by-gene approach to bacterial genomics. *Nat Rev Microbiol* 11:728-736.

Maini Rekdal, Vayu; Bess, Elizabeth N.; Bisanz, Jordan E.; Turnbaugh, Peter J.; Balskus, Emily P. (2019-06-14). "Discovery and inhibition of an interspecies gut bacterial pathway for Levodopa

metabolism"(https://www.ncbi.nlm.nih.gov/pmc/articles/PMC7745125) . Science. 364 (6445): eaau6323.

Makarova KS, Haft DH, Barrangou R, et al., (2011). Evolution and classification of the CRISPR–Cas systems. Nature Rev Microbiol.9:467–477.

Mališová, L., Jakubů, V., Pomorská, K., Musílek, M., & Žemličková, H. (2021). Spread of linezolid-resistant Enterococcus spp. in human clinical isolates in the Czech Republic. *Antibiotics*, *10*(2), 219.

Marraffini LA. 2015. CRISPR-Cas immunity in prokaryotes. *Nature* 526:55–61. doi: 10.1038/nature15386, PMID: 26432244

Martín, B.; Corominas, L.; Garriga, M.; Aymerich, T. 2009**.** Identification and Tracing of Enterococcus Spp. by RAPD-PCR in Traditional Fermented Sausages and Meat Environment. *J. Appl. Microbiol. 106*, 66–77.

Martín-Platero, A. M., Valdivia, E., Maqueda, M., & Martínez-Bueno, M. (2009). Characterization and safety evaluation of enterococci isolated from Spanish goats' milk cheeses. *International journal of food microbiology*, *132*(1), 24-32.

Matheeussen V, Loens K, Scott C, Di Lorenzo C, McCulloch E, Donoso et al., (2019). Quality of molecular detection of vancomycin resistance in enterococci: results of 6 consecutive years of Quality Control for Molecular Diagnostics (QCMD)

external quality assessment. Eur J Clin Microbiol Infect Dis.28:doi:10.1007/s10096-019-03591-2.

McBride SM, Fischetti VA, Leblanc DJ, Moellering RC Jr, Gilmore MS (2007) Genetic diversity among *Enterococcus faecalis*. PLoS One 2: e582.

McInnes RS, McCallum GE, Lamberte LE, van Schaik W. 2020. Horizontal transfer of antibiotic resistance genes in the human gut microbiome. *Curr Opin Microbiol*.53:35–43. doi:10.1016/j.mib.2020.02.0027.fu

Meade, E., Slattery, M. A., & Garvey, M. (2020). Bacteriocins, potent antimicrobial peptides and the fight against multi drug resistant species: resistance is futile?. Antibiotics, 9(1), 32.

Medeiros, A. W., Pereira, R. I., Oliveira, D. V. D., Martins, P. D., d'Azevedo, P. A., Van der Sand, S., ... & Frazzon, A. P. G. (2014). Molecular detection of virulence factors among food and clinical *Enterococcus faecalis* strains in South Brazil. Brazilian Journal of Microbiology, 45(1), 327-332.

Mercuro NJ, Davis SL, Zervos MJ & Herc ES (2018) Combatting resistant enterococcal infections: a pharmacotherapy review. Expert Opin Pharmacother 19: 979-992.

Mike Smith, Ph.D. Polymerase chain reaction (PCR),2023.

Miller WR, Murray BE, Rice LB, Arias CA. 2016. Vancomycin-Resistant Enterococci: Therapeutic Challenges in the 21st Century. Infect Dis Clin North Am. Jun;30(2):415-439.

Miller, W. R., Munita, J. M., & Arias, C. A. (2014). Mechanisms of antibiotic resistance in enterococci. Expert review of antiinfective therapy, 12(10), 1221-1236.

Miller, W.R.; Munita, J.M.; Arias, C.A. 2014. Mechanisms of antibiotic resistance in enterococci. *Expert Rev. Anti. Infect. Ther. 12*, 1221²1236.

Miller, W.R.; Murray, B.E.; Rice, L.B.; Arias, C.A. (2020).Resistance in Vancomycin-Resistant Enterococci. Infect. Dis. Clin. N. Am. 34, 751–771.

Mojica FJM, Díez-Villaseñor C, García-Martínez J, et al., (2005). Intervening sequences of regularly spaced prokaryotic repeats derive from foreign genetic Elements. J Mol Evol.60:174–182.

Monstein, H., M. Quednau, A.Samuelsson, S.Ahrne, B. Isaksson and J.Jonasson. 1998. Division of the genus *Enterococcus* into species groups using PCR-based molecular typing methods. Microbiology. 144.

Muhammad Farman1,2, Muhammad Yasir1,6, Rashad R. Al-Hindi2, Suha A. Farraj1, Asif A. Jiman-Fatani3,4, Maha Alawi3,5 and Esam I. Azhar1,6 (2019). Genomic analysis of multidrug-resistant clinical Enterococcus faecalis isolates for antimicrobial

resistance genes and virulence factors from the western region of Saudi Arabia, *Antimicrobial Resistance & Infection Control*, *8*, 1-11.

Naha, A., Miryala, S. K., Debroy, R., Ramaiah, S., & Anbarasu, A.(2020). Elucidating the multi-drug resistance mechanism of *Enterococcus faecalis* V583: a gene interaction network analysis. Gene, 748, 144704.

Nallapareddy, S. R., Duh, R. W., Singh, K. V., & Murray, B. E. (2002). Molecular typing of selected Enterococcus faecalis isolates: pilot study using multilocus sequence typing and pulsed-field gel electrophoresis. *Journal of Clinical Microbiology*, *40*(3), 868-876.

Nallapareddy, S. R., Wenxiang, H., Weinstock, G. M., & Murray, B. E. (2005). Molecular characterization of a widespread, pathogenic, and antibiotic resistance-receptive Enterococcus faecalis lineage and dissemination of its putative pathogenicity island. *Journal of bacteriology*, *187*(16), 5709-5718.

Nateghian, A., Fallah, F., Daghighi, Z., Goudarzi, H., Hashemi, A., & Robinson, J. L. (2016). Detection of virulence genes in resistant enterococci isolated from pediatric patients at high risk for nosocomial infections. Diagnostic microbiology and infectious disease, 85(2), 260-262.

Nautiyal, S., Jauhari, S., Joshi, G., & Kataria, V. K. (2016). Characterization of *Enterococcus* species in a tertiary care hospital. *IOSR Journal of Pharmacy, 6(8), 48-51*.

Nawaz, A., Ali, S. M., Rana, N. F., Tanweer, T., Batool, A., Webster, T. J., ... & Iqbal, A. (2021). Ciprofloxacin-Loaded Gold Nanoparticles against Antimicrobial Resistance: An In Vivo Assessment. Nanomaterials, 11(11), 3152.

Nellore, A., Huprikar, S., & AST ID Community of Practice. (2019).Vancomycin-resistant Enterococcus in solid organ transplant recipients: Guidelines from the American Society of Transplantation Infectious Diseases Community of Practice. Clinical transplantation, 33(9): e13549.

Nicolau DP (2008) : Carbapenems: a potent class of antibiotics. Expert Opin Pharmacother. Jan;9(1):23-37. [Article]

Nieto-Arribas, P., Seseña, S., Poveda, J. M., Chicón, R., Cabezas, L., & Palop, L. (2011). Enterococcus populations in artisanal Manchego cheese: Biodiversity, technological and safety aspects. *Food Microbiology*, *28*(5), 891-899.

Nikta Ahmadpoor, Roya Ahmadrajabi, Sarvenaz Esfahani, Zoya Hojabri, Mohammad Hassan Moshafi, and Fereshteh Saffari. (2021). High-Level Resistance to Erythromycin and Tetracycline and Dissemination of ,Resistance Determinants

among Clinical Enterococci in Iran, *Medical Principles and Practice*, *30*(3), 272-276.

Nishimasu H, Ran FA, Hsu PD, Konermann S, Shehata SI, Dohmae N, Ishitani R, Zhang F, Nureki O. 2014. Crystal structure of Cas9 in complex with guide RNA and target DNA. *Cell* 156:935–949. doi: 10.1016/j.cell.2014.02. 001, PMID: 24529477

Noroozi, N.,Momtaz, H., & Tajbakhsh, E. (2022). Molecular characterization and antimicrobial resistance of *Enterococcus faecalis* isolated from seafood samples. *Veterinary Medicine and Science*, *8,* 1104–1112.

Nowakiewicz A, Zi ´ołkowska G, Zi ¸eba P, Gnat S, Tros´cian´ czyk A, Adaszek Ł. 2017. Characterization of multidrug resistant E. faecalis strains from pigs of local origin by ADSRRS-fingerprinting and MALDITOF MS; evaluation of the compatibility of methods employed for multidrug resistance analysis. PLoSOne 12:e0171160.

O'Driscoll T, Crank CW. 2015. Vancomycin-resistant enterococcal infections: epidemiology, clinical manifestations, and optimal management. Infect Drug Resist 8:217–30.

Oerlemans EFM, Wuyts S, Bellen G, Wittouck S, De Boeck I, Ruban K, *et al*.,(2020). The Dwindling Microbiota of Aerobic Vaginitis, an Inflammatory State Enriched in Pathobionts with Limited TLR Stimulation. Diagnostics. 10: 879.

Okwen, M. C. (2016). Investigation, identification and comparison of variations in virulence of *Enterococcus faecalis* from secondary endodontic and periodontal infections (Doctoral dissertation, The University of Texas School of Dentistry at Houston).

Ostrowsky BE, Trick WE, Sohn AH, Quirk SB, Holt S, Carson LA, Hill BC, Arduino MJ, Kuehnert MJ, Jarvis WR. 2001 . Control of vancomycin-resistant enterococcus in health care facilities in a region. N Engl J Med. May 10;344(19):1427-33.

Oyamada Y, Ito H, Fujimoto K, Asada R, Niga T, Okamoto R, Inoue M, Yamagishi J.2006. Combination of known and unknown mechanisms confers high-level resistance to fluoroquinolones in Enterococcus faecium. J Med Microbiol 55:729–736.

Paiao, F. G., Arisitides, L. G. A., Murate, L. S., Vilas-Boas, D. T.,Vilas-Boas, L. A. and Shimokomaki, M. (2012).Detection of *Salmonella* spp, *Salmonella Enteritidis* and *Typhimurium* in Natural Infected Broiler Chickens by a Multiplex PCR- Based Assay. Brazilliann. *Journal of Microbiology* 44(1): 37-41

Palmer KL, Carniol K, Manson JM, et al.,(2010). High-quality draft genome sequences of 28 Enterococcus sp. isolates. J Bacteriol.192:2469–2470.

Palmer KL, Gilmore MS. 2010. Multidrug-resistant enterococci lack CRISPR-*cas*. *mBio* 1:e00227-10–e00227-19. doi: 10.1128/mBio.00227-10, PMID: 21060735.

Panesso D, Reyes J, Rincon S, Diaz L, Galloway-Pena J, et al. (2010) Molecular epidemiology of vancomycin-resistant Enterococcus faecium: a prospective, multicenter study in South American hospitals. J Clin Microbiol 48: 1562–1569.

Panthee, S., Paudel, A., Hamamoto, H., Ogasawara, A. A., Iwasa, T., Blom, J., & Sekimizu, K. (2021). Complete genome sequence and comparative genomic analysis of Enterococcus faecalis EF-2001, a probiotic bacterium. *Genomics*, *113*(3), 1534-1542.

Patricia Ruiz-Garbajosa,1* Marc J. M. Bonten,2,3 D. Ashley Robinson,4 Janetta Top,2,3 Sreedhar R. Nallapareddy,5 Carmen Torres,6 Teresa M. Coque,1 Rafael Canto´n,1 Fernando Baquero,1 Barbara E. Murray,5 Rosa del Campo,1 and Rob J. L. Willems2,3 Multilocus Sequence Typing Scheme for *Enterococcus faecalis* Reveals Hospital-Adapted Genetic Complexes in a Background of High Rates of Recombination pdates-warnings-fluoroquinolone-antibiotics-risks-mental-health-and-low-blood-sugar-adverse) . U.S. Food and Drug Administration (Press release). 10 July 2018. Retrieved 15 April 2019.

Paulsen IT, Banerjei L, Myers GS, Nelson KE, Seshadri R, Read TD, Fouts DE, Eisen JA, Gill SR, Heidelberg JF, Tettelin H, Dodson RJ, Umayam L, Brinkac L, Beanan M, Daugherty S, DeBoy RT, Durkin S, Kolonay J, Madupu R, Nelson W, Vamathevan J, Tran

B, Upton J, Hansen T, Shetty J, Khouri H, Utterback T, Radune D, Ketchum KA, Dougherty BA, Fraser CM. 2003. Role of mobile DNA in the evolution of vancomycin-resistant *Enterococcus faecalis*. Science 299:2071–2074.

Perera, B, Audi, S, Solomou, S, Courtenay, K, Ramsay, H (2020). M ental and physical health conditions in people with intellectual disabilities: comparing local and national data. British Journal of Learning Disabilities 48, 19–27.

Peters, B. M., Jabra-Rizk, M. A., Graeme, A. O., Costerton, J. W., & Shirtliff, M. E. (2012). Polymicrobial interactions: impact on pathogenesis and human disease. Clinical microbiology reviews, 25(1), 193-213.

Phumisantiphong, U., Siripanichgon, K., Reamtong, O. & Diraphat, P. (2017). A novel bacteriocin from *Enterococcus faecalis* 478 exhibits a potent activity against vancomycin-resistant enterococci. *PLoS One* 12, e0186415.

Pinholt M, Østergaard C, Arpi M, Bruun NE, Schønheyder HC, Gradel KO, Søgaard M, Knudsen JD. 2014 Incidence, clinical characteristics and 30-day mortality of enterococcal bacteraemia in Denmark 2006–2009: A population-based cohort study.

Poh LengWeng,1 Ramliza Ramli,2 Mariana Nor Shamsudin,1 Yoke-Kqueen Cheah,3 and Rukman Awang Hamat1, (2017). High Genetic Diversity of *Enterococcus faecium* and *Enterococcus*

faecalis Clinical Isolates by Pulsed-Field Gel Electrophoresis and Multilocus Sequence Typing from a Hospital in Malaysia. *BioMed research international*.

Pontinen A K, TOP J, Arredondo-Alonso S, et al., (2021). Apparent nosocomial adaptation of *Enterococcus faecalis* predates the modern hospital era[J]. Nature Communications, 12(1): 1523. DOI:10.1038/s41467-021-21749-5.

Poulsen LL, Bisgaard M, Son NT, Trung NV, An HM, et al. (2012) *Enterococcus* faeciumclones in poultry and in humans with urinary tract infections, Vietnam. Emerging infectious diseases 18: 1096-1100.

Praharaj I., Sujatha S., Parija S.C. 2013. Phenotypic & genotypic characterization of vancomycin resistant Enterococcus isolates from clinical specimens. Indian J. Med. Res.138:549–556.

Prescott, J. F. (2013). Beta-lactam antibiotics: cephalosporins. Antimicrobial therapy in veterinary medicine, 139-157.

Price VJ, Huo W, Sharifi A, Palmer KL. 2016. CRISPR-Cas and Restriction-Modification Act additively against conjugative antibiotic resistance plasmid transfer in *Enterococcus faecalis*. *mSphere* 1:e00064-16. doi: 10.1128/mSphere.00064-16, PMID: 27303749

Pursey E, Dimitriu T, Paganelli FL, Westra ER, van Houte S. 2022. CRISPR-Cas is associated with fewer antibiotic resistance genes

in bacterial pathogens. *Philos Trans R Soc Lond B Biol Sci*.377(1842):20200464. doi:10.1098/rstb.2020.0464

Radhouani H, Silva N, Poeta P, Torres C, Correia S, Igrejas G. 2014. Potential impact of antimicrobial resistance in wildlife, environment and human health.Front Microbiol 5:23.

Rambaran K. A., Seifert C. F. (2019). Ciprofloxacin vs. levofloxacin for prophylaxis in recipients of hematopoietic stem cell transplantation. *J. Oncol. Pharm. Pract.* 25 (4), 884–890. 10.1177/1078155218787286 .

Ramsey, M., Hartke, A., & Huycke, M. (2014). The physiology and metabolism of enterococci. *Enterococci: From Commensals to Leading Causes of Drug Resistant Infection [Internet]*.

Rathnayake I, Hargreaves M, Huygens F. 2011. Snp diversity of *Enterococcus faecalis* and *Enterococcus faecium* in a south east Queensland waterway, Australia, and associated antibiotic resistance gene profiles. *BMC Microbiol*. 11: 201.

Reinseth, I., Tønnesen, H. H., Carlsen, H., & Diep, D. B. (2021). Exploring the therapeutic potenital of the leaderless enterocins K1 and EJ97 in the treatment of vancomycin-resistant enterococcal infection. *Frontiers in Microbiology, 12, 248*.

Reygaert, W. C. (2018). An overview of the antimicrobial resistance mechanisms of bacteria. *AIMS microbiology, 4(3), 482-501*.

Rice LB. 2001. Emergence of Vancomycin-Resistant Enterococci. Emerg Infect Dis. 7(2):183–187.

Rice, L. B. (2012, February). Mechanisms of resistance and clinical relevance of resistance to β-lactams, glycopeptides, and fluoroquinolones. In *Mayo Clinic Proceedings* (Vol. 87, No. 2, pp. 198-208). Elsevier.

Rossetti, L., & Giraffa, G. (2005). Rapid identification of dairy lactic acid bacteria by M13-generated, RAPD-PCR fingerprint databases. *Journal of Microbiological Methods*, *63*(2), 135-144.

Ruiz-Garbajosa P, Bonten M J, Robinson D A, et al. (2006). Multilocus sequence typing scheme for *Enterococcus faecalis* reveals hospital-adapted genetic complexes in a background of high rates of recombination[J]. Journal of Clinical Microbiology, 44(6):2220-2228. DOI:10.1128/JCM.02596-05.

Ryo Yamaguchi , Takehito Yamamoto, Koh Okamoto, Sohei Harada, Miho Echizenya, Takeya Tsutsumi, Tappei Takada, 2023. Teicoplanin and vancomycin as treatment for glycopeptide-susceptible *Enterococcus faecium* bacteraemia: a propensity score-adjusted noninferior comparative study , *Journal of Antimicrobial Chemotherapy*, *78*(5), 1231-1240.

Sadowy E, Sienko A, Hryniewicz W (2011) Comparison of multilocus variable-number tandem-repeat analysis with

multilocus sequence typing and pulsed-field gel electrophoresis for *Enterococcus faecalis*. Pol J Microbiol 60: 335-339.

Saffari, F., Dalfardi, M. S., Mansouri, S., & Ahmadrajabi, R. (2017). Survey for correlation between biofilm formation and virulence determinants in a collection of pathogenic and fecal Enterococcus faecalis isolates. *Infection & chemotherapy*, *49*(3), 176-183.

Said, H. S., & Abdelmegeed, E. S. (2019). Emergence of multidrug resistance and extensive drug resistance among enterococcal clinical isolates in Egypt. Infection and drug resistance, 12,1113-1125.

Said, M. S., Tirthani, E., & Lesho, E. (2021). *Enterococcus infections*. StatPearls Publishing.

Sambrook J,MacCallumP, Russell D.Molecular cloning: (2005). A laboratorymanual. 3rd ed. Cold Spring Harbor, NY: Laboratory Press.

Sangeetha KT, Golia S, Vasudha CL. 2017. A study of aerobic bacterial pathogens associated with vaginitis in reproductive age group women (15–45 years) and their sensitivity pattern. International Journal of Research in Medical Sciences. 3: 2268–2273.

Santos, B. A., Oliveira, J. S., Cardoso, N. T., Barbosa, A. V., Superti, S. V., Teixeira, L. M., & Neves, F. P. (2017). Major globally

disseminated clonal complexes of antimicrobial resistant enterococci associated with infections in cancer patients in Brazil. Infection, Genetics and Evolution, 55, 56-62.

Sartelli, M., Weber, D. G., Ruppé, E., Bassetti, M., Wright, B. J., Ansaloni, L., ... & Moore, E. E. (2016). Antimicrobials: a global alliance for optimizing their rational use in intra-abdominal infections (AGORA). *World journal of emergency surgery*, *11*(1), 1-32.

Sattari-Maraji, A., Jabalameli, F., Farahani, N. N., Beigverdi, R., & Emaneini, M. (2019). Antimicrobial resistance pattern,virulence determinants and molecular analysis of *Enterococcus faecium* isolated from children infections in Iran. BMC microbiology, 19(1), 1-8.

Scheidegger, E. M. D., Fracalanzza, S. A. P., Teixeira, L. M., & Cardarelli-Leite, P. (2009). RFLP analysis of a PCR-amplified fragment of the 16S rRNA gene as a tool to identify Enterococcus strains. *Memórias do Instituto Oswaldo Cruz*, *104*, 1003-1008.

Schleifer KH, Kilpper-Balz R. 1984 Transfer of Streptococcus faecalis and Streptococcus faecium to the Genus Enterococcus norn. rev. as Enterococcus faecalis comb. nov. and Enterococcus faecium comb. nov. Int J Syst Bacteriol Int Union Microbiol Soc 34:31–34.

Seenaa, S.A. and Lamees A. Abdul-Lateef (2020). Detection of Virulence Genes of Enterococcus Faecalis Isolates In Hilla Ctiy.

Selle K, Barrangou R. 2015. Harnessing CRISPR–Cas systems for bacterial genome 672 editing. Trends Microbiol 23:225–232.

Seno, Y., Kariyama, R., Mitsuhata, R., Monden, K. & Kumon, H. (2005). Clinical implications of biofilm formation by *Enterococcus faecalis* in the urinary tract. *Acta Med Okayama* 59, 79-87.

Shabbir MA, Wu Q, Shabbir MZ, et al., (2018). The CRISPR- cas system promotes antimicrobial resistance in *Campylobacter jejuni*. *Fut Microbiol*.13(16):1757–1774. doi:10.2217/fmb-2018-0234

Shankar N., et al., 2001. Role of Enterococcus faecalis Surface Protein Esp in thePathogenesis of Ascending Urinary Tract Infection., Infect.Immun.., 2001, 69(7), 4366–4372 doi: 10.1128/IAI.69.7.4366-4372 .

Sharma,B. Kumar,A. and Sarin,J. (2016). Academic Stress, Anxiety, Remedial MeasuresbAdopted and Its Satisfaction among Medical Student, *A Systematic Review*, *International Journal of Health Sciences and Research*, 6(7), 368-376.

Sharqi, H. M., Hassan, O. M., & Obaid, A. S. (2021). Investigation of the Antibiotic-Resistant ESKAPE Pathogens in Ramadi

Hospitals, Iraq. Indian Journal of Forensic Medicine &Toxicology, 15(4), 3307.

Shazadi K, Ahmad SZ, Ahmad SS, Arshad N. 2021. *In vivo* prophylactic efficacy of Lactobacillus reuteri MT180537 against aerobic vaginitis. Microbial Pathogenesis. 160: 105197.

Shiadeh, S. M., Pormohammad, A., Hashemi, A., & Lak, P. (2019). Global prevalence of antibiotic resistance in blood-isolated *Enterococcus faecalis* and *Enterococcus faecium*: A systematic review and meta-analysis. *Infection and Drug Resistance*, *12*, 2713.

Shuan Tao, Huimin Chen1, Na Li, Yewei Fang, Yao Xu, Wei Liang. 2022.Association of CRISPR-Cas System with the Antibiotic Resistance and Virulence Genes in Nosocomial Isolates of *Enterococcus*. significance for public health[J]. Clinical Microbiology and Infection. 6939-6949.

Snustad, D. P., & Simmons, M. J. (2015). *Principles of genetics*. John Wiley & Sons.

Son R, Nimita F, Rusul G, Nasreldin E, Samuel L, Nishibuchi M. 1999.Isolation and molecular characterization of vancomycin resistant *Enterococcus faecium* in Malaysia. *Lett Appl Microbiol*; 29: 118-22.

Sorek, R., Kunin, V., Hugenholtz, P. (2008) CRISPR–a widespread system that provides acquired resistance against phages in bacteria and archaea. Nat. Rev. Microbiol. 6, 181–186.

Spratt, B. G., Hanage, W. P., & Feil, E. J. (2001). The relative contributions of recombination and point mutation to the diversification of bacterial clones. *Current opinion in microbiology*, *4*(5), 602-606.

Ste¸pien´ -Pys´niak D, Marek A, Banach T, Adaszek Ł, Pyzik E, Wilczyn´ ski J, Winiarczyk S. 2016. Prevalence and antibiotic resistance of Enterococcus strains isolated from poultry. Acta Vet Hung 64:148–163.

Stecher, B., & Hardt, W. D. (2011). Mechanisms controlling pathogen colonization of the gut. Current opinion in microbiology, 14(1), 82-91.

Stroud L, Edwards J, Danzing L, Culver D, Gaynes R. 1996 Risk factors for mortality associated with enterococcal bloodstream infections. InfectControlHospEpidemiol 17:576–580.

Stuart, C; Schwartz, S; Beeson, T; Owatz, C (February 2006). "Enterococcus faecalis: Its Role in Root Canal Treatment Failure and Current Concepts in Retreatment"

Sun et al. (2015) Z. Sun, W. Liu, Y. Song, H. Xu, J. Yu, M. Bilige, H. Zhang, Y. Chen Population structure of Lactobacillus helveticus isolates from naturally fermented dairy products based on

multilocus sequence typing J. Dairy Sci., 98 (2015), pp. 2962-2972

Svetitsky S, Leibovici L, Paul M. 2009. Comparative efficacy and safety of vancomycin versus teicoplanin: systematic review and meta-analysis. *Antimicrob Agents Chemother*, 53: 4069–79.

Takeuchi N, Wolf YI, Makarova KS, et al. 2012. Nature and intensity of selection pressure on CRISPR-associated genes. J Bacteriol.194:1216–25.

Tao Z, Zhang L, Zhang Q, Lv T, Chen R, Wang L, *et al*. 2019.The Pathogenesis of Streptococcus anginosus in Aerobic Vaginitis. Infect Drug Resist.12: 3745–3754.

Tateda, K., Ohno, A., Ishii, Y., Murakami, H., Yamaguchi, K., & Levofloxacin Surveillance Group. (2019). Investigation of the susceptibility trends in Japan to fluoroquinolones and other antimicrobial agents in a nationwide collection of clinical isolates: A longitudinal analysis from 1994 to 2016. *Journal of Infection and Chemotherapy*, *25(8), 594-604.*

Taur Y, Xavier JB, Lipuma L, Ubeda C, Goldberg J, Gobourne A, Lee YJ, Dubin KA, Socci ND, Viale A, Perales MA, Jenq RR, Van Den Brink MRM, Pamer EG. 2012 Intestinal domination and the risk of bacteremia in patients undergoing allogeneic hematopoietic stem cell transplantation. Clin Infect Dis 55:905–914.

Tedim AP, Ruiz-Garbajosa P, Corander J, Rodríguez CM, Cantón R, Willems RJ, Baquero F, Coque TM. 2015. Population biology of intestinal Enterococcus isolates from hospitalized and nonhospitalized individuals in different age groups. Appl Environ Microbiol.81(5):1820–31.

Terns MP, Terns RM. 2011.CRISPR-based adaptive immune systems. Curr Opin Microbiol.14:321–7.

Thompson, J. D., Higgins, D. G., & Gibson, T. J. (1994). CLUSTAL W: improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position-specific gap penalties and weight matrix choice. *Nucleic acids research*, *22*(22),4673–4680.

Tian Y, Yu H, Wang Z. (2019 Dec). Distribution of acquired antibiotic resistance genes among Enterococcus spp. isolated from a hospital in Baotou, China. *BMC Res Notes*;12((1)):27.

Tien BYQ, Goh HMS, Chong KKL, Bhaduri-Tagore S, Holec S, Dress R, et al.,(2017 Nov). Enterococcus faecalis Promotes Innate Immune Suppression and Polymicrobial Catheter-Associated Urinary Tract Infection. Freitag NE, editor. Infect Immun. 17;85(12):225–44.

Tong, Z., J. Ma, J. Tan, L. Huang, and J. Ling. 2016. ‘Effects of inactivated Enterococcus faecalis on the proliferation and

osteogenic induction of osteoblasts', Mol Med Rep, 14: 5125–33.

Turner KM, Hanage WP, Fraser C, Connor TR, Spratt BG (2007) Assessing the reliability of eBURST using simulated populations with known ancestry. BMC Microbiol 7: 30

van Harten, R. M., Willems, R. J. L., Martin, N. I. & Hendrickx, A. P. A. (2017). Multidrug-resistant enterococcal infections: New compounds, novel antimicrobial therapies? *Trends Microbiol*, doi:10.1016/j.tim.2017.01.004.

Vandepitte, J., Engbaek, P., Piot, P. and Heuck, C. (1991). Basic laboratory procedures in clinical bacteriology. Geneva, World Health Organization: 31-35.

Vu J, Carvalho J. 2011. Enterococcus: review of its physiology, pathogenesis, diseases and the challenges it poses for clinical microbiology. *Front Biol*.6:357–66.

Wan Y, Mills E, Leung RCY, Vieira A, Zhi X, Croucher NJ, Woodford N, Jauneikaite E, Ellington MJ, Sriskandan S. (2021 Dec). Alterations in chromosomal genes *nfsA*, *nfsB*, and *ribE* are associated with nitrofurantoin resistance in *Escherichia coli* from the United Kingdom. Microb Genom.7(12) [PMC free article: PMC8767348] [PubMed: 34860151].

Wang C, Fan A, Li H, Yan Y, Qi W, Wang Y, *et al*. (2020). Vaginal bacterial profiles of aerobic vaginitis: a case—control study. Diagnostic Microbiology and Infectious Disease. 96: 114981.

Wang FY, Fang B, Qiang XH, Yu TO, Zhong JR, Cao J, et al. (2016 May). The Efficacy and Immunomodulatory Effects of Ulinastatin and Thymosin α1 for Sepsis: A Systematic Review and Meta-Analysis. BioMed Research International. Hindawi; 31;2016(2):1–8.

Wei Hong Tay, Ronni A.G. da Silva, Foo Kiong Ho, Kelvin K.L. Chong, Alexander Ludwig,Kimberly A. Kline, 2021-09. Enterococcus faecalis persists and replicates within epithelial cells in vitro and in vivo during wound infection, *bioRxiv*.

Weiner LM, Webb AK, Limbago B, Dudeck MA, Patel J, Kallen AJ, Edwards JR, Sievert DM. 2016 Antimicrobial-Resistant Pathogens Associated With Healthcare-Associated Infections: Summary of Data Reported to the National Healthcare Safety Network at the Centers for Disease Control and Prevention, 2011–2014. Infect Control Hosp Epidemiol. 37(11):1288–1301.

Weinstock DM, Conlon M, Iovino C, Aubrey T, Gudiol C, Riedel E, *et al*. (2007). Colonization, bloodstream infection, and mortality caused by vancomycin-resistant enterococcus early after allogeneic hematopoietic stem cell transplant. Biology of Blood and Marrow Transplantation. 13: 615–621.

Weng PL, Ramli R, Shamsudin MN, Cheah YK, Hamat RA. 2013. High genetic diversity of Enterococcus faecium and Enterococcus faecalis clinical isolates by pulsed-field gel electrophoresis and multilocus sequence typing from a hospital in Malaysia. Biomed Res Int 2013:938937.

Werner G, Fleige C, Fessler AT, Trung NV, An HM, et al. (2012) Improved identification including MALDI-TOF mass spectrometry analysis of group D streptococci from bovine mastitis and subsequent molecular characterization of corresponding *Enterococcus* faecium and *Enterococcus* faecium isolates. Vet Microbiol 160: 162-169.

Wheatley RM, MacLean RC. 2021. CRISPR-Cas systems restrict horizontal gene transfer in Pseudomonas aeruginosa. *ISME J*.15(5):1420–1433. doi:10.1038/s41396-020-00860-3

Wilkie, T. M., and M. Simon. 1991. Cloning multigene families with degenerate PCR primers. Methods in Enzymology 2:32-41.

Willems, R. J., Homan, W., Top, J., van Santen-Verheuvel, M., Tribe, D., Manzioros, X., ... & Bonten, M. J. (2001). Variant esp gene as a marker of a distinct genetic lineage of vancomycinresistant Enterococcus faecium spreading in hospitals. *The Lancet*, *357*(9259), 853-855.

Williams, J. G., Kubelik, A. R., Livak, K. J., Rafalski, J. A., & Tingey, S. V. (1990). DNA polymorphisms amplified by arbitrary

primers are useful as genetic markers. *Nucleic acids research*, *18*(22), 6531-6535.

Winn, W. C., Allen, S. D., Janda W. M. and Koneman and E. W. (2006). Koneman,s color atlas and textbook of diagnostic microbiology. 6th ED. Lippincott Williams and Wilkins, USA. 234-241.

World Health Organization. *Global guidelines for the prevention of surgical site infections*. World Health Organization, 2016.

Wu, D., Lu, J., Liu, Y., Zhang, Z., & Luo, L. (2020). Positive effects of COVID-19 control measures on influenza prevention. *International Journal of Infectious Diseases*, *95*, 345-346.

Xu Y, Li Z. 2020. CRISPR-Cas systems: overview, innovations and applications in human disease research and gene therapy. Comput Struct Biotechnol J.18:2401–2415. doi:10.1016/j.csbj.2020.08.031

Yasufuku T, Shigemura K, Shirakawa T, Matsumoto M, Nakano Y, Tanaka K, et al., (2011). Mechanisms of and risk factors for fluoroquinolone resistance in clinical Enterococcus faecalis isolates from patients with urinary tract infections. J Clin Microbiol. 49:3912–3916.

Yilema, A., Moges, F., Tadele, S., Endris, M., Kassu, A., Abebe, W.,& Ayalew, G. (2017). Isolation of enterococci, their antimicrobial

susceptibility patterns and associated factors among patients attending at the University of Gondar Teaching Hospital. BMC infectious diseases, 17(1), 1-8.

Zalipour, M., Esfahani, B. N., Halaji, M., Azimian, A., & Havaei, S. A. (2019). Molecular characterization of vancomycin-resistant Enterococcus faecalis among inpatients at Iranian University Hospitals: clonal dissemination of ST6 And ST422. *Infection and drug resistance*, 3039-3047.

Zhang, X., Paganelli, F. L., Bierschenk, D., Kuipers, A., Bonten, M. J., Willems, R. J., & Van Schaik, W. (2012). Genome-wide identification of ampicillin resistance determinants in Enterococcus faecium. *PLoS genetics*, *8*(6), e1002804.

Zhen X, Lundborg CS, Sun X, Hu X, Dong H. 2019. Economic burden of antibiotic resistance in ESKAPE organisms: a systematic review. Antimicrob Resist Infect Control.8:137.

Zheng JX, Li H, Pu ZY, Wang HY, Deng XB, Liu XJ, Deng QW, Yu ZJ. 2017. Bloodstream infections caused by Enterococcus spp:A 10-year retrospective analysis at a tertiary hospital in China. J Huazhong Univ Sci Technolog Med Sci. Apr;37(2):257-263.

Zheng Z, Zhang Y, Liu Z, et al. (2020). The CRISPR-Cas systems were selectively inactivated during evolution of Bacillus cereus group for adaptation to diverse environments. *ISME J*.14(6):1479–1493. doi:10.1038/s41396-020-0623-5

Zhenget J.,al., 2020. Characterization of biofilm formation byEnterococcus faecalisisolates derived from urinary tract infections in China., J Med Microbiol. 67, 60-67 doi:10.1099/jmm.0.000647.

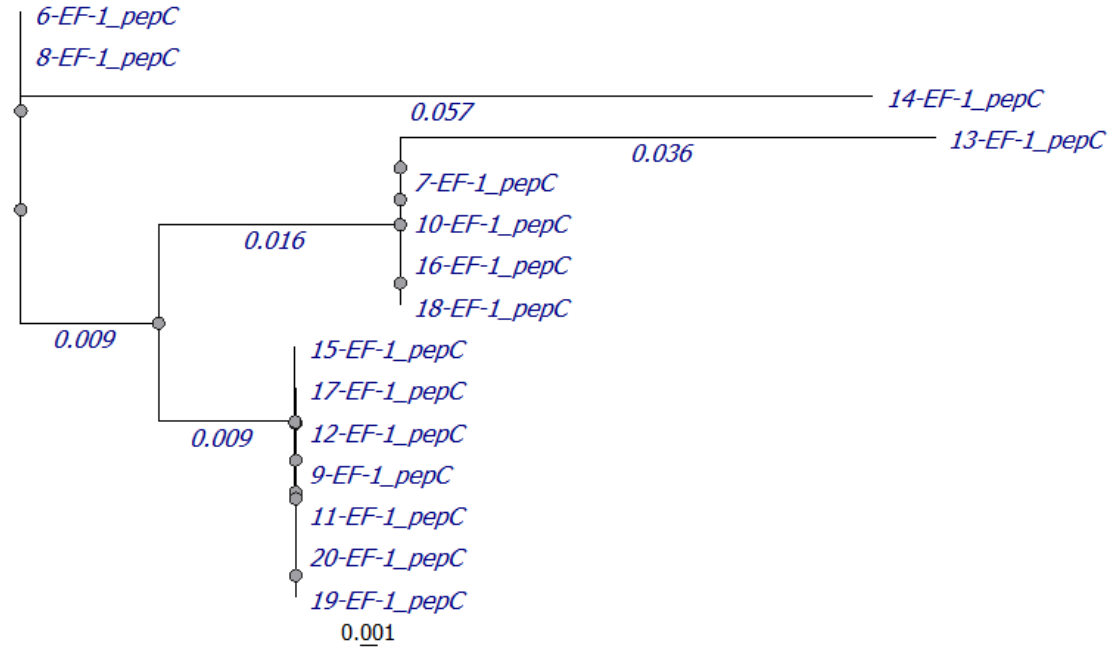
Zoletti, G. O., Pereira, E. M., Schuenck, R. P., Teixeira, L. M., Siqueira Jr, J. F., & dos Santos, K. R. N. (2011). Characterization of virulence factors and clonal diversity of Enterococcus faecalis isolates from treated dental root canals. *Research in microbiology*, *162*(2), 151-158.

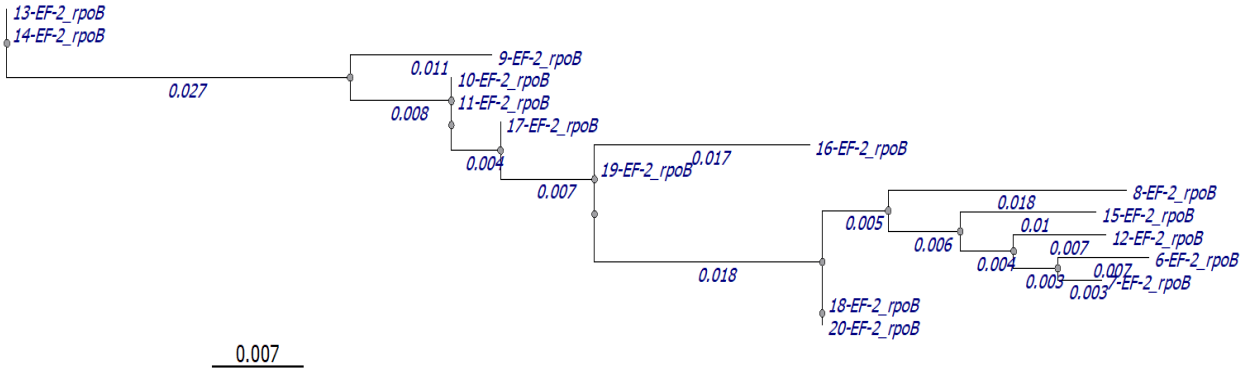
Zou, J. 2016. 'The opportunistic pathogen Enterococcus faecalis resists phagosome acidification and autophagy to promote intracellular survival in macrophages', Cell Microbiol , 18: 831–43.

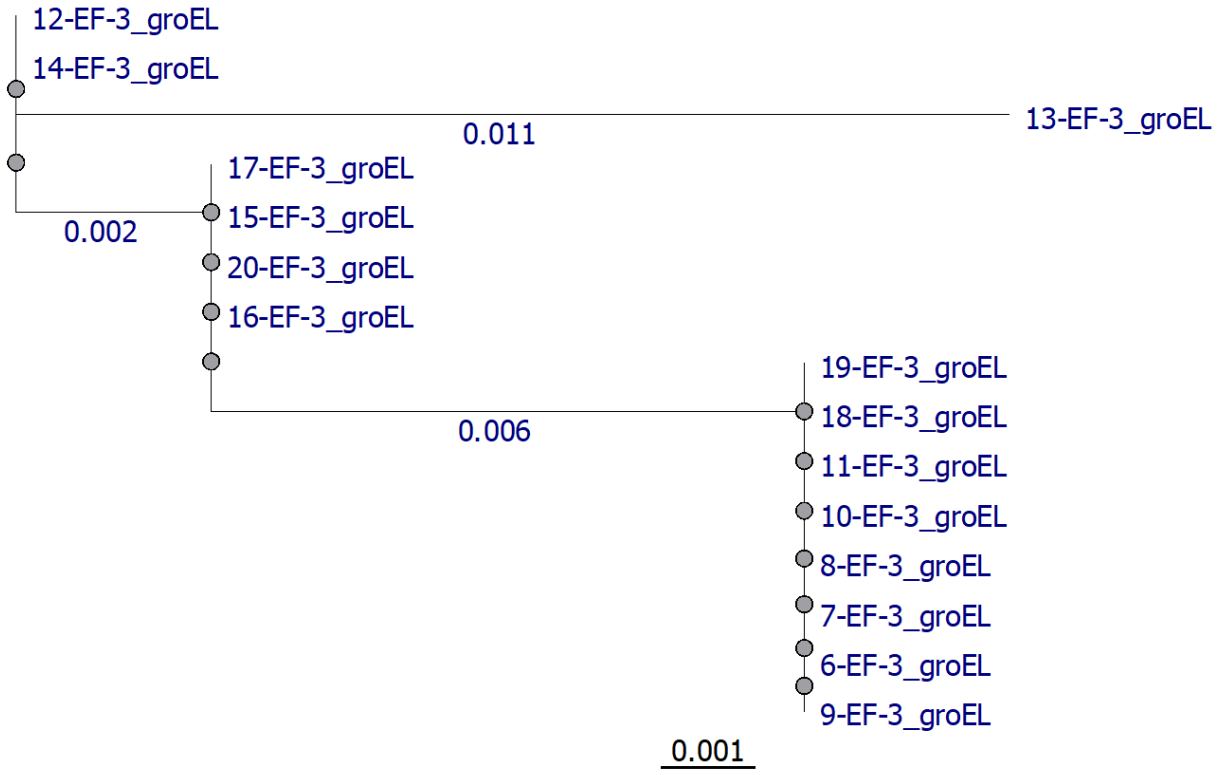
Zou, J., and N. Shankar. 2014. 'Enterococcus faecalis infection activates phosphatidylinositol 3-kinase signaling to block apoptotic cell death in macrophages', Infect Immun, 82: 5132–42. Abstract/FREE Full Text Google Scholar.

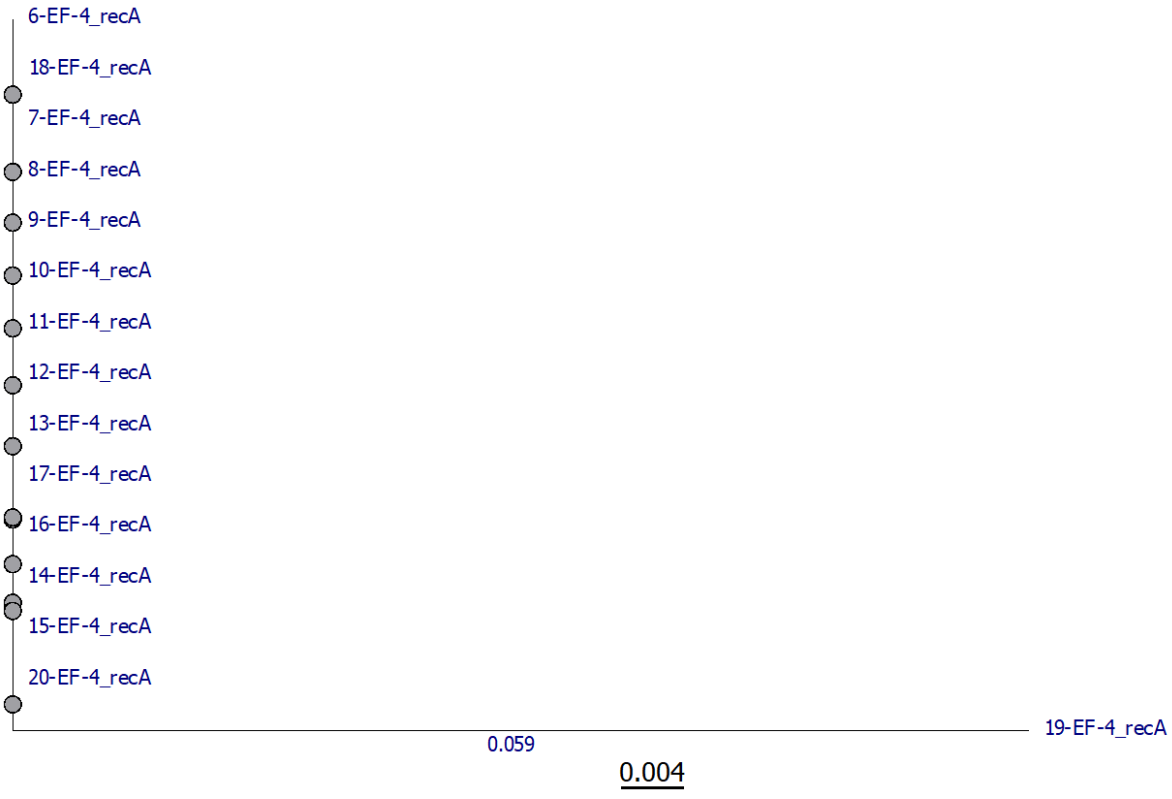
Zusso M., Lunardi V., Franceschini D., Pagetta A., Lo R., Stifani S., et al. (2019). Ciprofloxacin and levofloxacin attenuate microglia inflammatory response via TLR4/NF-kB pathway. *J. Neuroinflammation*16 (1), 148. 10.1186/s12974-019-1538-9.

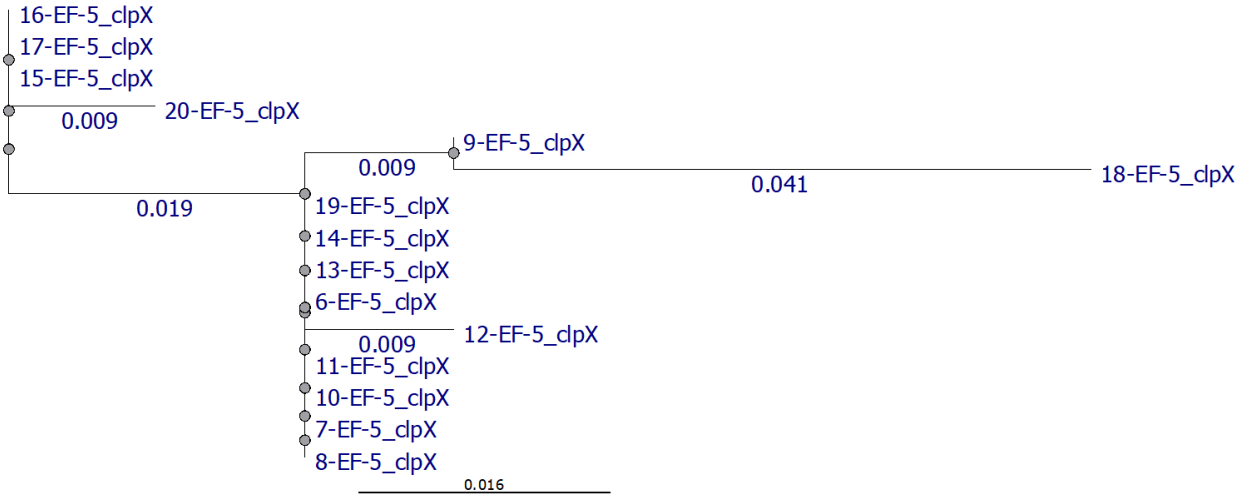
Appendix (1): Phylogram analysis based on (*pepC*, *rpoB*, *groEL, recA, clpX*) sequences from 15 *Enterococcus faecalis* isolates by maximum likelihood method.






12-EF-3_groEL
14-EF-3_groEL
0.011
13-EF-3_groEL
17-EF-3_groEL
0.002
15-EF-3_groEL
20-EF-3_groEL
16-EF-3_groEL
19-EF-3_groEL
0.006
18-EF-3_groEL
11-EF-3_groEL
10-EF-3_groEL
8-EF-3_groEL
7-EF-3_groEL
6-EF-3_groEL
9-EF-3_groEL
0.001


6-EF-4_recA
18-EF-4_recA
7-EF-4_recA
8-EF-4_recA
9-EF-4_recA
10-EF-4_recA
11-EF-4_recA
12-EF-4_recA
13-EF-4_recA
17-EF-4_recA
16-EF-4_recA
14-EF-4_recA
15-EF-4_recA
20-EF-4_recA
0.059
19-EF-4_recA
0.004


16-EF-5_clpX
17-EF-5_clpX
15-EF-5_clpX
0.009
20-EF-5_clpX
0.009
9-EF-5_clpX
0.041
18-EF-5_clpX
0.019
19-EF-5_clpX
14-EF-5_clpX
13-EF-5_clpX
6-EF-5_clpX
0.009
12-EF-5_clpX
11-EF-5_clpX
10-EF-5_clpX
7-EF-5_clpX
8-EF-5_clpX
0.016

الخلاصة

لقد تمّ من خلال هذه الدراسة جمع 105 عيّنة سريرية من مرضى يعانون من عدوى مختلفة كالتهاب المسالك البولية والتهاب المهبل والتهاب الجرح وتجرثُم الدم , والذين تمَّ إدخالهم إلى مستشفيينِ رئيسيينِ في مدينة الحلة , الأولى هي مستشفى الحلة التعليمي الجراحي والثانية هي مستشفى الامام الصادق عليه السلام , للفترة الزمنية الممتدة من (اغسطس 2022إلى نوفمبر 2022) .

وخضعت جميع أنواع العينات للزراعة الهوائية على أوساط مختلفة , فوجد أنّه من اجمالي (105) من العيّنات المزروعة ظهر منها 70 (66,6%) هي بكتيرية إيجابية , أمّا الكمية المتبقية من البكتيرية نفسها والتي تبلغ 35 (33,3%) لم يلحظ عليها أي نمو, علماً أنّ جميع العيّنات تمّ زرعها على وسط (chromogenic agar _ وسط انتقائي) , وتم تحديد 15 (14,2%) عينة إيجابية على أنّها (*E. faecalis*) فقد تمَّ اخضاع جميع عيّنات (*E. faecalis*) المعزولة من مصادر مختلفة في المختبر لاختبار الحساسية للمضادات الحيوية , وذلك عن طريق نشر قرص Kirby-bauer- - المعدلة , فوجدنا أنّ عُزلات (*E. faecalis*) الخمسة عشر عزلة هي 100% مقاومة للمضادات الحيوية (ciprofloxacin – clindamycin – kanamycin) , حيث أنّ أربع عشرة عزلة (93.3%)مقاومة(Imipenem) وأنّ ثلاث عشرة عزلة 86,6 % كانت مقاومة (Nitrofurantoin & Refampin) واثنتا عشرة عزلة % 80مقاومة (Erythromycin) و إحدى عشرة عزلة 73,3 % مقاومة (Vancomycin) وعزلتان اثنتان 13,3% مقاومة (Levofloxacin) وعزلة واحدة 6,6% مقاومة (Teicoplanin & piperacillin) .

وبعد ذلك خضعت هذه العزلات لطريقة الكشف الجزئي باستخدام أساس محدد يعتمد على جين (D- alanine D-alanine ligase) كمؤشر جيني للتعرف وبصورة مؤكدة على (*E. faecalis*) بواسطة (PCR) وأظهرت النتائج أنّ (15%) كانت موجبة ل (ddl) .

وتمَّ إجراء العلاقة بين خمس عشرة عزلة *E.faecalis* باستخدام RAPD-PCR .

فأظهر التمهيدي M13 تعدد الأشكال بين اختبارالعزلات فولّدَ (7-14) نطاقاً يتراوح بين (95 - إلى - 3000) زوج قاعدي .

كما وأنّ الرسم التخطيطي للمسافة الجينيّة بين جميع المعزولين بناءً على تعدد الأشكال المتولدة عن (RAPD- PCR) بعد استخدام التمهيدي (M13) باستخدام (UPGMA) حيث قسّم المخطط (cladogram) جميع العزلات الخمس عشرة إلى مجموعتين , فقد احتوت المجموعة الأولى (A) على عزلة واحدة فقط (EF17) والمجموعة الثانية (B) تحتوي على أربع عشرة عزلة , مقسمة (14) عزلة إلى مجموعتينِ فرعيّتينِ , المجموعة الفرعية الأولى مقسمة إلى فرعينِ ,

المجموعة الفرعية الاولى احتوت إحداها على عزلتين (EF12-EF13) , بينما احتوت المجموعة الفرعية الثانية على ثلاث عزلات (EF18-EF19-EF20) حيث أنّ المجموعة (B) أظهرت ارتباطاً وراثياً وثيقاً وبنفس المسافة الوراثية بين بعض العزلات , ممّا أظهر انتشاراً نسيلياً , وأنّ علاقتهم الوراثية الوثيقة القائمة على معامل الاختلاف إلى تباين جيني منخفض بينهما .

ففي هذه الدراسة تمَّ استخدام طريقة الكتابة المتسلسلة ذات البؤرة المتعددة (MLST) للتحقيق في القدرة التّميزية , والتكاثر , والعلاقة الوراثية بين خمس عشرة (15) عزلة *E.faecalis* . تعتمد على بيانات التسلسل التي تمّ الحصول عليها من (MLST)لتحديد الهياكل السكّانيّة التي تحلل مدى اختلال التوازن بين الآليات للعلاقة التطورية بين خمس عشرة (15) عزلة , على جينات التدبير المنزلي الخمسة المستخدمة بشكل متكرر لتحليل (*pep C, clpX ,recA ,rpoB,&groEL*) وفي الدراسة الحالية تم استخدام MLST لاستكشاف التركيب السكاني وتطور 15 عزلة (*E.faecalis*) من عيّنات سريرية مختلفة والتي قد توفّر معلومات أفضل فيما يتعلق بخصائصها البيولوجية , ولبدأ التحليل تمّ احتساب تنوع التسلسل لجينات التدبير المنزلي الخمسة ,

فقد تمّ تنفيذ هذه الخطوة لقياس ما إذا كانت هذه المواقع المختارة لديها تميّز كافٍ في الكتابة . ويتراوح عدد الآليات في هذه المواضيع الجينيّة من (2 – 7) مقارنة بتنوعات تسلسل النيوكليوتيدات متشابهة ولكن منخفضة بشكل عام , مما يعيد تكوين هذه الجينات .

أيضاً تمّ اكتشاف الدراسة الحالية (ST) في أن تكون فريدة من نوعها في المنطقة , ويمكن أن يساعد المزيد من التحليل وربما التسلسل الجيني لهذه السلالات في تحديد خصائص هذه السلالات .

وفقاً للملف الشخصي الأليلي , وجد أن المتغير الأليلي (SNP ,الادراج والحدف) بين العزلات في الحالة كان (*rpoB*) أكثر تنوعاً أو تحرراً من جينات التدبير المنزلي الأربعة الأخرى على عكس (*recA*) الذي كان أقل تبايناً . ويبدو أن الجين المختار لمخطط (MLST) الحالي يمثل تعدد الأشكال العام الذي شوهد في جينات التدبير المنزلي للإشريكية الفاسقة .

تم الاستدلال على نسالة هذه (ST) , وفي شجرة نسألة (MLST) لجميع سلالات (*E.faecalis*) إلى الحد الأقصى مثل نهج غطاء المحرك من تسلسل متسلسل . فقد أظهرت جميع عزلات (*E.faecalis*) سلالة متعددة الفصائل وكشفت عن ثلاث مجموعات متميّزة ,

المجموعة Aتحتوي على عزلة واحدة (EF13) والمجموعة (B) تحتوي على عزلة واحدة (EF14) والمجموعة C تحتوي على 13 عزلة , تنتشر أنظمة (CRISPR-Cas) على نطاق واسع بين التعانق والبكتيريا , والتي تحمي هذه الكائنات الحية من العناصر الوراثية المتنقلة مثل العاثيات والبلازميدات و اللينقولات تتم تضمين آلية عمل هذه الأنظمة في ثلاث خطوات للتكيف والتعبير والتداخل أقترح تحليل الجينوم .

إنّ أنظمة (CRISPR-Cas) تتفاعل مع العناصر المتنقلة يحتوي (*E.faecalis*) على نوع واحد من (CRISPR-Cas) وهو من النوع الثاني , وهنالك ثلاثة مواضع ل (CRISPR) في جينومات (*E.faecalis*) والتي تشمل (CRISPR1-Cas,CRISPR2-Cas,CRISPR3-Cas).

في هذه الدراسة حددنا حدوث مواضع CRISPR-Cas في بكتيريا (*E.faecalis*) المعزولة من مصادر معدية مختلفة (البول , المهبل , الجرح) توزيع وتكرار ثلاثة عناصر مختلفة في عزلات (*E.faecalis*) بشكل عام , ثم تحديد CRISPR2 في 8 (53.3%) من العزلات متبوعة ب (CRISPR1-Cas,CRISPR3-Cas) (33.3%,20%)على التوالي , وأظهرت الدراسة الحالية إنّ وجود مواضع CRISPR كان متغيّراً بين عزلات *E.faecalis* كانت العزلات أكثر عرضة لإيواء CRISPR2 اليتيم من CRISPR1-Cas,CRISPR3-Cas.

وزارة التعليم العالي والبحث العلمي

جامعة بابل/كلية الطب

فرع الاحياء المجهرية

التوصيف الجزيئي لنظام CRISPR-Cas والتنميط المتسلسل متعدد المواقع (MLST) لعزلات *Enterococcus faecalis* في محافظة بابل

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

من قبل
شيماء عبد الجبار سعيد الحسيني
بكالوريوس احياء مجهرية
جامعة بغداد -2009

اشراف

الأستاذ الدكتو رة
لميس عبد الرزاق عبد اللطيف

1445 ه **2023م**