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Multidrug Resistant *Enterococcus Faecalis* isolated from Root Canals and Its Relationship with The Presence of Some Virulence Genes

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ABSTRACT

Background: Enterococcus bacteria are normal flora found in the gut of different organisms, this bacterium has the potential to become an opportunistic pathogen and harm the host's extra intestinal sites. Dental infections also associated with Enterococci.

Objective: In order to shed light on multidrug resistance *E. faecalis* in root canals infections and also this research was carried out to investigate the percentage of *asa-1*, *cyl* genes *A* in the *E. faecalis* isolates from root canal samples by using polymerase chain reaction technique (PCR)**.**

Materials and Methods: During the period of November 2021 to January 2022, root canal samples were collected from (40) patients, (9) men and (31) women, and then were cultured on Pfizer specific *Enterococcus* media, a selective medium for the isolation and growth of *Enterococcus spp*, after that antibiotic sensitivity test was done on all *E. faecalis* isolated. And then molecular detection was done to detect the *E. faecalis*, asa-1 and Cyl A genes.

Results: The results showed percentage of *E. faecalis* isolates from root canal was 55% (22 isolates) and all isolates were positive for *E. faecalis* gene, also 100% of isolates were positive for Cyl-A gene and 86.36% of isolates were positive asa-1 gene. The antibiotics sensitivity result showed that 100% of *E. faecalis* isolates were sensitive to Imipenem and Linezolid, whereas 100% of *E. faecalis* isolates were resistant to Tetracycline and Trimethoprim.

Conclusion: Most of isolates were multidrug resistant and also almost all the isolates had asa-1 and Cyl-A genes together.

Keywords: Virulence gene, PCR, Root canal, Antibiotic.

INTRODUCTION

Enterococcus faecalis as Gram-positive bacteria are often seen individually, in pairs, or in short chains under the microscope. That have colonies on blood agar that are about 1-2 mm in diameter and are facultative anaerobes, non-motile, and often non-hemolytic. As a result of fermentation without gas generation, Enterococci create lactic acid. The responses of catalase and oxidase in *Enterococcus* are negative. So capable of growing at an ideal temperature range of 35–37°C, pH 4.8–9.6, and medium containing 6.5% salt[(1)].

Enterococcus bacteria has the capacity to develop into an opportunistic pathogen and affect the host's extra intestinal locations despite being in the gut of many organisms as normal flora[(2)].

Enterococci are linked to dental infections as well. Endocarditis, one of the most medically challenging diseases, might result from the oral infection if it develops into a bacteremia [(3)]. The oral cavity may act as a reservoir for superinfection microorganisms, including *E. faecalis*, *E. faecium* and *E. coli*, as assessed by anti-biotyping studies [(4)].

E. faecalis has been associated to the occurrence of several endodontic diseases, including primary and chronic endodontic infections. In original infections, asymptomatic chronic periradicular lesions are more commonly seen than acute periradicular periodontitis or abscesses [(5)].

Antibiotic resistance affects pathogenicity, which makes it more challenging to treat enterococcal infections medically since there are fewer treatment options available [(6)].Virulence factors play a role in pathogenesis by controlling adhesion and colonization, invasion into host tissues, and the production of toxin and enzymes, all of which facilitate microorganism adherence to root canal walls and surfaces, making it difficult to remove them even after instrumentation. They can also harm the host in a number of different ways by triggering the periapical tissue reaction to become more pronounced [(7)].

A pheromone-induced *E. faecalis* surface protein called "aggregation substance" (AS) encourages the formation of mating clumps during bacterial conjugation. As a component of the bacterial pheromone-responsive genetic exchange system, aggregate material aids plasmid transfers by promoting effective donor-recipient contact between Enterococcal bacteria. Aggregation components facilitate adhesion to a variety of eukaryotic cell surfaces in vitro and promote uptake by human intestinal cells that have been cultured [(8)].

Target cells such as erythrocytes, macrophages, polymorphonuclear cells, and other eukaryotic cells can all be lysed by cytolysin. It has been demonstrated that cytolysin facilitates the invasion by harming the host tissue [(9)]. Together with Aggregation Substance (AS), it will prevent the development of other bacteria [(10)].

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Hyaluronidase is a degradative enzyme that can harm tissue. Hyaluronidase works by depolymerizing the mucopolysaccharides in connective tissue.

Its purpose is to make the host tissue more conducive to the spread of bacteria and their toxins. Hyaluronidase helps other bacteria move from the root canal to the periapical lesions, aggravating the condition. Other bacteria are stimulated by hyaluronidase to manufacture the toxins they already possess, increasing the damage [(10)].

AIM OF THE STUDY

To shed light on multidrug resistance *E. faecalis* in root canals infections and to investigate the percentage of *asa-1*, *cyl* genes *A* in the *E. faecalis* isolates from root canal samples by using polymerase chain reaction technique (PCR)**.**

MATERIALS AND METHODS

Study Samples:

Root canal samples were collected during the period of November 2021 to January 2022 to shed light on the role of antibiotic sensitivity and virulence factors of *E. faecalis*.

A. Bacterial isolation and identifyct-ion:

Root canal samples were taken from 40 patients with infected root canals between the ages of 16 and 48 year, 9 men, and 31 women, between November 2021 and January 2022. A professional dentist inserted a sterile paper point and special files into the infected root canal for at least 5 to 15 seconds (to prevent saliva infiltration, sterile cotton pellets were utilized to cover the samples).

For further research, the paper points and files were collected in sterile Eppendorf tubes with 1 ml of sterile brain heart broth for each, and delivered as quickly as possible to the microbiology lab in a refrigerated box. After being vortexed for 5 seconds, the Eppendorf content was culture on Pfizer selective media [(11)].

B. Molecular detection:

Following the manufacturer's instructions, DNA was extracted from pure bacterial colonies using a genomic DNA micro extraction kit (Geneaid, China).

In order to assess the quality of samples for later uses, Nanodrop was employed to measure the extracted DNA's concentration. Precisely followed by the PCR-based genotyping identification of the *E. faecalis*, *asa-1*, and *cyl* genes.

C. Polymerase chain reaction (PCR):

1. Primers Selection

The primers chosen for this investigation are those shown in Table (**1**).

Table (1): The primers and their sequences used in conventional PCR

Gene	Sequence 5 → 3	Size (bp)
E. Feacalis [(12)]	**F:** TCAAGTACAGTTAGTCTTTATTAG **R:**ACGATTCAAAGCTAACTGAATCAGT	940
Cyl A [(13)]	**F :**ACTCGGGGATTGATA GGC **R:** GCTGCTAAAGCTGCGCTT	688
Asa-1 [(13)]	**F:** GCACGCTATTACGAACTATGA **R:** TAAGAAAGAACATCACCACGA	375

2. PCR Amplification

After being defrosted, the extracted DNA, primers, and PCR premix were quickly vortexed to help them sink to the bottom of their tubes.

A 25-ul PCR mixture was prepared, consisting of 5 ul of PCR premix, 1 ul of each primer (forward and reverse), 3 ul of DNA template, and the remaining 15 ul of sterile deionized distilled water [(14)]. The thermocycler PCR device was used to amplify DNA in accordance with the PCR protocol after briefly mixing the PCR reaction tubes (Table **2**).

Table (2): Program PCR amplification of *E.feacalis, Asa-1, Cyl A, Hyl* genes

Gene	Initial denaturation	Denaturation	Annealing	Extension	Final Extension	cycle
E.Feacalis	94ºC For 4 min.	94ºC For 40 sec.	56ºC For 40 sec	72ºC For 40 sec	72ºC For 5 min.	30
Cyl A	95ºC For 2 min.	95ºC For 30 sec.	49.5ºC For 30 sec	72ºC For 2 min	72ºC For 10min	35
Asa-1	94ºC For 7 min.	94ºC For 1 min.	56.5ºC For 1 min	72ºC For 1 min	72ºC For 7 min.	30

3. Agarose Gel Electrophoresis

It was possible to validate the positive *E. faecalis* gene result by electrophoresing 1.5 percent agarose gel with ethidium bromide staining for 50 minutes at 75 volts. A UV (ultraviolet) transilluminator was also used to photograph the agarose gel.

C. Antibiotic sensitivity test (Disk diffusion method):

The **Kirby-Bauer** [15] technique was used as follows:

1. Preparation of Mueller-Hinton plates

The manufacturer's instructions were followed when making Mueller-Hinton agar (MHA); the medium was cooled to 45–50°C before being put onto plates and allowed to set to a depth of about 4 mm on a flat surface. Until the agar had dried, the plates were stored at 4°C. After that, they were utilized.

2. Preparation of inoculum (Turbidity standard)

Colonies from overnight cultures of the tested isolates were transferred to a tube with 5 ml of normal saline and adjusted to McFarland standard tube No. 0.5 to create a culture with 1.5*108 C.F.U./ml to make the inoculums.

3. Inoculation of the test plates

By dipping a sterile swab into the inoculums, plates were infected. By vigorously spinning and pushing the swab against the tube's edge above the liquid level. After that, the swab was applied to the medium's surface three times, each time rotating the plate through at a 60-degree angle. The swab was then wrapped around the agar surface's edge.

The inoculums were then allowed to dry for a short period of time at room temperature with the lid closed. The antibiotic discs (Table **3**) were applied to the inoculation plate using sterile forceps. On the agar surface, the discs should be gently pushed down. The plates were kept anaerobically warm (37°C) for 18 to 24 hours.

4. Reading the results

The inhibitory zone widths were measured in millimeters following incubation (mm). By comparing the breadth of the inhibition zone for each antimicrobial drug to the typical inhibition zone, the diameter of the inhibition zone was translated into categories of sensitive (S), intermediate (I), and resistant (R) (Table **3**).

Table 3: Diameter interpretive standards of inhibition zone (CLSI) [16]

Antimicrobial /agent	Disc potency (µg)	Diameter of bacteria resistant		
		Resistance	Inter-mediate	Sensitive
APX (Amoxiclav)	25	<16	-	>17
C (Chloramphenicol)	30	<12	13-17	>18
CIP (Cipr ofloxacin)	10	<15	16-20	>21
CN (Gentamicin)	10	<12	13-14	>15
E (Erythromycin)	15	<13	14-22	>23
IPE (Imipenem)	10	<13	14-15	>16
RIF (Rifampin)	5	<16	17-19	>20
SXT (Trimethoprim)	25	<15	16-18	>19
TE (Tetracycline)	10	<14	15-18	>19
VA (Vancomycin)	30	<14	15-16	>17
Linezolid	30	<20	21-22	>23

Statistical analysis

System-SAS (2012) application was used to calculate the qualitative data, which were presented as frequency and percentage.

Ethics approval:

The Ethics Committee at the Department of Biology (University of Baghdad) approved the study protocol (Reference: CSEC/1021/0102) in October 30, 2021. This work has been carried out in accordance with The Code of Ethics of the World Medical Association (Declaration of Helsinki) for studies involving humans.

RESULT

Isolating and identifying Enterococcus spp. were the major goal of the sample collection. Under the microscope, the Gram-positive cocci with ovoid or spherical morphologies that were grouped alone, in pairs, or in short chains were used to identify each bacterial isolate (Figure **1**). On Pfizer Selective Enterococci medium, the colonies were spherical, grey, and 2mm in diameter, with a black tip in the center and black zones surrounding the colony (Figure **2**).

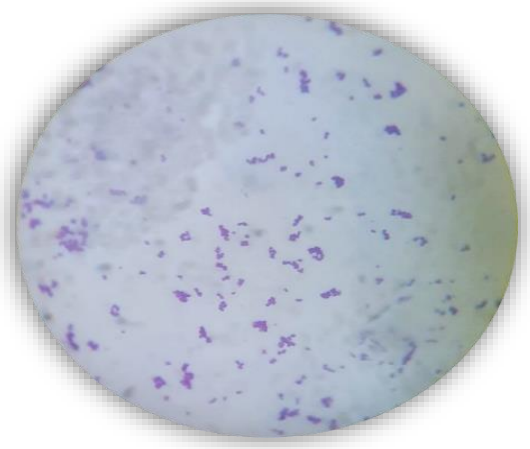

Figure (1): *Enterococcus spp*. under microscope

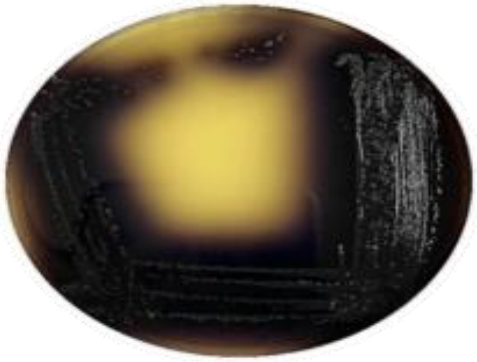

Figure (2): *Enterococcus spp*. on Pfizer Selective Enterococci medium

According to the findings of PCR testing for the *E. faecalis* gene, 22 (55%) of the isolates from root canals samples were found to be positive. The DNA ladder was used as a reference in the current investigation, which discovered that the *E. faecalis* gene band was located at 940 bp (Figure **3**). No smear, which would have indicated DNA degradation, was seen, and the bands were distinct, single, and not distributed.

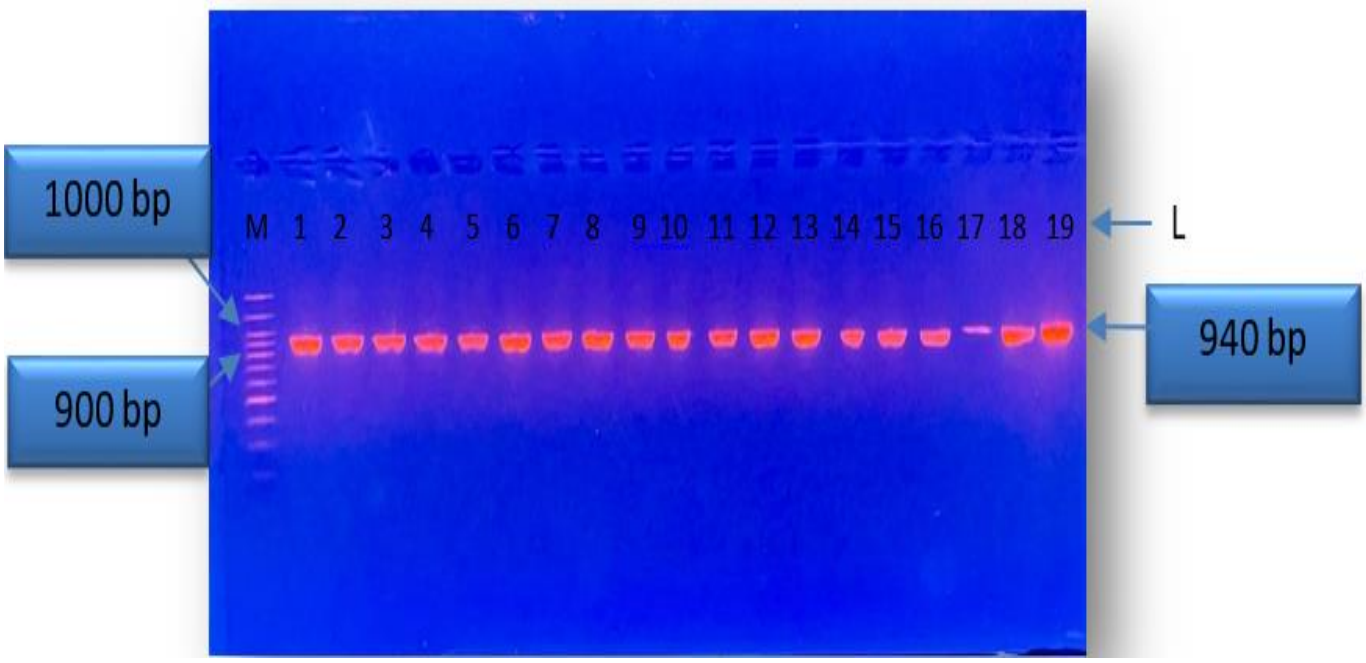

Figure (3): Agarose gel 1.5% electrophoresis of PCR amplified products for *E. faecalis* gene stained with ethidium bromide (75volt/cm for 50min). Lane M: l00bp Ladder. Lane L 1-19: positive bands of 940bp.

Molecular identification of *cyl* and *asa-1* genes

The virulence factors cyl A and asa-1 genes in *E. faecalis* were determined using the PCR technique on the 22 *E. faecalis* isolates. The current investigation discovered that the bands for the cyl and asa-1 genes were identified at 688 and 375 base pairs, respectively, when compared to the DNA ladder (Figure **4, 5**). The bands seemed crisp, single, and undispersed, and there was no smear that may have indicated DNA degradation.

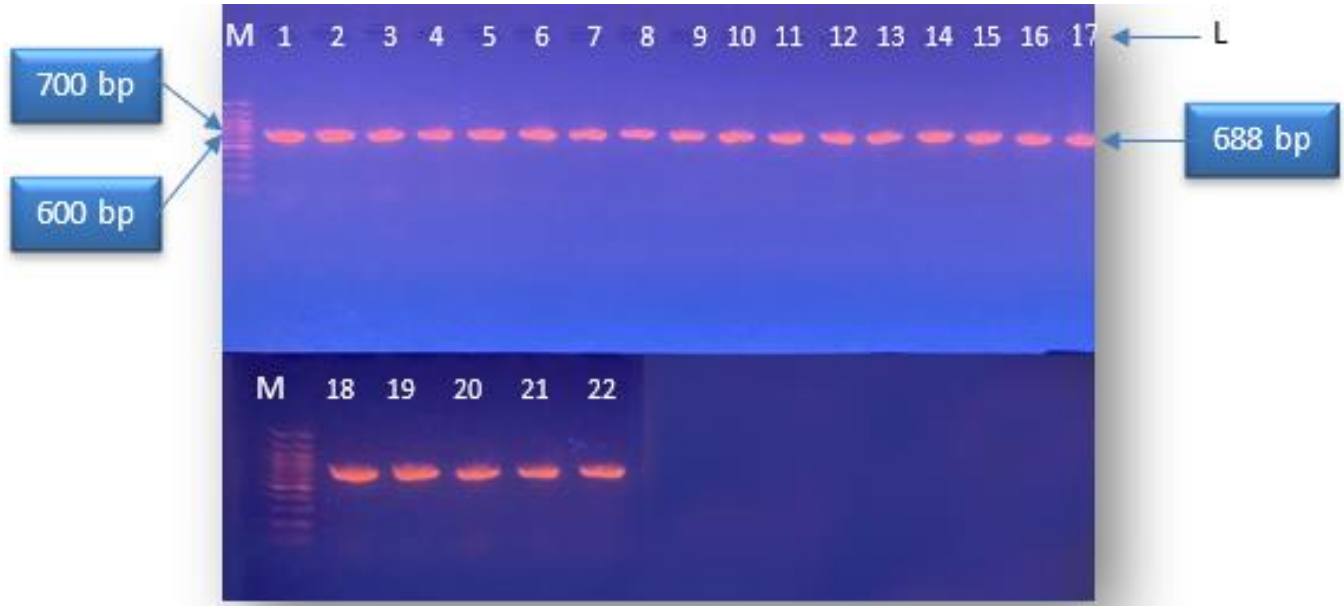

Figure (4): Agarose gel 1.5% electrophoresis of PCR amplified products for *cyl A* gene stained with ethidium bromide (75volt/cm for 50min). Lane M: l500bp Ladder. Lane L 1-22: positive result with positive bands of 688bp.

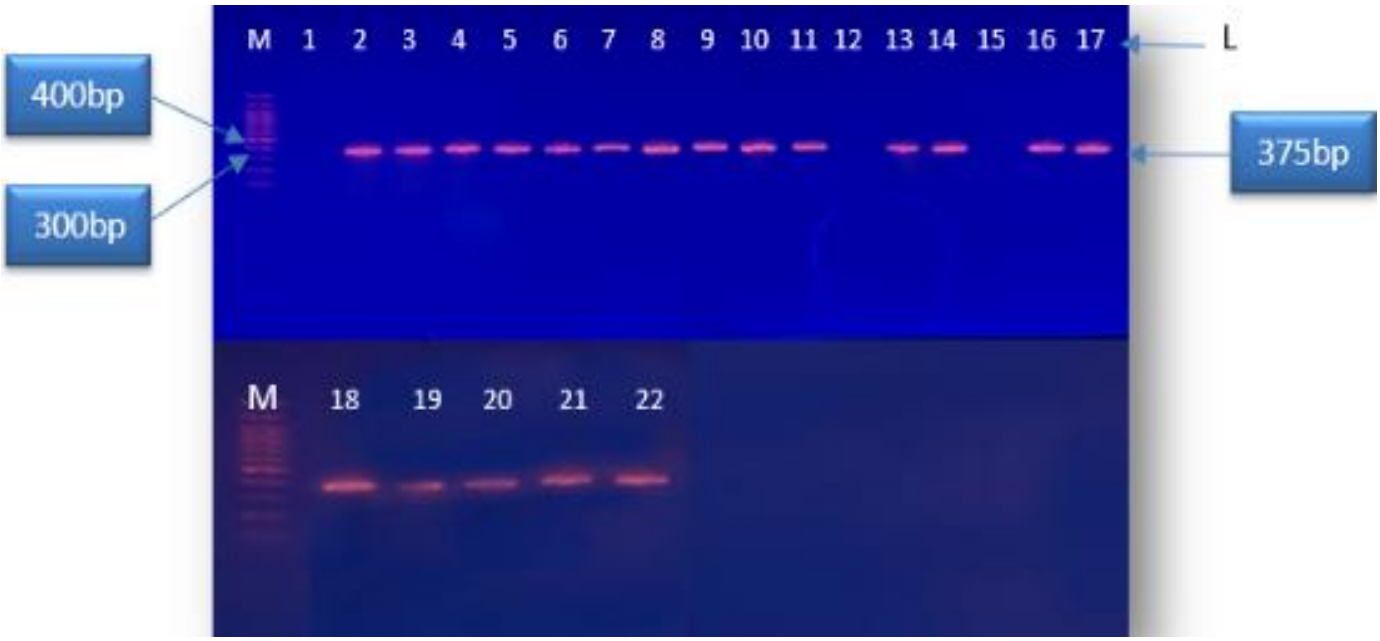

Figure (5): Agarose gel 1.5% electrophoresis of PCR amplified products for *asa-1* gene stained with ethidium bromide (75volt/cm for 50min). Lane M: l500bp Ladder. Lane L 2-11, 13, 14 and 16-22: positive result with positive bands of 375bp, and 1,12, and 15: negative result for *asa-1* gene.

Antibiotic sensitivity of root canal isolates:

The antibiotics previously listed in Table (**3**) were tested for *E. faecalis* sensitivity using the disk diffusion method. The result showed in Figure (**6**).

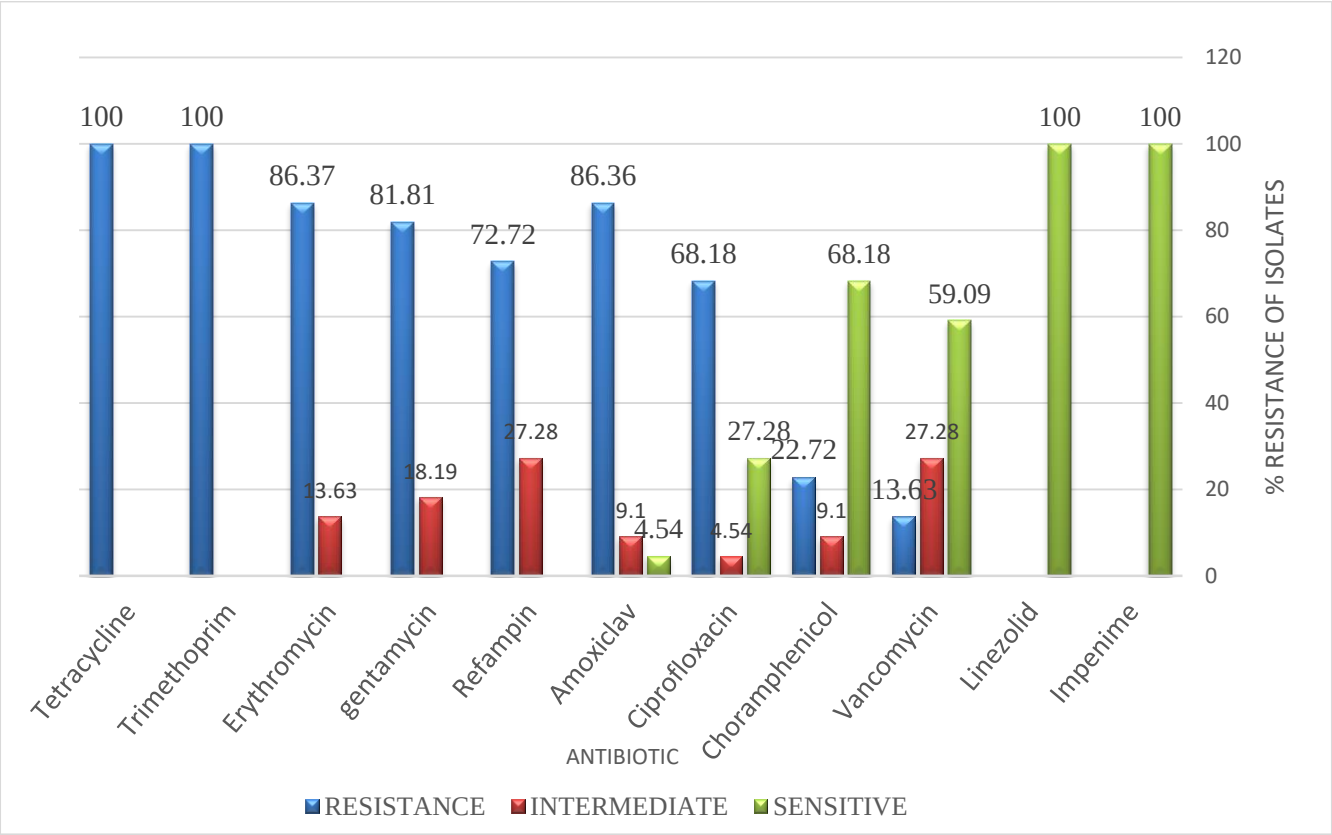

Figure (6): Antibiotic susceptibility for *E. faecalis* isolates from root canal sample against antibiotics.

DISCUSSION

The percentage of *E. faecalis* isolates from root canal was 55% (22 isolates) and all isolates have *E. faecalis* gene when detected by electrophoresizing 1.5% agarose gel with ethidium bromide staining for 50 minutes at 75 volts. This result was higher than the findings of the study by **Barbosa-Ribeiro *et al.*** [17], and similar result that found by **Mobarez and Kafil** [18] found that 56.12% of the sample had *E. faecalis* gene. The small differences between isolation percentages may be because of the number of specimens, the differences in the source of isolates, their geographical regions and differences in the identification methods.

Enterococcus faecalis isolates from root canals are often characterized by their development of biofilms, which aid the bacteria in maintaining and surviving in the anaerobic or micro aerobic conditions inside the root canal. Because the infection of the root canal will cause pain, discomfort, fever, as well as a longer period for treatment, this infection can provide a number of problems for both the patient and the dentist. Resistance to antibiotics one of the most common issues patients who have root canal infections caused by this bacterium deal with is *E. faecalis*, which also plays an important role in the infection of the root canals [19].

The antibiotics previously listed in Table (**3**) were tested for *E. faecalis* sensitivity using the disk diffusion method, the result showed that 100% of *E. faecalis* isolates were sensitive to Imipenem and Linezolid, whereas 100% of *E. faecalis* isolate were resistant to Tetracycline and Trimethoprim. Most *E.faecalis* were resistant to Erythromycin, Gentamicin, Amoxiclav, Rifampin and Ciprofloxacin (86.37%, 81.81%, 86.36%, 72.72% and 68.18%), respectively) except for a very few that were intermediate, Thus, they are considered as (MDR). As well as 59.09% of *E. faecalis* isolates showed sensitivity to Vancomycin and 68.18% of isolates was sensitive to Chloramphenicol where very few percentages of isolates were intermediate or resistant to these two antibiotics, Figure (**6**). The 22 *E. faecalis* isolates were subjected to the PCR method to identify the virulence factor *cyl A* and *asa-1* genes in *E. faecalis*.

The result showed 22 (100%) isolated had the *cyl A* gene and 19 (86.36%) isolates had the *asa-1* gene. only 19 isolated had the cyl A and *asa-1* genes.

Studies carried out by **Karimi *et al.*** [20], **Aghdam *et al.*** [21], and **Mobarez and Kafil** [18], who showed that 54%, 33% and 38% of *asa-1* gene were found in *E. faecalis* isolates, respectively. Study done

by **Saffari *et al.*** [22] showed that 63.6 % of *E. faecalis* have *cyl A* gene.

The body's natural defense against sickness brought on by enterococci is controlled by the surface expression of the aggregation substance, which also boosts the virulence of many opportunistic diseases. **Bhatty *et al*.**[23] also found in their investigation that the aggregating material considerably increases the development of biofilms in vitro.

Cyl-A, also known as hemolysin, is bactericidal to other Gram-positive bacteria and has hemolytic effects on people. Neutrophils and macrophages are destroyed by cytolysin, making them immune system-evading [24].

CONCLUSION

Antibiotic sevirulence genes may be prevalent is some isolates and absent from other depending on many factors such as the source of isolation.

Conflict of interest: Nil
Source of funding: Nil

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