6.0 Introduction

The current clinical challenges posed by enterococci include the increased incidence of nosocomial infections and the lack of enterococcal activity of several compounds due to intrinsic resistance or by the acquisition of resistance genes. Moreover, resistance to recently approved antimicrobial agents with anti enterococcal activity (e.g. daptomycin) [1-4] has been documented in E.faecalis and other enterococcal species. This forecasts the need for new therapeuttic options for enterococcal infections in the future. As discussed earlier, E. faecalis is classified as one of the most resistant pathogenic organisms, detected in infected root canals [5]. As a new generation of prospective antibiotics, CAMPs offer broad-spectrum antimicrobial activity. They possess the attractive evolutionary property of action like that of the naturally occurring peptides, so that bacteria may find it difficult to develop resistance [6-8].

In order to prove the in vivo efficacy of CAMPs, an animal infection model is of paramount importance in preclinical drug discovery before its recommendation in humans for clinical use. There is a need for appropriate models for the testing of the intracellular efficacy of antimicrobials against E. faecalis. Several in vitro models that use various cells and cell lines are available for the study of efficacy of antimicrobials [9]. However, only a few in vivo models have been developed. Mice are one of the most common and convenient mammalian models for studying the pathogenesis of bacterial infection [10] because of their well-characterized genome, easy husbandry, and low cost. The above mentioned features have allowed the mice to become one of the best tools for drug evaluation and infectious studies.
6.1 Methodology

6.1.1 Study Organism

All experiments were performed with *E. faecalis*, obtained from the clinical laboratory at Sri Ramachandra University and maintained in brain heart infusion medium at 37°C under aerobic conditions. To prepare the inoculums for the study, *E. faecalis* strains were grown overnight at 37°C in 5 ml BHI broth and then the overnight culture was diluted to 1:100 times and incubated at 37°C for 16 to 20 hours. The culture was concentrated by centrifugation at 14,000 g (for 15 min at 26°C). The bacterial pellet was suspended in 2 ml of PBS and the O.D was measured at 600nm and adjusted to 0.5 McFarland. A bacterial suspension of 5 x 10⁸ CFU was found to be optimal for inducing a reproducible, longer-term, non acute infection in mice [11]. The number of bacteria injected per mouse was confirmed by the broth dilution of the inoculum and viable plate counts.

6.1.2 Animal model

Adult female BALB/c mice were obtained from Biogen, Bangalore, India. Animals were housed in groups on soft bedding with food and water available (*ad libitum*) in a temperature-controlled environment with a light-dark cycle of 12:12 h. All animals were allowed to habituate to the housing facilities for at least one week prior to the study initiation. Guidelines of “Guide for the Care and Use of Laboratory Animals” (Institute of Laboratory Animal Resources, National Academic Press 1996; NIH publication number #85-23, revised 1996) were strictly followed throughout the study. All experimental procedures were approved by the Institutional Animal Ethics Committee (IAEC), Sri Ramachandra University, constituted as per the directions of the Committee for the Purpose of Control and Supervision of Experiments on
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Animals (CPCSEA), India. IAEC clearance was obtained (IEC approval No: IEC-NI/09/DEC/13/37). 4 to 6 week old female BALB/c mice weighing between 20 to 25 g were used for the study. Mice were observed at regular intervals for morbidity and mortality.

6.1.3 Preparation of antimicrobial agents

Control drug: Vancomycin for injection was procured from Himedia. A stock solution of 4mg/mL was prepared in sterile pyrogen-free saline. The mice were treated with a dose of 20mg/Kg body weight [12].

Test drug: Peptide (VSL2) was used as the test drug. Three different concentrations of peptides were used for the study. Low dose- 5mg/Kg, Mid dose- 10mg/kg and High dose- 20mg/kg was prepared using sterile pyrogen-free saline.

6.1.4 Murine thigh infection

The thigh infection was induced by intramuscular injection of 0.1 ml of the inoculum into the left thigh of each mouse 1.5 h prior to initiation of treatment. VSL2 was administered by intravenous injection of 0.2 ml in three doses ranging from 5, 10, and 20mg/kg with six mice per dose level and vancomycin at 20 mg/kg body weight of mice. Mice were euthanized 24 h after dose administration and thighs were removed, homogenized and plated for determination of viable bacteria. Organs from mice were collected and subjected to histopathological evaluation [12]. The steps involved in the development of infection are shown in figure: 6.1.
6.1.5 CFU determination

The harvested tissues were homogenized, and 100µL of the homogenized sample was diluted in 900 µL of sterile saline and serially diluted till the dilution of $10^{-5}$. Aliquots (50 µL) from the last two dilutions were plated on the BHI agar plate and incubated at 37°C for 24 hours and the colonies were counted.

6.1.6 Histopathologic evaluation

All the organs from the infected animals were harvested and processed for histopathological evaluation. Samples of dissected organs were stored in the fixative solution (10% neutral buffered formalin for 48 h) and cut longitudinally at a thickness of 5 µm. [13]. Staining was done with hematoxylin and eosin and verified by the
veterinary pathologist. Statistical analysis for the experiments was performed using one way ANOVA.

6.2 Results

6.2.1 Murine thigh infection

Figure 6.2 shows the process of development of infection in mice and figure 6.3 shows the effect of intravenously administered peptides (VSL2) to BALB/c mice infected intramuscularly with E. faecalis. VSL2 was administered 1.5 h after infection as a single dose over a range from 5, 10, 20 mg/kg and vancomycin at a single dose of 20mg/kg of mice. The bacterial density taken from control animals without treatment was determined to be $3 \log_{10}$ CFU/thigh. The maximal effect on bacterial count reduction was observed at the high dosage of VSL2 (20mg/kg) wherein $1 \log$CFU/ml was observed (figure. 6.3). This dosage of peptides showed good efficacy when compared to the positive control group treated with vancomycin, which exhibited $2 \log_{10}$ CFU/thigh. The E$_{C50}$, or dose resulting in 50% of maximal bacterial killing, was 20 mg/kg. Death of animals was not observed in any of the groups.

6.2.2 Histopathology examination (HPE)

Figure 6.4 shows the histopathological evaluation of organs (Heart, Liver, Kidney, Spleen and Brain) collected from infected animals after treatment with VSL2. HPE of collected organs from all the animals after staining with hematoxylin and Eosin (H&E) did not reveal any pathology.
Figure: 6.2 Viable bacterial colonies after treatment with VSL2/Vancomycin

![Viable bacterial colonies after treatment with VSL2/Vancomycin](image)

Figure: 6.3 Bacterial counts in thighs of mice infected with *E. faecalis*

![Bacterial counts in thighs of mice infected with *E. faecalis*](image)
Figure: 6.4 Histopathological evaluations of organs harvested from mice infected with *E. faecalis*

**Vancomycin 20mg/kg**

BRAIN | HEART | KIDNEY | LIVER | SPLEEN
---|---|---|---|---

**Vehicle control (Saline)**

BRAIN | HEART | KIDNEY | LIVER | SPLEEN
---|---|---|---|---

**VSL2 5mg/kg**

BRAIN | HEART | KIDNEY | LIVER | SPLEEN
---|---|---|---|---

**VSL2 10 mg/kg**

BRAIN | HEART | KIDNEY | LIVER | SPLEEN
---|---|---|---|---

**VSL2 20 mg/kg**

BRAIN | HEART | KIDNEY | LIVER | SPLEEN
---|---|---|---|---
Chapter 6

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6.3 Discussion

Enterococci have emerged as increasingly important pathogens because of the acquired resistance to glycopeptides and other agents [14,15]. Research on antimicrobials to overcome resistance in gram-positive bacteria has led to the discovery of a number of promising new compounds. In addition, many gram-positive strains resistant to multiple antimicrobial agents have emerged in addition to the existing resistant strains. New compounds for the effective treatment of infections caused by multiresistant gram-positive species are urgently needed [16-18]. For this reason, CAMPs were chosen as one of the treatment regimens. CAMPs are promising agents due to their mechanism of action; particularly its rapid killing and concentration-dependent activity. CAMPs have already been shown to be a potent bactericidal inhibitor of resistant gram-positive bacteria by in vitro methods. Recently, infection models using various kinds of mice and guinea pigs have been developed to assess the antimicrobial activity of antimicrobial agents [19-21]. In the present study, we used BALB/c mice as in vivo models to study the antimicrobial activity of peptides against E. faecalis.

The infection model described in the present study involved the use of BALB/c mice to study the efficacy of CAMPs against E. faecalis by an intramuscular infection model. This model required a high dose of bacteria (5 \times 10^8 CFU) to cause infection in mice. We observed that CAMPs were more efficacious than vancomycin against infections with E. faecalis. Histopathology of organs collected from the mice after treatment with VSL2 towards infection also revealed normal architecture of organs, thereby re confirming the biocompatibility of peptides in vivo.
6.4 Conclusion

Intravenous injection of VSL2 after 2 hours of *E. faecalis* infection inhibited the growth and kept the infection under control. HPE of tissues collected from the drug treated mice after 24 hours did not show any pathologic changes or signs of toxicity. Hence, the peptide showed good antibacterial efficacy *in vivo* and were also not toxic to mice.

6.5 References


