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COMBATTING ANTIBIOTIC RESISTANCE IN CHRONIC PERIODONTITIS BY DISSEMINATION OF PLASMID IN *ENTEROCOCCUS FAECALIS*

Usha Subbiah[1*], Angeline Julius[2], Athira Ajith[1] and Sonaa Elango[3]

[1]Human Genetics Research Centre, Sree Balaji Dental College and Hospital, Bharath Institute of Higher Education and Research, Chennai, Tamil Nadu, India.
[2]Centre for Materials Engineering and Regenerative Medicine, Bharath Institute of Higher Education and Research, Chennai, Tamil Nadu, India.
[3]Department of Life Science, School of Natural Sciences, University of Suwon, Wau-ri, Bongdam -eup, Hwaseong-si, Gyeonggi - do, Republic of Korea.

***Corresponding Author: Usha Subbiah**
Human Genetics Research Centre, Sree Balaji Dental College and Hospital, Bharath Institute of Higher Education and Research, Chennai, Tamil Nadu, India.

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ABSTRACT

This study aimed to investigate the basis of plasmid-mediated resistance to antimicrobials in *Enterococcus faecalis* isolated from unstimulated saliva and subgingival plaque. In 50 chronic periodontitis samples, *E. faecalis* (n=34) was isolated from unstimulated saliva and subgingival plaque. The resistant isolates were screened from disc diffusion assay and MIC determination by using the agar dilution method. The bacterial transformation was performed to ensure the susceptibility of the plasmid in the clinical isolates. A conjugation experiment was carried out using the solid agar mating method. In addition, plasmid curing assay was performed to determine plasmid-mediated antibiotic resistance. Salivary and subgingival plaque isolates had maximum resistance to ampicillin (92%), metronidazole (90%) and linezolid (95%). The plasmid-encoded antibiotic resistance reversal was proved from the plating, where acridine orange had a remarkable plasmid curing efficiency of 82% and 87% on ampicillin, and on linezolid resistance 89% and 81% of saliva and plaque *E. faecalis* respectively. The present study's findings might allow the eradication of this plasmid-mediated resistance in pathogens during antibiotic adjuvant in periodontal therapy.

KEYWORDS: Ampicillin, Antibiotic resistance, *Enterococcus faecalis*, Linezolid, Plasmid curing, subgingival plaque.

INTRODUCTION

Enterococcus faecalis is a Gram-positive, facultative anaerobic coccus that is often found in hospitals and communities across the world. In humans, it is responsible for up to 90% of enterococcal infections.[1] *Enterococcus faecalis* has been linked to chronic periodontitis in individuals with post-treatment apical periodontitis in dentistry.[2] 51.8 percent of periodontitis patients had it retrieved from their periodontal pockets.[3] It causes periodontal disease and appears to be resistant to antibiotics in highly infected subgingival locations.[4] Antimicrobial therapy of dental infections has been linked to the evolution of bacterial resistance in both sick and healthy oral flora. The oral microflora may thus operate as a source of antibiotic resistance genes, with the genes responsible for such resistance in normal oral flora being acquired from and passed on to temporary colonisers of the location, resulting in mouth infections.

The bacterium obtains antibiotic resistance genes by spontaneous mutation or genetic exchange with other bacteria. It can withstand one or more antibiotics. Bacteria grow resistant to several distinct antibiotic families as a result of various resistance characteristics. Systemic antibiotics such as tetracycline, clindamycin, ciprofloxacin, metronidazole, and -lactams have been routinely utilised for periodontal treatment. *E. faecalis* is resistant to a broader spectrum of antimicrobials, including aminoglycosides, glycopeptides, beta-lactams, and quinolones, as well as a recent increase in linezolid resistance. A substantial proportion of subgingival *E. faecalis* is resistant to common antimicrobials including tetracycline, erythromycin, clindamycin, and metronidazole.[5]

Horizontal gene transfer (HGT).[6] and the transfer of antibiotic-resistant genes from *E. faecalis* to Staphylococcus aureus[7] show that *E. faecalis* is capable

of sharing resistance. The fundamental mechanism for the transmission of antibiotic resistance is genetic interchange, often known as horizontal gene transfer (HGT).[8] Plasmids contain the majority of the genetic factors that confer antibiotic resistance. Extrachromosomal DNA sequences are frequently transferrable to other bacteria and are responsible for the establishment of antibiotic resistance.[9]

Multidrug resistance caused by plasmids is the major problem in the treatment of infectious illnesses. Through their extensive host range plasmids, Staphylococci, Streptococci, and Enterococci shared multiple antibiotic resistance genes that propagated throughout these various bacteria species.[10] *E. faecalis* was found in periodontitis patients' saliva and subgingival samples more frequently[11], and it carries the hemolysin-bacteriocin plasmid as well as resistance plasmids.[12] Through HGT, it gains unique genetic features in periodontal pathogens, including multidrug resistance (MDR) determinants.[13]

This has a significant influence on bacterial evolution and genome flexibility, as well as the distribution of resistance genes. Small hydrophobic peptide sex pheromones specific for different types of plasmids are excreted by pheromone sensitive plasmids in *E. faecalis* to interact and deliver interbacterial signals. As a result, plasmids provide light on the plasmid transfer process in *E. faecalis* and have the potential to cause periodontal inflammation and tissue destruction.[14]

Because little is known about enterococcal plasmids in periodontitis, the goal of this study is to isolate *E. faecalis* and determine whether or not it has a plasmid in saliva and subgingival plaque from individuals with chronic periodontitis. Another goal was to identify the phenotypic identification of antibiotic resistance, plasmid curing, and transformation efficiency in *E. faecalis* from chronic periodontitis patients. As a result, the purpose of this work is to investigate the role of plasmid in multidrug resistance issues in clinical *E. faecalis*.

MATERIALS AND METHODS

Study design

The study included saliva (n=50) and subgingival plaque (n=50) from chronic periodontal patients. Individuals presenting as outpatients at the Sree Balaji Dental College and Hospital, Pallikaranai, Chennai, India, were recruited. Chronic periodontal patients gave their written informed permission. The study was authorised by the Ethical Committee Institutional Review Board with the following inclusion and exclusion criteria (SBDCH/IEC/09/2016/20, SBDCH/IEC/01/2018/7).

Criteria's for unstimulated saliva collection

Inclusion and exclusion criteria

Patients with chronic periodontitis in the age bracket of 30 to 50 years, of either gender, and patients with chronic periodontitis with at least 12 scoreable teeth were included in the study. Alcoholism, smokers or former smokers who have quit within the last 5 years, pan/gutka/tobacco chewers, phenytoin, calcium channel blockers, cyclosporin users, aggressive periodontitis within the last 6 months to 1 year, antibiotic therapy or immunosuppressant medication within the last 6 months, scaling and polishing within the last 30 days, pregnancy or lactation are all exclusion criteria.

Criteria's for Subgingival plaque collection

Inclusion criteria: All research participants had chronic mild periodontitis and were between the ages of 30 and 50 years old, regardless of gender. In 16, 36, and 46, there were 12 scorable teeth, periodontal pocket depths of 3 and 5 mm, and clinical attachment loss of 3-4 mm. Subjects who had subgingival periodontal therapy in the previous 6 months, systemic disorders or serious uncontrolled medical disorders, history of antibiotic use in the previous 6 months, alcoholism, smokers or former smokers who had quit less than 5 years ago, pan/ gutka/ tobacco chewers, pregnancy or lactation were all excluded.

Isolation and phenotypic Identification

Whole unstimulated saliva collection

According to the previous study[15], whole unstimulated saliva samples (50) were taken from chronic periodontal patients attending the Department of Periodontitis. The samples were kept at 20°C for a week before being used for analysis. For the identification of facultative anaerobic bacteria from saliva, unstimulated saliva samples were plated on Brain heart infusion agar (Himedia, India), Enterococcus Differential agar base (Himedia, India), and Tryptone soya serum bacitracin vancomycin agar (Himedia, India) and incubated for 3 days at 37°C in 10% CO2.

Subgingival plaque collection

All 50 individuals underwent an intra-oral examination, and clinical data such as plaque index and gingival index[16] were recorded. After carefully removing supragingival plaque with a clean cotton roll, subgingival plaque was collected using a sterile Gracey curette from the buccal surface of all first permanent molars. The plaque was placed in 1 mL of sterile sodium thoioglycolate (ST) broth right away (pH 7.8). The materials were subsequently sent to SBDCH's Human Genetics Laboratory with the required safeguards.

The subgingival plaque was immediately incubated in ST broth with 10% CO2 for 4 hours before being micro-centrifuged for 10 minutes at 4,000 rpm. For identification of *E. faecalis* from subgingival plaque samples, the pellet was resuspended in remaining supernatant and plated on Brain heart infusion agar (Himedia, India), Enterococcus Differential agar base, and Tryptone soya serum bacitracinvancomycin agar (Himedia, India), and incubated for 3 days at 37°C in 10% CO2. In our lab, conventional morphological and

gram stains were used to provide a preliminary phenotypic diagnosis of the isolates.

Molecular Identification

Following the manufacturer's instructions, genomic DNA was extracted from the isolates' cell pellets using the HiPurA Bacterial Genomic DNA Purification Kit (Himedia Biotech, India). The universal primers 27F 5' AGAGTTTGATCMTGGCTCAG 3' and 1492R 5' TACGGYTACCTTGTTACGACTT 3' were used to amplify the bacterial 16S rRNA gene from DNA. The amplified PCR products were sequenced using the automated sequencer ABI 3100 with Big Dye Terminator Kit v. 3.1 at MACROGEN sequencing firm in Seoul, Republic of Korea. By using BLAST, the sequences acquired were compared to those in the National Center of Biotechnology Information database.

Antimicrobial Susceptibility Test

The antibiogram for *E. faecalis* was carried out using Bauer and Kirby's technique[17], which followed CLSI recommendations. The antibiotic solutions of amoxicillin, cefixime, ceftriaxone, cefuroxime, chloramphenicol, ceftoxime, clindamycin, gentamyicin, meropenem, metronidazole, tetracycline, levofloxacin, cefoperazone, and sulbactam, ampicillin, methicillin, vancomycin, linezolid, and erythromycin (ranging from In a nutshell, 0.5 McFarland standard inoculum from an overnight *E. faecalis* culture was equally dispersed over the Mueller Hinton plates' whole surface. The antibiotic discs were adhered to the swabbed plates and incubated for 18 hours at 37°C in ambient air. The plates were examined after incubation for uniform culture development and the establishment of inhibitory zones around the discs was measured in centimetres. For accuracy, the exam was done three times.

MIC determination

MIC determination (for values ranging from 4 to 50 ug/ml) was performed utilising agar dilution techniques on the aforesaid resistant isolates that were screened through disc diffusion assay (Therese et al., 2006). 105 cells per millilitre of *E. faecalis* solution were injected separately onto Mueller Hinton agar plates containing antibiotics. The inoculation plates were incubated for 48 hours at 37 degrees Celsius. The MICs for the aforementioned bacterial species were then determined by identifying the lowest concentration of antimicrobial agent that resulted in no observable growth.

Transformation assay

The bacterial transformation was used to ensure the susceptibility of the plasmid of clinical isolates. According to the calcium chloride technique, competent cells for E. coli DH5 (Himedia Biotech) and *E. faecalis* without plasmid (Sasham Biologics, Chennai) were produced. Briefly, *E. coli DH5* and *E. faecalis* were cultured overnight at 37°C in a shaking container containing 3 ml of Luria-Bertani (LB) medium. The overnight culture was added to LB medium that had been pre-warmed to 37 degrees Celsius. To achieve an OD of 0.35-0.4 at 600 nm, the culture was incubated at 37 °C with shaking and refrigerated for maximum transformation efficiency. The cells pellet was resuspended in ice-cold 50 mM CaCl2, and centrifuged at 4 °C.

The *E. faecalis* isolate plasmid was introduced to the aforementioned competent cells, and they were heat shock for 2 minutes at 42°C before being put in ice for 10 minutes. After 1 hour of incubation, LB broth was added. Transformed colonies were seen when the materials were distributed on the appropriate plates and cultured overnight. Transformant cfu/ plasmid DNA (g) was used to determine transformation efficiency, with Transformant cfu = No. of bacterial colonies dilution ratios original transformation volume/ plated volume.[18]

Conjugation assay

The solid agar mating technique was used for the conjugation experiment.[19] Equal amounts of donor (clinical E. faecalis) and recipient E. coli J53 (Himedia Biotech) overnight cultures, as well as *E. faecalis* without plasmid, were incubated for 1 hour at 37°C. Transconjugants were chosen onto the corresponding antibiotics in LB agar after the conjugated samples were plated for 18 hours at 37°C.

Plasmid curing

The ampicillin resistant salivary *E. faecalis* isolates with the highest percentage of resistance (92%) and linezolid resistant plaque isolates with 95% resistance from the antimicrobial sensitivity study were chosen for the plasmid curing assay to see if the antibiotic resistance was plasmid-mediated. The alkaline lysis procedure, as defined by FavorPrep Plasmid Extraction Mini Kit, was used to extract and profile plasmids for the aforesaid antibiotic (ampicillin and linezolid) resistance isolates (Favorgen Biotech Corp, Taiwan). Plasmid bands were separated on a 0.8 percent (w/v) gel using an Agarose gel electrophoresis device (EPS Biosolutions, Chennai), and the size of the plasmid was determined using a 1-50 kb DNA marker (SibEnzyme) and plasmid profiles were documented using Gelstan 4X. (The Medicare Scientific Supplies, Chennai).

Brown's technique of plasmid curing was used, which included the use of acridine orange (0.1 mg/mL), SDS (15%), promethazine (0.3 mg/mL), and a high temperature of 42°C. After that, the samples were incubated at 37°C for an overnight period. To see if the plasmid was properly cured, the DNA was extracted and validated on an agarose gel electrophoresis. The colonies grown in Muller Hinton agar plates were decreased in the presence of antibiotics (ampicillin at MIC 10 g/ml for salivary isolates, linezolid at MIC 10 g/ml for plaque isolates) to confirm that the strain had lost antibiotic resistance following plasmid removal. The number of colonies with cured phenotypic per 100 colonies

examined was used to calculate the % curing effectiveness.[20]

Data Analysis

The findings of the plasmid mediated antibiotic resistance transfer experiment were evaluated using the ANOVA test to determine the significance of the results of the plasmid mediated antibiotic resistance transfer experiment, and the P-value was determined. A statistically significant P value of 0.05 was used.

RESULTS AND DISCUSSION

Strain characterization: Gram's staining and 16s rRNA sequencing for all isolated bacterial strains were performed. Based on the results of gram's staining, 24 strains (12 Gram-positive and 12 Gram-negative) were found to be unique in shape, size and morphology from one another.

Distribution of *E. faecalis*

A total of 34 *E. faecalis* were isolated from saliva and subgingival plaque of 100 chronic periodontal patients. They were distinguished based on their colony morphology on 3 selective agar plates and were gram positive. The distribution of *E. faecalis* (n=21) from plaque was detected on Enterococcus Differential agar and Tryptone soya serum bacitracin vancomycin agar and, from saliva the distribution of *E. faecalis* (n=13) was in Enterococcus Differential agar and Brain heart infusion agar plates. These bacterial strains were confirmed by 16s rRNA sequencing with universal primers. The obtained *E. faecalis* sequence was matched with the existing sequence using BLAST programme and submitted to GenBank with the accession number MF972884.

Detection and Antimicrobial susceptibility of Enterococcus faecalis

The resistance pattern of the isolates to different antibiotics was shown in Table 1. Among 19 antimicrobials tested with varying concentrations ranged from 5 to 1000 ug/ml, 13 salivary *E. faecalis* isolates were 77% sensitive to ceftriaxone, levofloxacin, methicillin, linezolid, 92% to cefuroxime. The majority of the following antibiotics showed their resistance to amoxicillin, gentamyicin, cefixime, metronidazole, erythromycin, cefoperazone and sulbactam, erythromycin (77%), chloramphenicol, cefatoxime and vancomycin (85%), and ampicillin (92%) for salivary *E. faecalis*. Meanwhile plaque *E. faecalis* showed their sensitivity to tetracycline and methicillin (90%) and resistance to cefuroxime, gentamyicin, erythromycin (76%), ampicillin (80%), cefixime, vancomycin (85%), amoxicillin, chloramphenicol, clindamycin and sodium azide (86%), metronidazole (90%) and linezolid (95%). The resistant antimicrobials for salivary and plaque *E. faecalis* were screened for MIC since the isolates suggested to harbour plasmid mediated resistance activity. The MIC of various antibiotics was determined by using a narrow range of concentrations from 4 to 50 µg/ml are shown in Table 1 and Table 2.

Table 1: Multiple resistance profiles to antimicrobials by *E. faecalis*(n=13) isolated from saliva of chronic periodontal patients.

S.No	Antibiotics	No.of Isolates by Disc diffusion		No.of strains with MIC (µg/ml)					
Sensitive (%)			Resistant (%)	4	10	15	25	40	50
1	Amoxicillin	3(23)	10(77)	0	3	2	2	1	3
2	Cefixime	3(23)	10(77)	2	0	2	1	4	1
3	Ceftriaxone	10(77)	3(23)	0	0	0	0	0	3
4	Cefuroxime	12(92)	1(8)	0	0	0	2	2	4
5	Chloramphenicol	2(15)	11(85)	0	1	0	3	4	3
6	Cefatoxime	2(15)	11(85)	0	0	2	3	3	3
7	Clindamycin	4(31)	9(69)	2	3	4	0	0	0
8	Gentamyicin	3(23)	10(77)	0	0	1	2	3	4
9	Meropenem	7(54)	6(46)	0	0	0	0	3	3
10	Metronidazole	3(23)	10(77)	0	0	2	1	3	4
11	Tetracycline	9(69)	4(31)	3	1	0	0	0	0
12	Levofloxacin	10(77)	3(23)	0	0	0	0	0	3
13	Cefoperazone and sulbactam	3(23)	10(77)	0	0	1	2	3	4
14	Ampicillin	1(8)	12(92)	0	4	2	3	2	1
15	Methicillin	10(77)	3(23)	3	0	0	0	0	0
16	Vancomycin	2(15)	11(85)	0	0	0	2	5	4
17	Linezolid	10(77)	3(23)	0	0	0	0	1	2
18	Erythromycin	3(23)	10(77)	0	0	1	3	2	4
19	Sodium azide	8(62)	5(38)	0	0	0	0	2	3

Table 2: Multiple resistance profiles to antimicrobials by *E. faecalis* (n=21) isolated from subgingival plaque of chronic periodontal patients.

S.No	Antibiotics	No.of Isolates by Disc diffusion		No. of strains with MIC (µg/ml)					
		Sensitive (%)	Resistant (%)	4	10	15	25	40	50
1	Amoxicillin	3(14)	18(86)	0	0	8	3	4	3
2	Cefixime	4(19)	17(81)	0	0	0	6	4	7
3	Ceftriaxone	12(57)	9(43)	0	0	0	0	3	6
4	Cefuroxime	5(24)	16(76)	2	5	3	2	3	1
5	Chloramphenicol	3(14)	18(86)	0	0	0	0	10	8
6	Cefatoxime	9(43)	12(57)	0	0	0	1	3	8
7	Clindamycin	3(14)	18(86)	6	4	1	3	2	2
8	Gentamyicin	5(24)	16(76)	0	2	6	1	4	3
9	Meropenem	6(29)	15(71)	0	3	0	1	2	9
10	Metronidazole	2(10)	19(90)	0	6	4	5	3	1
11	Tetracycline	19(90)	2(10)	2	0	0	0	0	0
12	Levofloxacin	6(29)	15(71)	0	0	1	6	2	6
13	Cefoperazone and sulbactam	15(71)	6(29)	0	0	0	0	3	3
14	Ampicillin	4(19)	17(80)	0	0	0	10	4	3
15	Methicillin	19(90)	2(10)	2	0	0	0	0	0
16	Vancomycin	4(19)	17(81)	0	0	0	8	3	6
17	Linezolid	1(5)	20 (95)	0	8	3	4	4	1
18	Erythromycin	5(24%)	16(76)	0	0	4	2	6	4
19	Sodium azide	3(14%)	18(86)	0	0	5	4	2	7

Salivary *E.faecalis* isolates showed the highest resistance rate (92%) for ampicillin (MIC ≥10 µg/ml). Meanwhile cefatoxime (MIC ≥15 µg/ml) and vancomycin (MIC 25 µg/ml) showed >80% resistance. The plaques isolates showed the highest resistance to metronidazole (90%) and linezolid (95%) with the MIC ≥ 10 ug/ml. Then other antimicrobials >80% to clindamycin (MIC 4ug/ml), amoxicillin and sodium azide (15 ug/ml), cefixime, ampicillin and vancomycin (25 ug/ml), chloramphenicol (40 ug/ml) when compared to other antimicrobials tested.

Transformation

The plasmid DNA of *E. faecalis* was transformed into *E. faecalis* with the transformation efficiency of $1.48X10^6$ cfu/ ug DNA and to DH5*α* was 2.88 $X10^6$ cfu/ ug DNA. The transformation efficiency of the isolate was found to be higher with DH5*α* than *E. faecalis* and plasmid failed to conjugate through *E.coli*J53.

Plasmid Elimination

To determine the relationship between a genetic trait and resistance carriage of the specific trait, the plasmid of salivary and plaque *E. faecalis* was analysed. Resistant (ampicillin and linezolid) isolates were subjected to a minimum of three independent plasmid extractions for confirming the presence of the plasmids. Nearly 70% of the isolates harboured plasmid. Plasmid (48.5 kb)was observed for subgingival plaque isolates (Figure 1)and salivary *E. faecalis* plasmid was reported in our earlier study.[21] It was understood that the plasmid pattern was not identical between salivary and plaque isolates but was identical among themselves based on the molecular size variation by comparing with standard plasmids pUC 18 and positive control *E. faecalis*. Table 3 and 4 shows the plasmid curing efficiency of curing agents on ampicillin and linezolidresistant isolates.

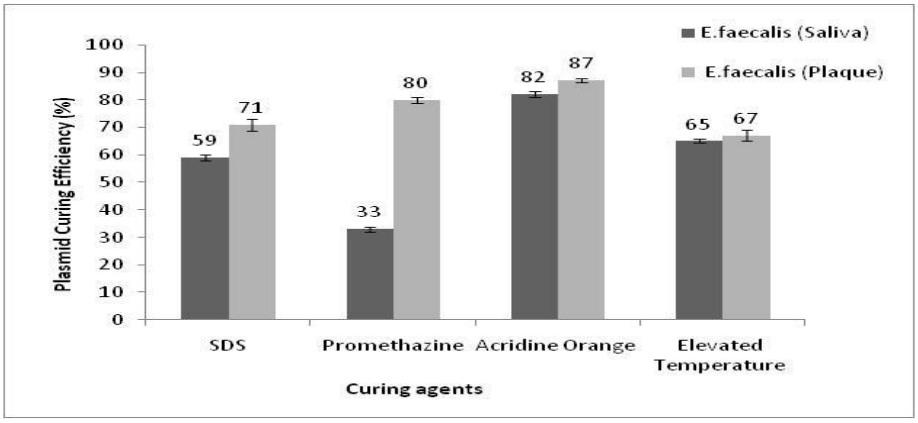


Figure 1: Plasmid curing efficiency for salivary and sub gingival plaque ampicillin resistant *E. faecalis* isolates.

Table 3: Distribution of ampicillin resistant *E. faecalis* before and after curing by curing agents.

S.No	Curing agents	No. of Salivary *E. faecalis* colonies		
		Before	After	Plasmid Curing Efficiency (%)
1	SDS (10%)	34	20	59
2	Promethazine (300µg)	36	12	33
3	Acridine Orange (100 µg)	28	23	82
4	Elevated Temperature (42◦C)	37	24	65
S.No	**Curing agents**	**No. of Plaque *E. faecalis* colonies**		
		Before	**After**	**Plasmid Curing Efficiency (%)**
1	SDS (10%)	28	20	71
2	Promethazine (300µg)	45	36	80
3	Acridine Orange (100 µg)	52	45	87
4	Elevated Temperature (42◦C)	39	26	67

Table 4: Distribution of linezolid resistant *E. faecalis* before and after curing by curing agent.

S.No	Curing agents	No. of Salivary *E. faecalis* colonies		
		Before	After	Plasmid Curing Efficiency (%)
1	SDS (10%)	34	17	50
2	Promethazine (300µg)	36	10	28
3	Acridine Orange (100 µg)	28	25	89
4	Elevated Temperature (42◦C)	37	31	84
S.No	**Curing agents**	**No.of Plaque *E.faecalis* colonies**		
		Before	**After**	**Plasmid Curing Efficiency (%)**
1	SDS (10%)	28	16	57
2	Promethazine (300µg)	45	31	69
3	Acridine Orange (100 µg)	52	42	81
4	Elevated Temperature (42◦C)	39	31	79

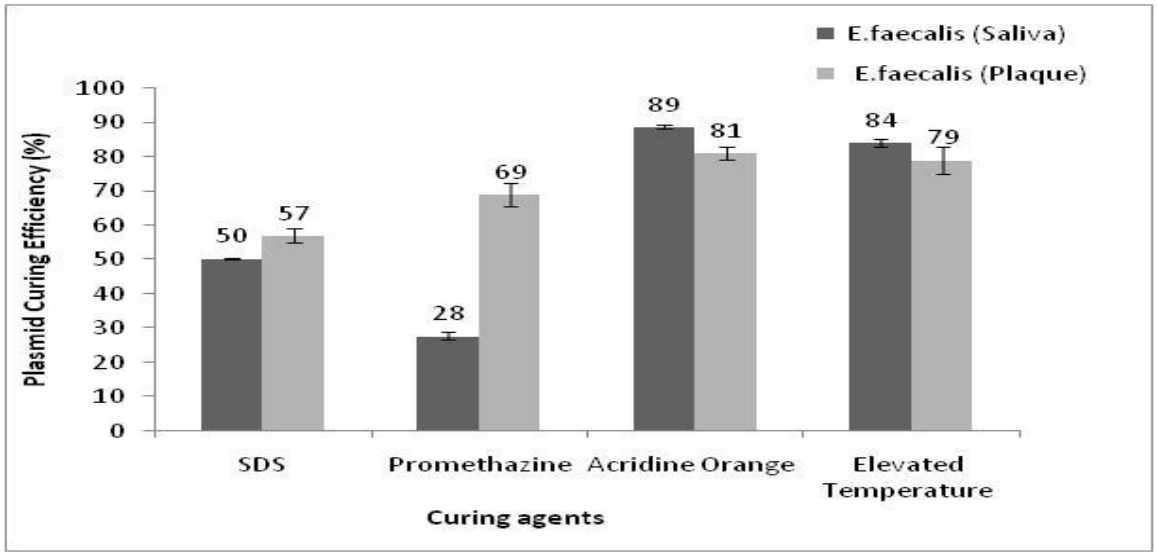
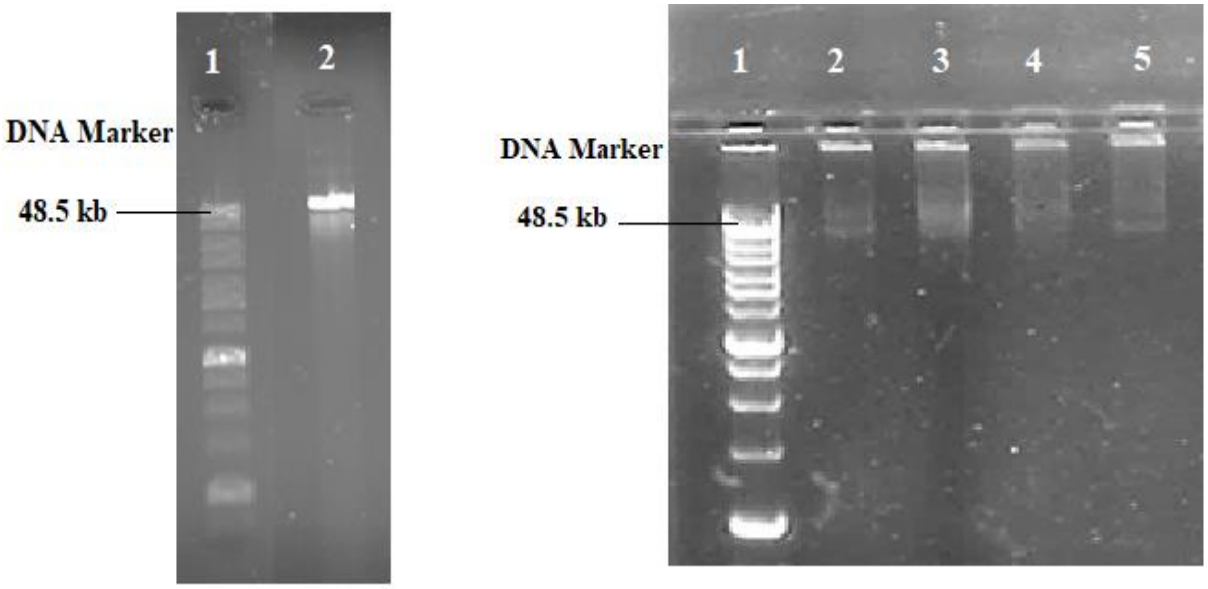


Figure 2: Plasmid curing efficiency for salivary and sub gingival plaque linezolid resistant *E.faecalis* isolates.

After curing, the isolates were grown lesser in the corresponding screening agar. The distribution of colonies after curing was less and the percentage of curing efficiency was higher to ampicillin and linezolid (Table 3 and 4, Fig. 1 and 2).It is clear from the table that the acridine orange had a remarkable plasmid curing efficiency>81%, on ampicillin and linezolid cultures of saliva and plaque when compared to other curing compounds such as SDS and elevated temperature. The curing effect of promethazine was drastically reduced to 28% and 33% in ampicillin and linezolid resistant salivary *E. faecalis* but not in plaque isolates. Figure 3, the demonstrates.



3. a. **3.b.**

Figure 3a and 3b. Plasmid curing status of *E. faecalis.*

Figure 3. a. Lane 1- 50 kb DNA marker, Lane 2 - Plasmid of untreated (control) *E. faecalis* with the band size of 48.5 kb.

Figure 3. b. Lane 1- 50 kb DNA marker, Lane 2 – Plasmid of ampicillin resistant *E.faecalis* (saliva) treated with Acridine Orange, Lane 3 - Plasmid of ampicillin resistant *E.faecalis* (plaque) treated with Acridine Orange, Lane 4 - Plasmid of linezolid resistant *E.faecalis* (saliva) treated with Acridine Orange, - Plasmid of linezolid resistant *E.faecalis* (plaque) treated with Acridine Orange.

In the present study, *E. faecalis* was found predominant in both saliva and subgingival plaque. The antibiotype among the *E. faecalis* was highly resistance (above 90%) to ampicillin, metronidazole and linezolid with the MIC ≥10 µg/ml when compared to other antimicrobials. From our result, it is understood that the association of *E. faecalis* not only cause nosocomial and community-acquired infections but also acquired its resistance in saliva and subgingival plaque of chronic periodontitis. *E. faecalis* was one of the most challenging bacteria to eradicate in recent decades.[6] Its prevalence in saliva and especially in root canals of teeth concealed from the antibiotics and immune system[1] had become a challenging bacteria to eradicate in the recent decades.[22]

The maximum resistance of periodontal to ampicillin, metronidazole and linezolid and the identification of resistance plasmid confirmed that these isolates gained plasmid mediated resistance. This was supported by the localization of these genes on mobile genetic elements.[23] Linezolid had recently approved for the treatment of vancomycin and methicillin-resistant *Enterococcus and staphylococcus* infections (including bacteremia).[24] Its resistance to linezolid might be by harbouring *cfr* gene transferable plasmid.[25] Meanwhile *optr A* was found first in China by Wang et al in 2015, widely spread in Chinese *E. faecalis* strains from human and animal origin. This dissemination of *optrA* was among different types of enterococcal plasmids especially in *E. faecalis*. The chloramphenicol and linezolid resistant isolates might carry low molecular weight *cfr* and *optrA* in *E. faecalis* of subgingival plaque.[26] This suggested that the aquisition of the resistance in plasmid might be easily transferred through natural transformation itself but not via conjugation as they failed to conjugate between *E.faecalis*. This might tolerate chloramphenicol and linezolid selective pressure. Reports also indicated that the linezolid resistance genes *cfr* and *optrA* were transferable in resistance plasmid to other antimicrobial classes such as macrolide–lincosamide–streptogramin B, aminoglycoside and phenols.[27] Further clinical *E. faecalis* isolates acquired its resistance from ampicillin and erythromycin (>69%) as they do not normally produce β-lactamases.[28] This resistance from salivary *E. faecalis* might be acquired from plasmids of oral gram-negative bacteria of saliva. The resistance of enterococci to β-lactam might be caused by the production of β-lactamase, which is encoded by the *TEM* gene, or modification in the penicillin-binding proteins.

The transformation efficiency of *E. faecalis* plasmid indicated that the transfer might be through natural transformation, but not through conjugative pheromone-inducible plasmid transfer.[29] The successful natural transformation of plasmid harboured *E. facecalis* to plasmid less *E. faecalis* suggested that clinical *E. faecalis* might horizontally transfer the linezolid resistance gene encoded plasmid. This indicated that the clinical *E. faecalis* might also contain non-conjugative plasmid that carries resistance gene besides pheromone responsive plasmid. Besides ceftriaxone, tetracycline, cefoperazone and sulbactam, methicillin, cefatoxime (≤43%) other

antibiotics tested displayed a higher percentage (>70%) of resistance.

From plasmid curing analysis, the reduction in plasmid harbouring cells pointed out that they might contain plasmid responsible for ampicillin and linezolid resistance, which were stably lost upon plasmid elimination. The high efficiency of plasmid curing corresponds to the reduction in colonies by the curing agents including elevated temperature. The higher curing efficiency for acridine orange indicated that the acridine orange intercalated between the base pair of the plasmid. Besides, other curing agents such as SDS and elevated temperature also showed their curing effect. But Keyhani et al. 2006[30] reported that except sarkosyl, SDS and acridine orange remained unsuccessful in gram-positive *E. faecalis* plasmid curing. The findings of the present study showed a relation between the antimicrobial resistance and the occurrence of the plasmid in *E. faecalis*. The curing efficiency for all curing agents was above 89% in linezolid resistant colonies might reduce the linezolid resistance gene and interfere with the growth of this plasmid harbouring bacteria. The curing of R-plasmids might interfere with the transfer of resistant genes and allowing spontaneously arising plasmid less segregants to become predominant. Such reversal of resistance is associated with the ability of the agent to inhibit the efflux pump of pathogens. Therefore, routine surveillance for the presence of plasmid mediated resistance is urgently warranted.

CONCLUSION

The present study indicates that the plasmids are the major reason for the development and spread of resistance among *Enterococcus* sp. Further attention could be given to the plasmid mediated resistance genes as targets for combating the maintenance and spread of antibiotic resistance.

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CONFLICT OF INTERESTS

The authors have no conflicts of interest to declare.

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