4.0 Introduction

Enterococci frequently cause a wide variety of infections in humans and commonly infect the urinary tract, bloodstream, abdomen [1,2], endocardium, biliary tract, burn wounds and in situ foreign devices [3-6]. Enterococci are also implicated in infections of the root canal system. However, they make up a small proportion of the initial flora, dominated by gram-negative species [7,9]. In contrast, it has been reported that enterococci are frequently isolated from obturated root canals of teeth that exhibit chronic periapical pathology [8,10]. Enterococci are normal human commensals adapted to the nutrient-rich, oxygen-depleted, ecologically complex environments of the oral cavity, gastrointestinal tract and vagina [14]. For bacteria to act as pathogens, they must first colonize the host and this involves a series of interactive events. Initially, there is a loose physical association of the organism with the surface of a tissue, which allows stronger and more permanent bonds to be established through the binding activities of microbial cell surface adhesins to complementary receptors on the host surface. Once the microbial cells are bound, they must be able to utilize available nutrients, compete or cooperate with other bacterial species in the immediate environment and with host defence mechanisms before accumulation of microorganisms can occur by cell division and growth. Once these mechanisms are established at multiple sites, the host becomes colonized [15]. It is well established that viable bacteria can be recovered from the root canal system after it has been treated by effective chemo-mechanical instrumentation [12]. *E. faecalis*, which is responsible for 80–90% of human enterococcal infections, is the dominant enterococcus species [10] and commonly the only species recovered from the obturated root canal [11]. These facts indicate that *E. faecalis* has a pathogenic role in chronic endodontic treatment failure. It has been suggested that *E. faecalis* virulence
may be related to resistance to intracanal medicaments [12] and its ability to survive in the root canal as a single organism, without the support of other bacteria [13].

_E. faecalis_ can proficiently invade dentinal tubules and it is therefore probable that microbes within dentinal tubules surviving chemo-mechanical instrumentation and intracanal medication could colonize the tubules and re-infect the obturated root canal [20]. _E faecalis_ has been often isolated from teeth with endodontic failed treatments [18]. Consequently, recent laboratory studies have focused on evaluating the effectiveness of root canal irrigants and medicaments against _E faecalis_. Many of these studies have grown the bacterial strains as planktonic cultures (bacteria in suspension) [19]. However, planktonic bacteria do not act in accordance with the _in vivo_ growth conditions found in an infected tooth, in which bacteria grow as a biofilm on the dentinal walls [20]. Bacterial biofilm is made by bacteria in sessile form, embedded in a polysaccharide matrix. This structure makes it more difficult for drugs to reach the bacteria. Therefore, all studies about the “clinical” action of endodontic irrigants should be conducted with bacteria in the “biofilm form.” However, very few studies with regard to the action of antimicrobial irrigants against biofilm have been published [21]. Hence, in this chapter, we have attempted to evaluate the efficacy of CAMPs against _E faecalis_, grown as a biofilm and the possibility of use of CAMPs in the treatment of root canal infection.

4.1 Methods

4.1.1 Isolation and characterization of resistant strains from root canal treatment (RCT) failed cases.

Root canal treatment failed tooth samples were collected from the Department of Endodontics, SRU. Strains (_E.faecalis_) isolated from those tooth samples which were
resistant to root canal treatment were isolated, characterized and the activity of VSL2 & VS2 on those strains were determined by micro dilution method.

4.1.2 Microdilution method

Bacterial cells (resistant strains) grown overnight were diluted in BHI broth to a density of $10^5$ CFU/ml. Then, 100µL of this culture was aliquoted into the wells of a 96-well flat-bottom microtiter plate and peptides (VSL2 & VS2) were added to each well at varying concentrations and mixed well. The plate was then incubated at 37°C for 24 hours. After incubation, the optical density at 600 nm was measured using a microtiter plate reader and the MIC was determined (MIC is defined as the lowest concentration of a drug that inhibits the measurable growth of an organism after overnight incubation).

4.1.3 Time Kill Assay

An overnight culture of *E. faecalis* in BHI broth was diluted to reach a concentration of $1 \times 10^6$ CFU/mL. Peptides were added according to their MICs (10µM) and incubated at various time intervals (3h, 6h, 12h, & 24h)[22]. After incubation, the viable bacterial counts were done by taking 100µL aliquot from treated and untreated cultures. Then, 10 fold dilutions were prepared in BHI agar plates and incubated for 24 hours at 37°C. The inhibitory effect was calculated based on the CFUs counted on the plates.
4.1.4 Quantification of microbial load on ex vivo dentinal tubules model by real-time polymerase chain reaction

4.1.4.1 Preparation of tooth samples

Freshly extracted tooth samples were collected (IEC approval No: IEC-NI/09/DEC/13/37) and immersed in 5.25% sodium hypochlorite to remove soft surface tissues and organic debris. The tooth samples were horizontally sectioned into coronal, middle and apical sections using carbide disc [23]. The root canal of each specimen was enlarged with a No: 10 round bur. The smear layer including organic and inorganic debris was removed by placing the samples in an ultrasonic bath containing 17% ethylenediaminetetraacetic acid, followed by rinsing with 5.25% NaOCl each for 5 min. The processed tooth samples were then sterilized by autoclaving at 121°C for 30 min.

4.1.4.2 Contamination of blocks

Sterilized tooth samples were immersed in *E. faecalis* suspension and incubated for 7 days at 37°C. Samples which were not incubated with organisms served as negative control to confirm the sterility of samples. Medium was changed every day. After incubation, the samples were gently washed with saline to remove the excess culture from the surface of the sample. Subsequently, 10 samples were used for each group and the inoculated samples were grouped as follows:

Group 1: Tooth samples treated with VSL2 & VS2 (10µM)

Group 2: Tooth samples treated with positive control (vancomycin 10µM)

Group 3: Tooth samples without any treatment served as negative control
Group 4: Tooth samples treated with positive control (2% CHX).

After the incubation, the dentinal chips were harvested at 400 μm depth and the microbial load was quantified using real-time (RT) polymerase chain reaction (PCR) from the chips (figure: 4.1).

4.1.4.3 Real-time PCR

DNA was isolated from the harvested dental chips by phenol chloroform isoamyl alcohol method. All the PCR reactions were done in 20 μl on detection system. Microbial load of *E. faecalis* present in dentinal tubule was determined using real time PCR (ABI 7900 HT). The assay is based on the detection of the 16s rRNA gene. Universal 16s rRNA primers were used for the amplification. The forward primer (5’GATTAGATACCCCTGGTAGTCC 3’) reverse primer (5’CCCGGGAACGTATTCACCG 3’) sequences were used for quantification of *E. faecalis*[24]. The assay was performed using SYBR green. The following PCR conditions were adopted to amplify the target: To amplify the product, 50°C for 2 min, 95°C for 10 min, 95°C for 15 s and 60°C for 1 min for 35 cycles. The efficacy of the compounds against *E. faecalis* was calculated based on the cycle threshold (CT) value [25].

Percentage of reduction = CT value of treated sample/CT value of untreated Sample ×100
4.1.5 Quantitative assessment of bacterial viability in dentin by confocal laser scanning microscopy (CLSM)

The dentin block with a length of 4 mm was horizontally sectioned from each tooth at 1mm below the cementoenamel junction. The root canals inside the blocks were enlarged to a size of Gates Glidden Drill #6. Each dentin block was fractured by making a thin groove in the middle of the specimen by using low speed hand piece with a small bur. Then, the specimens were fractured by a blade and hammer into semi cylindrical halves. The processed dentin was infected with *E. faecalis* for 7 days. The contaminated specimens were washed with sterile water and were randomly divided into four groups: CHX, VSL2, VS2 and untreated. The specimens were disinfected with each drug and incubated for 24 hours at 37°C. After the disinfection, the dentin specimens were stained with fluorescein diacetate (FDA), which emits green fluorescence in live cells and propidium iodide (PI), which emits red
fluorescence in dead cells, whose membranes are disrupted and bound to
deoxyribonucleic acid (DNA). The uninfected specimens were stained under the same
protocol and used as negative controls. Fluorescence from the stained cells was
viewed by using confocal laser scanning microscopy (CLSM). Further, by staining
with FDA and PI, the % of live and dead cells was computed.

4.1.6 Morphological changes and biofilm assay by scanning electron microscopy

*E. faecalis* was grown on Whatman No: 1 filter paper strips incubated overnight at
37°C. After incubation, the strips were treated with peptides (VSL2&VS2) at their
MICs (10µM) for 24 h and followed by immersion in neutralizing broth to stop the
action of CAMPs [26]. The strips were dried and coated with gold. The observations
were made on supra 55, Carl Zeiss scanning electron microscope at a magnification of
100 KX.

4.1.7 FACS-based analysis for PI uptake

Cells grown overnight (*E.faecalis*) were subcultured to an OD 600 of 0.35. The cells
were harvested (4,000 rpm; 10 min; 4°C), washed and resuspended in buffer (5 mM
glucose, 5 mM HEPES, pH 7.2) to 10^8 CFU/ml. *E.faecalis* suspension (10^8 CFU/ml)
was incubated in 480 µl of buffer containing peptides VSL2 & VS2 (10µM) at
varying time points (15,30& 60 min) and followed by incubation with 5µL of
propidium iodide (1 mg/ml) for 15 min at room temperature. The suspension was then
subjected to fluorescence-activated cell sorter (FACS) analysis. The cells suspended
in buffer with and without PI served as controls.

Post hoc multiple comparisons were used to compare the results at a significance level
of p< 0.05.
4.2 Results

4.2.1 Time kill assay

The activity of VSL2 & VS2 on root canal treatment failed resistant strains of *E. faecalis* was found to be 50µM (figure:4.2). Time kill assay demonstrated that VSL2 & VS2 showed faster killing of *E. faecalis* when compared to the positive control ampicillin. Killing kinetics of the peptides at their MIC showed reduction in CFU after a treatment of 3 hours, compared to the positive control ampicillin (20µg) that showed significant killing only at the end of 12 hours (figure:4.3).

**Figure: 4.2 Activity of CAMPs against *E.faecalis* strains isolated from root canal treatment failed tooth samples**

![Graph showing optical density at 600nm against concentration of CAMPs (VSL2 and VS2)](image-url)
4.2.2 Quantification of microbial load on *ex vivo* dentinal tubule model by real-time PCR

Antibacterial activity of VSL2 and VS2 was tested against *E. faecalis* in dentinal tubule model by real-time PCR. The activity of peptides was comparable to vancomycin and the peptides showed high inhibitory activity at 400 μm depth after 24 hour of treatment (10μM). VSL2, vancomycin, VS2 and CHX showed 97%, 98% and 99% reduction of *E. faecalis* after 24 h treatment, respectively (figure: 4.4). Comparison between the treated groups were conducted using the Kruskal-Wallis/analysis of variance on rank and were found to be statistically not significant at p>0.05.
Figure: 4.4 Quantification of microbial load on *Ex Vivo* dentinal tubule model after treatment with antibacterial agents at their MICs using Real Time PCR

4.2.3 Quantitative assessment of bacterial viability in dentin by CLSM

The efficacy of AMPS against *E. faecalis* that penetrated into the dentinal tubules were assessed by CLSM (figure: 4.5a). The results showed 58% and 70% dead cells in VSL2 and VS2 treated tooth samples when compared with untreated samples which showed only 6% of dead cells (figure:4.5b). The antibacterial effect of all the compounds was statistically analyzed using one way ANOVA and multiple comparisons were performed using post hoc tests. The results were not statistically significant at p<0.05.
Figure: 4.5 a Confocal laser scanning microscopy of \textit{E. faecalis} infected dentinal tubules treated by different antibacterial agents after viability staining

CONTROL

CHX (2%)

VSL2 (10µM)

VS2 (10µM)
Figure: 4.5 b Percentage of live and dead cells distributed in dentinal tubules after treatment with antibacterial agents at their MICs

4.2.4 Determination of the effect of peptides on microbe biofilm formation and cell lysis as observed by electron microscopy

Biofilm assay showed that treatment with the AMPS (VSL2 and VS2) leads to significant reduction in microbial load as compared with the untreated controls. SEM analysis showed that peptide treatment significantly decreased the degree of attachment of viable bacterial cells to matrix (Whatman paper), thereby demonstrating their potency to inhibit biofilm formation (as shown in figure: 4.6). The morphological changes in the organism are shown in figure 4.7. Increase in roughness of cell wall and rupture of the cell membrane were also observed after treatment with CHX and CAMPs. On the other hand, no membrane damage was observed in the untreated samples.
Figure: 4.6 Biofilm assay by scanning electron microscopy
Figure: 4.7 Scanning electron micrographs of \textit{E. faecalis} showing cell wall damage in CAMPs treated cells.
4.2.5 FACS-based PI uptake assay

This assay was used to study the kinetics of inner membrane permeabilization by two selected peptides (VSL2 & VS2). As shown in figure 4.8, CAMPs were good in inducing membrane permeabilization. VSL2 & VS2 showed very fast kinetics of inner membrane permeabilization. On treatment for 15 min at their MIC (10µM), VSL2 permeabilized only 34% of *E. faecalis* cells, whereas VS2 & CHX permeabilized 48.6 & 82%. At 30 min VSL2, VS2 exhibited 73% and CHX exhibited 82% permeability. After 60 min VSL2 permeabilized 82%, VS2 and CHX permeabilized 80% and 90% of cells.

**Figure: 4.8 Kinetics of PI Uptake by CAMPs treated *E. faecalis* using FACS**

![Graph showing kinetics of PI uptake by CAMPs treated *E. faecalis* using FACS](image)
4.3 Discussion

Endodontic treatment involves eradication of microbes invading the root canal and thereby, teeth conservation. Use of high concentration of irritants and packaging of medicaments into the root canal are the two main modes of treatments of root canal infection. Usage of irritants is limited due to their toxicity profile. Calcium hydroxide, the most widely used intracanal medicament, leads to incomplete eradication of the infecting microbes causing reinfection and tooth decay. Medicaments that are used commonly for root canal treatment also get distributed to the whole body from the root apex and hence, may cause allergic reactions [27]. Treatment failure occurs despite the usage of these irritants due to the resistant microbes harboring the root canal. These agents can damage the surrounding tissues over long residence in the root canal. The reason for the endodontic treatment failure has been attributed to restricted or inadequate residence time of the powerful antiseptics in the root canal. It has also been attributed to its toxicity, resulting in incomplete cleaning of the root canal. Therefore, it leaves resident microbes behind, which can colonize & cause reinfection [12]. Hence, complete removal of microbe from root canal is expected to provide good results in combating majority of endodontic infections by a new class of agents which can overcome these limitations. Based on these observations from clinics, the investigators tested the hypothesis that CAMPs owing to their peptidic nature and biocompatibility could serve as useful intracanal medicaments for eradication of microbes from infected root canals. Thus, CAMPs might prove to be one of the best possible alternatives. The investigators also showed that designed CAMPs could serve as useful intracanal medicaments and eradicate *E. faecalis* at 400 μm depth. An initial screening study showed that out of 11 peptides tested, two of them demonstrated significant activity against *E. faecalis*, the most important
pathogen involved in root canal infections. These peptides (VSL2 & VS2) showed activity against *E. faecalis*, *S. aureus* and *C. albicans*, but not against *Streptococcus* which was confirmed thrice by repeating the experiments. Recent studies on vancomycin resistant *Streptococcus* have shown the presence of vanB gene in clinical isolates to be responsible for this resistance. Probably, these factors could also be a reason for null activity of our peptides on *Streptococcus* [30]. Time kill assay showed that at a MIC of 10 µM, the CAMPs could kill *E. faecalis* cells at a faster rate (3 hrs) as compared to the positive control ampicillin that did not show microbe reduction even after 12 and 24 hours of treatment. Biofilm is a structured consortium of bacteria embedded in a self-produced polymer matrix consisting of polysaccharide, protein and etc [28, 31]. This biofilm reduction, as determined by SEM, showed that peptides prevented the aggregation of *E. faecalis*. This assay is also significant, since the formation of biofilm is an important causative factor in dental infection [16] and it was inhibited by the tested CAMPs.

The morphological changes documented by SEM analysis revealed that microbe death by cell membrane damage could probably be the major mechanism of action for the given antibacterial peptides. Similar observations were also reported earlier for other CAMPs [32, 33]. The efficacy of CAMPs against the organisms invading the root canal was also proved by CLSM. *Ex vivo* study on dentinal tubule model showed a drastic reduction in microbial load at 400µm depth in root canal. Real-time PCR for 16s rRNA quantification asserted the efficacy of the CAMPs as an intracanal medicament. It was also observed that the CAMPs needed to be in place for only 12 hours, beyond which permanent dentures could be affixed, thereby showing the high efficacy and fast response of the medicaments.
4.4 Conclusion

The chosen peptides, VSL2 & VS2 showed better antibacterial property against *E. faecalis* (ATCC 29212) as shown by *ex vivo* tooth model. The peptides also caused bacterial cell membrane damage which was confirmed by SEM. These peptides were also shown to have good antibacterial efficacy against strains isolated from root canal treatment failed tooth samples, resistant to root canal irrigants. Hence, they can be developed as intracanal medicaments to treat root canal infections caused by *E. faecalis*. 
4.5 References


