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Molecular genotyping of *enterococcus faecalis* isolated from clinical sources by BOX-PCR and ERIC-PCR

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***Abstract*---**Gram-positive bacteria, *Enterococcus faecalis*, cause serious nosocomial infections such as UTI, bloodstream infections, and endocarditis. The study's aimed to isolate and identify *Enterococcus faecalis* from various clinical specimens in Al-Diwanyiah province hospitals ,testing the sensitivity of isolates to antibiotics as well as to genotyping of *E.faecalis* isolates by BOX –PCR or ERIC-PCR Analysis. The study extended from October 2021 to January 2022. Biochemical tests, Vitek 2 and 16S rRNA analysis identified 37 bacteria as *Enterococcus faecalis*. Five clusters (I-V) and about 13 polymorphic variants showed among 20 isolates by ERIC PCR. Whereas, four cluster (I-IV) and 14 polymorphic variants were showed by BOX PCR. The results showed that that there is a slight difference between ERIC and BOX-PCR analysis for *Enterococcus faecalis* isolated from clinical samples.

***Keywords*---**Clinical specimens, *Enterococcus faecalis*, Antibiotic resistance, ERIC PCR, BOX PCR.

Introduction

Enterococcus faecalis(*E. faecalis*) is a Gram-positive bacterium that is facultative anaerobic and does not generate spores. It can be seen as short chains, pairs, or single cells under the microscope. As normal commensals, they live in the human intestine (AL-Saadi , 2013). Healthy people aren't at risk from infection from this pathogen, but patients in intensive care units with serious illnesses or compromised immune systems are at risk. It is most commonly responsible for serious nosocomial infections such as urinary tract infections, endocarditis, bacteremia, intra-abdominal abscesses, and intra-pelvic abscesses, all of which

are associated with the bacterium *E. faecalis*. (Bhardwaj *et al.*, 2013). Many antibiotics, including penicillin, ampicillin, piperacillin, and vancomycin, are inherently resistant to *E. faecalis* (Kristich *et al.,* 2014) . Methods such as pulsed field gel electrophoresis (PFGE) for E. faecalis source surveillance and tracing are molecular typing methods that can be used (Weng *et al.*, 2013), amplified fragment length polymorphism (AFLP)(Weterings *et al.*, 2021), RAPD (random amplified polymorphic DNA) (Pourakbari *et al.*, 2013), multi-locus sequence typing (MLST)(Werner *et al.*, 2012),and two of the repetitive elements are BOX elements and enterobacterial repetitive intergenic consensus (ERIC) sequences(Bachtiar et al., 2015; Syrmis et al., 2004), can be used. The Enterococcus genome has a huge number of repetitive sequences that are randomly scattered across DNA. ERIC-PCR produces a distinct pattern for each strain, which is considered a distinct type. Dispersed repeat sequences are relatively short non-coding sequences found throughout the prokaryotic and eukaryotic genomes. BOX-PCR and ERIC-PCR primers are complementary to these repetitive sequences, allowing for specialized binding and distinct BOX-PCR fingerprint patterns, as well as ERIC-PCR with reproducibility(Ahmadi *et al.*, 2019).For processing a large number of isolates, genotyping technologies such as BOX-PCR provide a higher throughput and are less expensive than sequencing analysis(Nayak et al., 2011). For genotyping, researchers have utilized a variety of molecular approaches, the most powerful of which is Enterobacterial Repetitive Intergenic Consensus (ERIC) -PCR. This procedure is faster, easier, and less expensive than previous genomic typing methods(Wei et al., 2017) .The aim of this research is molecular genotyping Of *Enterococcus faecalis* Isolated from clinical sources by BOX-PCR and ERIC-PCR .

Methods

Isolation and Biochemical Identification of *Enterococcus faecalis*

Thirty seven *Enterococcus faecalis* obtained from 150 clinical samples collected from patients in Hospitals of Al-Diwanyiah province within three months (October 2021 to January 2022) and from various clinical sources including; 50 urine, 30 high vaginal swab, 15 seminal fluid, 10 root canal, 20 blood, 25 from stool, and 10 root canal, no bacteria 0(0.0%) isolated from root canal samples as shown in TABLE 1.

On blood agar, every samples was grown, bile esculin agar and chromogenic agar medium; twenty-four hours of incubation at 37°C. The isolates were diagnosis based on the standard biochemical and microbiological methods. These isolates then subjected to VITEK2 system for confirmed detection of *E.faecalis* .Also they subjected to molecular detection method using specific primer based on 16S rRNA gene as a genetic marker for confirmed identification of *E. faecalis* by PCR.

Table 1
E. faecalis isolated from different clinical sources

Source	Total No.	Positive samples	%
Urine	50	18	36
Stool	25	6	24
Seminal fluids	15	3	20

Vaginal swab	30	8	26.66
Root canal	10	0	0
blood	20	2	10
total	150	37	24.66
X^2/ P value	9.29/ 0.098*		

* No significant difference at P<0.05

Molecular Detection of *E. faecalis* by 16SrRNA Gene

DNA Extraction of *Enterococcus faecalis*

The DNA was extracted from fresh growth of *Enterococcus faecalis* using a DNA extraction kit (Geneaid, Taiwan). The procedure was created in accordance with the manufacturer's protocol. A NanoDrop was used to measure the concentration of DNA for both quality and quantity (Thermo Scientific NanoDrop, USA).

Polymerase Chain Reaction Amplification of 16SrRNA Gene

The primer 3 plus program was used to design the primers used to amplify regions of the 16S rRNA gene. Preparation of the PCR reaction included Mastermix (Promega, USA) with total volume (25uL) of the reaction mixture, which included 5uL of DNA template, 2uL(10pmol) for each of the primers, 12.5 µl GoTaq ®Green PCR master, and 3.5µl PCR water. The thermocycler conditions were 95 Celsius degree for two minutes of the first denaturation, 30 cycles including denaturing at 95 Celsius degree for 30 seconds, annealing at 54 Celsius degree for 30 seconds, The first extension was at 72 C for one minute, and the final extension was at 72 C for five minutes. Ethidium bromide stain was added to 1.5 percent agarose gels to separate DNA bands by electrophoresis, and UV Transilluminators were used to see DNA fragments in the PCR products (Wised, Korea).

Antibiotic resistant of *E. faecalis*

The modified Kirby-Power disc diffusion method was used to assess antibiotic resistance in *E. faecalis* isolates. Selective antibiotics such as vancomycin, ampicillin, gentamicin, rifampin, ciprofloxacin, nitofuranthion, norfloxacin, levofloxacin, tetracycline, streptomycin, imipenem, and piperacillin are used to demonstrate their effect on *E. faecalis* isolates and use mueller-hinton agar medium. The results were compared using Clinical Laboratory Standards Institute (CLSI, 2020) criteria.

Molecular typing of *Enterococcus faecalis*

E.faecalis isolates were activated by culturing them in brain heart infusion broth for twenty-four hours at 37 degrees Celsius. DNA was extracted using a particular extraction kit (Geneaid -Taiwan) in accordance with the manufacturer's directive. Eric PCR and BOX PCR analysis were performed for Genotyping of *E.faecalis* isolates as mentioned in previous studies(Ahmadi *et al.*, 2019).

Table 2 describe the primer sequences and the size of the amplicons. PCR product was analyzed in a 1.5 percent agarose gel with 3µl of ethidium bromide stain in TBE buffer, and a UV Transilluminator was used to visualize the PCR products.

Table2
Polymerase Chain Reaction (PCR) Primers That were Used in This Study

Target Genes	Primer Sequence(5′-3′)	Product Size (bp)	References
ERIC	ERIC2-F : AAGTAAGTGACTGGGGTGAGCG ERIC1-R : ATGTAAGCTCCTGGGGATTCAC	50-2000	Ahmadi *et al.*, 2019
BOXA1R	R: CTACGGCAAGGCGACGCT	250-2000	Ahmadi *et al.*, 2019
16SrRNA	341F :CCT ACG GGA GGC AGC AG 907R :CCG TCA ATT CMT TTG AGT TT	600	

Results
Identification of *E.faecalis*

Thirty seven isolates were diagnosed as *Enterococcus faecalis* from (150) clinical specimens that collected from patients in Hospitals of Al-Diwanyiah province, through the use of conventional biochemical methods and the Vitek 2 system; include 18 of urine , 6 of stool , 3 of seminal fluid, 8 isolate of high vaginal swab and 2 of blood. In addition, as describe in Fig.1 , The 16SrRNAgene was used to confirm the identity of 20 isolates.

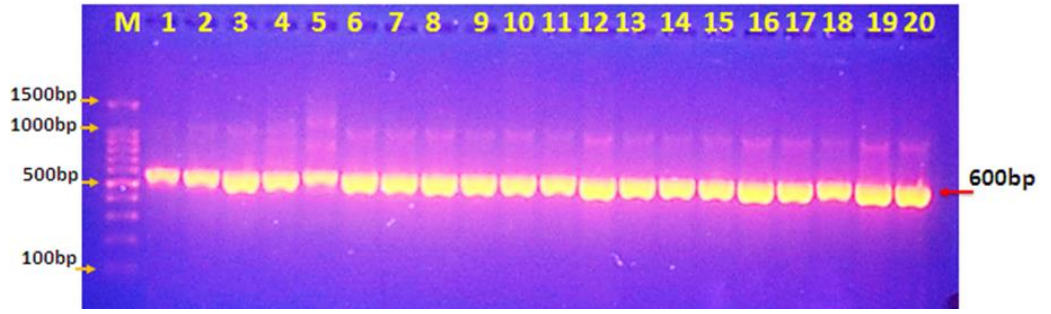

Figure 1: An figure of agarose gel electrophoresis that describe PCR product analysis of 16S rRNA gene in *Enterococcus faecalis* bacteria with (600 bp) PCR product size, where ladder (100-1500bp), the number of isolates is represented by the lane (1-20)

Antibiotic resistant

E. faecalis isolates from clinical sources were tested for antibiotic resistance and sensitivity, and the results are shown in the table(3).Vancomycin (VA) showed 34 (91.89 %) sensitivity rate, 1(2.7%) intermediate rate , and 2 (5.4 %) resistance rate .Ampicillin (AM) showed 37 (100 %) sensitivity rate . Gentamicin (CN) showed 35 (94.59 %) resistance rate and 2 (5.4 %) intermediate rate. Piperacillin (PRL) showed 37 (100 %) sensitivity rate. Ciprofloxacin (CIP) showed 22 (59.45

%) sensitivity rate, 0 (0.0%) intermediate rate and 15 (40.54 %) resistance rate. Levofloxacin showed 22 (59.45) sensitivity rate , 7 (18.91 %) intermediate rate and 8 (21.62%) resistance rate. Norfloxacin (NOR) showed 21(56.75%) sensitivity rate , 5 (13.51 %) intermediate rate and 11 (29.72%) resistance rate . Nitrofurantion (F) showed 37 (100 %) sensitivity rate. Tetracycline (TE) showed 1 (2.7 %) sensitivity rate , 0 (0.0 %) intermediate rate and 36 (97.29%) resistance rate .Rifampin (RA) showed 4 (10.81%) , 0 (0.0%) intermediate rate and 33 (89.18) resistance rate . Streptomycin (S) showed 2 (5.4%) sensitivity rate , 2 (5.4%) intermediate rate and 33 (89 .18 %) resistance rate .Imipenem (IMP) showed 37 (100%) sensitivity rate .It was found that the best antibiotics in terms of sensitivity are Vancomycin, Ampicillin, Nitrofurantion, imipenem and piperacillin

Table 3
The ratio of resistance, intermediate and sensitivity to antibiotics

Type of antibiotic	Sensitive (S)	Intermediate (I)	Resistance (R)
vancomycin (VA)	34 (91.89 %)	1 (2.7 %)	2(5.4%)
Ampicillin (AM)	37(100 %)	0 (0%)	0 (0%)
Gentamicin (CN)	0 (0 %)	2 (5.4%)	35(94.59%)
Ciprofloxacin (CIP)	22 (59.45 %)	0 (0%)	15(40.54%)
Levofloxacin (LEV)	22 (59.45 %)	7 (18.91%)	8 (21.62%)
norfloxacin (NOR)	21 (56.75 %)	5 (13.51%)	11 (29.72%)
nitrofurantoin (F)	37 (100 %)	0 (0%)	0 (0%)
Tetracycline (TE)	1 (2.7 %)	0 (0%)	36 (97.29%)
Rifampin (RA)	4 (10.81 %)	0 (0%)	33 (89.18%)
Streptomycin	2 (5.4 %)	2 (5.4%)	33 (89.18%)
Imipenem (IMP)	37 (100 %)	0(0%)	0(0%)
piperacillin (PRL)	37 (100 %)	0(0%)	0(0%)
X^2/ P value	350.12/ 0*		

* Highly significant difference at P<0.05

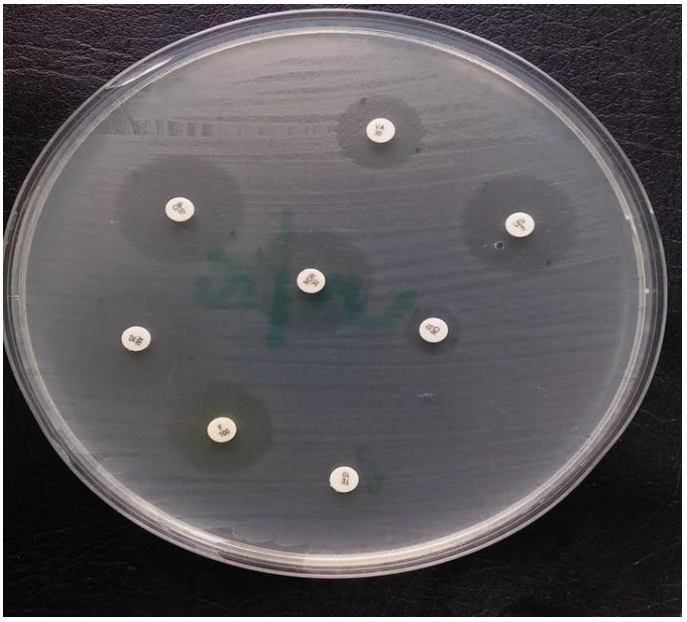

Figure (2): Antibiotic sensitivity test for *E. faecalis* isolates on Muller Hinton agar

Genotyping of *E.faecalis* isolates by ERIC PCR analysis

ERIC PCR technique have been widely used to identify Enterococcus species and characterize its genetic variance. Using the ERIC PCR method, the genotyping of *E. faecalis* isolates could be done (Martín-Platero *et al.*, 2009) . ERIC PCR results are shown in Table (4) , Figure (3) and (4). Among the 20 clinical isolates of *Enterococcus faecalis*, 5 clusters (I-V) and about 13 polymorphic variants were showed that were classified as follows: group 1 had three polymorphic variants, group 2 had two polymorphic variants, group three had three polymorphic variants ,the fourth group had three polymorphic variants, and the fifth group had two polymorphic variants.

Isolates No. 6,8,7,2,1 are located in the cluster I and have 3 polymorphic variants, isolates No. 3,4 are located in the cluster II and have 2 polymorphic variants, isolates No. 5,16,13,17,20 are located in the cluster III and possess 3 polymorphic variants and isolates No. 9,10,12,19 are located in the cluster IV and possess 3 polymorphic, while samples No. 11, 14, 15 and 18 are located in the cluster V and possess 2 polymorphic variants.

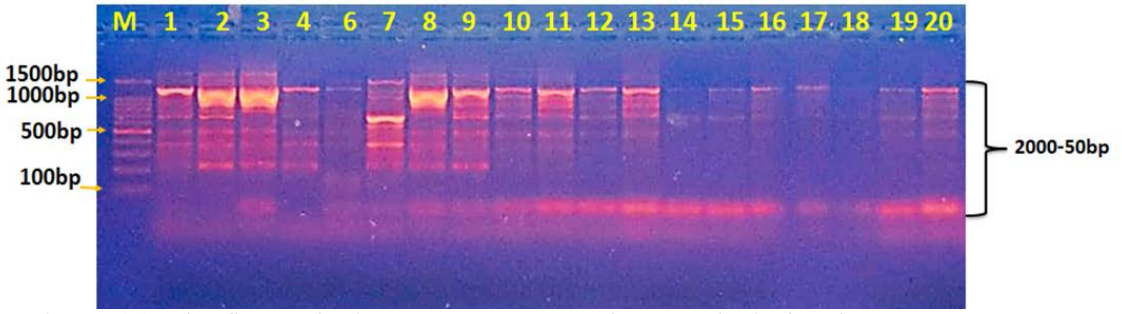

Figure (3): The figure depicts RAPD PCR product analysis for the ERIC repeat DNA region in *Enterococcus faecalis* isolates. The letter M (Marker ladder 100-1500bp). The lane (1-20) showed Polymorphic genomic loci at product sizes ranging from 50-2000 bp.

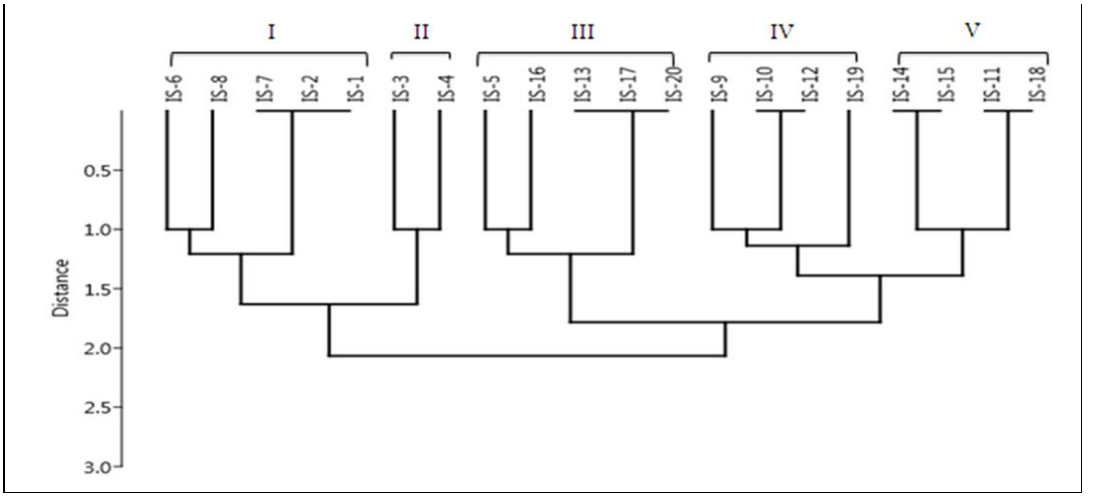

Figure (4): RAPD-PCR dendrogram tree analysis of the ERIC gene in *E. faecalis* isolates (Paleontological Statistics version 4.0). The cluster analysis (algorithm Ward's method) revealed 5 cluster variants among 13 polymorphic variants in 20 *E. faecalis* clinical isolates.

Table (4)
Cluster analysis and polymorphism variations for *E. faecalis* isolates using ERIC-PCR

Cluster No.	Isolate No.	No. polymorphic variants
I	6,8,7,2,1	3
II	3,4	2
III	5,16,13,17,20	3
IV	9,10,12,19	3
V	14,15,11,18	2
Total:5	20	13

Genotyping of *E.faecalis* isolates by BOX PCR

Box PCR results are display in Table (5) , Figure (5) and (6) . Among the 20 clinical isolates of *Enterococcus faecalis*, four cluster (I-IV) showed and about 14 polymorphic variants were among the isolates that were classified as follows: cluster I contained two polymorphic variants, cluster II contained six polymorphic variants, and cluster III contained four polymorphic variants, the fourth cluster contained two polymorphic variants.

Isolates No. 11, 12 , 13 , 14 , 15 , 17 ,4 are located in the cluster I and have two polymorphic variants, isolates No. 5 ,6,10,7,16,19,18 are located in the cluster II and have six polymorphic variants, isolates No. 8,3,2,20 are located in the cluster III and possess four polymorphic variants , isolates No. 9,1 are located in the cluster IV and possess two polymorphic.

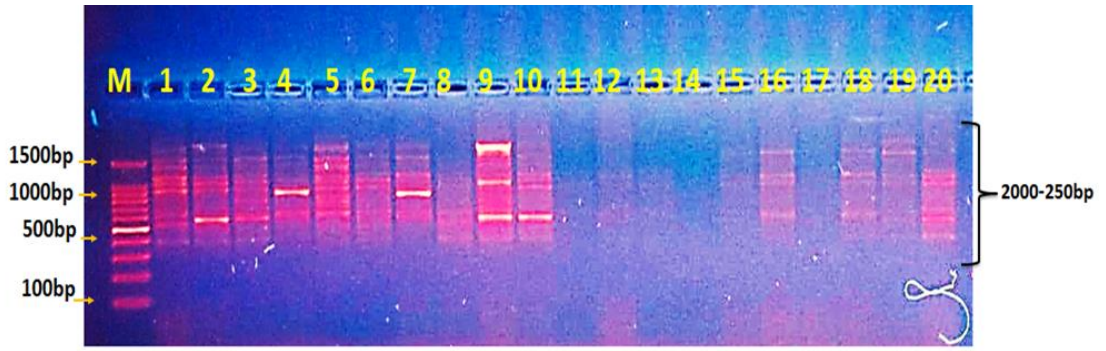

Figure (5) : The figure depicts RAPD PCR product analysis for the BOX repeat DNA region in *Enterococcus faecalis* isolates. The letter M (Marker ladder 100-1500bp). The lane (1-20) Polymorphic genomic loci were discovered at product sizes ranging from 50 to 2000 bp.

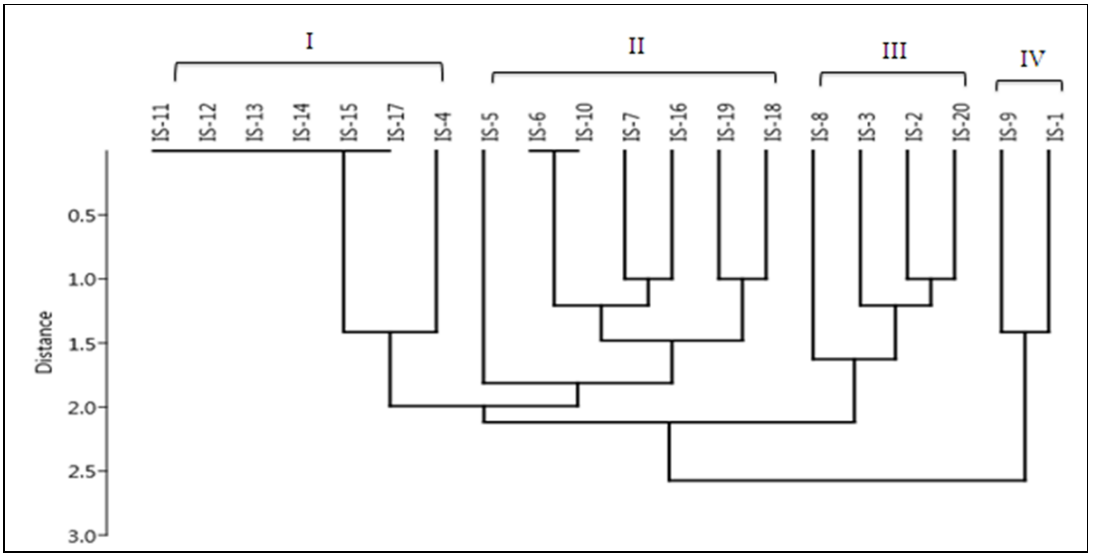

Figure (6): RAPD-PCR dendrogram tree analysis for BOX gene in *E. faecalis* isolates utilizing (Paleontological Statistics version 4.0). The cluster analysis (algorithm Ward's technique) revealed four cluster variations among 14 polymorphic variants in 20 *E. faecalis* clinical isolates.

Table (5)
Cluster analysis and polymorphism variations for *E. faecalis* isolates using BOX-PCR

Cluster No.	Isolate No.	No. polymorphic variants
I	11, 12 , 13 , 14 , 15 , 17 ,4	2
II	5 ,6,10,7,16,19,18	6
III	8,3,2,20	4
IV	9,1	2
Total:4	20	14

Discussion

In the present study, *Enterococcus faecalis* isolated and identified from clinical samples depending on standard biochemical tests and molecular identification by 16S rRNA gene. Because the 16SrRNA gene is present in all bacteria, it is used as a diagnostic tool for bacteria. Furthermore, the function of the 16SrRNA gene has remained constant over time, implying that random sequence changes are a precise measure of development (Patel, 2001). The 16SrRNA gene has been proven to be one of the most effective tools for identification microorganisms such as streptococci and enterococci (Lal *et al.*, 2011).

The results of our study showed that 34 isolates (91.89%) were sensitive to vancomycin, and it is almost similar to the results obtained by the researcher (Khalid, 2016), who found that all isolates of *E. faecalis* isolated from urine were sensitive to vancomycin at a rate of (96%), While (Kadim et al., 2015) discovered that *E. faecalis* isolated from pus was sensitive to Vancomycin at rate (15.6

percent). In this study, the results revealed that 37(100%) isolates were sensitive to Ampicillin , That were agreement with results obtained by (Rams et al., 2013) who found that, all *E. faecalis* isolates were sensitive to Ampicillin at rate (100 %), It is also consistent with the results obtained (hassan, 2012) who found all *E. faecalis* isolates were sensitive to Ampicillin at rate (100 %).The results of this study showed that all isolates of *E. faecalis* are sensitive to piperacillin 37 (100%). While in the study conducted by (Conceição *et al.*, 2012), the sensitivity of *E.faecalis* isolates to piperacillin was (26.5%) by the disc diffusion method.

Antibiotics in the β-lactam family act as suicide substrates for penicillin-binding proteins, or (PBPs) that suppress bacterial growth. They suppress persistent cell wall production by inactivating PBPs after they are altered by a β-lactam antibiotic (Hugonnet *et al.*, 2016). In this study, the results revealed that 35(94.59%) isolates were resistance to Gentamicin, The present result is almost similar to what was reached by(Hassan, 2012), who found that isolates of *E. faecalis* isolated from different clinical cases are resistant to gentamicin by(89.3 %). The results of our study differ with the result obtained by (AL-Yassary, 2011) , where 60% of E. faecalis isolates were resistant to Gentamicin.The resistance of enterococci, especially *E. faecalis*, to Aminoglycosides has been increasing recently because they possess modified enzymes such as (2-phosphotransferase - 6-Acetyl transferase), which play an important role in the resistance of the isolates to Gentamicin(Brooks et al., 2004) .In this study, the results showed that 33(89.18%) are resistant to Streptomycin . The result of this study differs with the result obtained by (AL-Yassary, 2011), in which the resistant of *E.faecalis* isolates to Streptomycin was rate 55%. The results of current study are almost in agreement with the results obtained by (Hussain, 2020), wherein the rate of streptomycin resistance in *E. faecalis* isolates was (82.9 %). Aminoglycosides alone are ineffective in the treatment of transmissible enterococcal infections Insufficiently active across the cytoplasm, enterococci are innately resistant to low levels of Aminoglycosides. They are usually combined with inhibitors of cell wall production, which may facilitate their uptake. To achieve high peak serum levels of aminoglycosides, daily dose regimens have been advocated because they provide greater activity than typical drug delivery methods(Clewell et al., 2014). In this study, Among the E.faecalis isolates tested, 15 (40.54 %) were Ciprofloxacin-resistant, 22 (59.45) were sensitive to Ciprofloxacin. This study is not agreement with(Hussain, 2020) that showed, antibiotic susceptibility testing of the total isolates of *E. faecalis* from various clinical source , 25.5% were resistant to Ciprofloxacin. these results were not agreement with results obtained by (Al-Khamasi, 2014) who found that strains of *E. faecalis* were resistant to Ciprofloxacin in rate (60 %). In this study, 22 (59.45%) of *E.faecalis* were sensitive to Levofloxacin and 8 (21.62%) were resistance to Levofloxacin .This study agreement with (AL-Yassary, 2011)showed that, antibiotic susceptibility testing of the total isolates of *E. faecalis* from various clinical source , 55 % were sensititive to levofloxacin . these results were not agreement with results obtained by (Seo & Lee, 2013), who found that strains of *E. faecalis* were resistant to levofloxacin in rate (4.8%). In this study, 21 (56.75%) isolates of *E.faecalis* were sensitive to norfloxacin , 11 (29.72%) were resistance to Norfloxacin . This result does not correspond to the results of the study obtained by (Lee, 2013), in which the rate of resistance of *E.faecalis* to the Norfloxacin was 58%. The result of our study agrees with the results obtained by (Seo & Lee, 2013), in which the percentage of

resistance of *E.faecalis* to Norfloxacin was 26.8%. Also, the result of our study does not agree with the result (AL-Yassary, 2011), in which(80 %) of the *E.faecalis* were sensitive to Norfloxacin. The results of our study showed that 37 (100%) of *E.faecalis* were sensitive to Nitrofurantion . While in the result obtained by (AL-Yassary, 2011), 85 percent of the *E. faecalis* isolates tested were sensitive to nitrofurantion, and this slightly differs from the results of our study. The results of this study differ with the results of the study obtained by (Hussain, 2020), in which 89.3% of *E. faecalis* isolates were resistant to Nitrofurantion. The result of our current study is in agreement with a study with the result obtained by (Seo & Lee, 2013), in which *Enterococcus faecalis* does not possess resistance againstNitrofurantion

In this study, 36 (97.29%) isolates of *E.faecalis* were resistant to Tetracycline. The results were almost agreement with results of Kadim, (2015) who found that all *E. faecalis* isolates were resistant to Tetracycline. This study agreement with result of Zalipour *et al.* (2019) who found that, the highest antibiotic resistance was seen against tetracycline (93.5%). In this study, the results showed that 33(89.18%) are resistant to Rifampin and 4(10.81%) were sensitive to Rifampin. This study's results are nearly identical with results of the study obtained by (Al-Khamasi, 2014) , in which Enterococcus faecalis resistant to Rifampin in rate 80%. Our study results found all isolates are sensitive to Imipenem 37 (100%) .This result is similar to the result obtained (Hassan, 2012) , (Saadi, 2007) and (Seo & Lee, 2013) in which all isolates of *E.faecalis* were sensitive to Imipenem in rate (100%).

In our investigation, ERIC PCR identified only five clusters, but in a study by (Ahmadi *et al.*, 2019), ERIC PCR classified *Enterococcus faecalis* into 15 clusters. There are clusters with a single isolate, which varies from our results because there is no single isolate in the five clusters. Another study by (Zalipour et al., 2019) discovered 14 clusters of ERIC, and these clusters comprise single isolates, which differs from the results of our study. Also, our results were different with the results of a study conducted by (ALNAKSHABANDI *et al.*, 2020), the ERIC-PCR analysis categorized isolates into two major clusters, with cluster A accounting for 28% of the total and further subdivided into groups; 8% of isolates belonged in cluster A1 and 20% in cluster A2. Cluster B accounted for 72% of the isolates. Six isolates (33.3 percent) were found to be part of B1, while 66.6 percent of the isolates in this cluster belonged to B2.

In our study among 20 clinical isolates of *E. faecalis*, four groups (I-IV) and about 14 polymorphic variants were showed and this is inconsistent with the result of the study obtained by (Ahmadi *et al.,* 2019), where *E. faecalis* was classified into 25 groups using BOX PCR, but in our study, BOX PCR revealed four groups. Also, in the study of (Ahmadi *et al.*, 2019), there are groups with one isolate, which differs from the results of our study because there is no single isolate in the four groups.

Conclusion

The current study showed most *Enterococcus faecalis* isolated from different clinical samples from patients in hospitals have polymorphic variants and the

effectiveness of ERIC PCR and BOX PCR in detecting theses polymorphism .Also effectiveness of PCR in detecting housekeeping gene (16SrRNA).

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