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Levels of Intravenous Enterococcus faecalis That Cause Heart Colonization

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Levels of Intravenous *Enterococcus faecalis* That Cause Heart Colonization

by

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A thesis submitted in partial satisfaction of
the requirement for the degree
Master of Science in Endodontics

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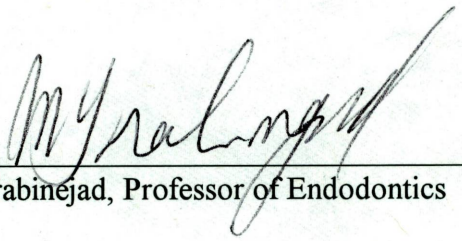

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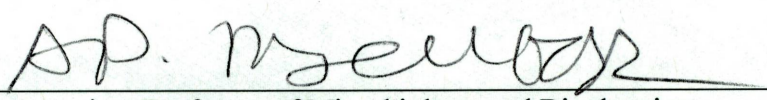

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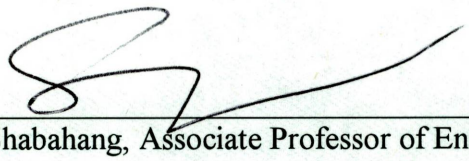

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I would like to dedicate this work to the late Dr. Charles Kean who was not only a mentor, but also a friend.

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ABSTRACT OF THE THESIS

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Master of Science, Graduate Program in Endodontics
Loma Linda University, September 2004
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While previous studies have shown that presence of bacteria in systemic circulation can cause infective endocarditis, there is no information on the specific amount of bacteria necessary to cause this condition. The purpose of this study was to establish the minimum level of circulating bacteria that will cause colonization of damaged heart valves in rats. Fifty-two Sprague-Dawley rats were anesthetized and their heart valves were damaged using an established protocol. A clinical isolate of *Enterococcus faecalis* was transformed with a plasmid bearing the *luxF* and Chloramphenicol cassettes to label the bacteria with the light-emitting protein. After four weeks, the rats were re-anesthetized and intravenously injected with various concentrations of the labeled *E. faecalis* ranging from 1×10^2 to 1×10^8 cells in a 100 µl volume. The animals were sacrificed one week after infection and the hearts were excised and incubated overnight in brain heart infusion containing chloramphenicol. The presence or absence of luminescent bacteria was examined under a low light imager and recorded. All animals injected with 1×10^8 *E. faecalis* cells consistently demonstrated colonization of their heart valves. Variable incidence of colonization was also observed in animals injected with 1×10^3 to 1×10^7 cells. None of the rats injected with 5×10^2 or 1×10^2 bacterial cells demonstrated colonization of their heart valves. This study has

established for the first time the lowest level of bacteria, which can cause colonization of damaged heart valves in rats.

CHAPTER ONE

LITERATURE REVIEW

The Role of Bacteria in Systemic Diseases

The concept of local or systemic disease occurring secondary to a localized chronic infection has been called the focal infection theory, and its origin can be traced back to the time of Hippocrates (Thoden van Velzen *et al.*, 1984). In the eighteenth century, Stoll suggested a relationship between some systemic diseases and infected tonsils (Noda *et al.*, 1979). In 1801 Benjamin Rush reported that a patient suffering from arthritis was cured after having a tooth extracted (Anderson, 1971). Recently, interest in the association between dental diseases with systemic disease has been renewed. Associations between dental diseases and systemic outcomes are potentially important because of the high occurrence of dental diseases. If this extremely common source of chronic infection leads to an increased morbidity and mortality rate, the public health impact of oral disease on millions of people would be substantial.

Recent studies demonstrate an association between dental and systemic diseases, including systemic infections, cardiovascular disease, pregnancy outcomes, respiratory diseases and diabetes. Wu and co-workers (2000) examined the relation between periodontal health and various cardiovascular risk factors such as serum total and high-density lipoprotein cholesterol, C-reactive protein, and plasma fibrinogen. The results of their study show a significant relation between indicators of poor periodontal status and increased C-reactive protein and fibrinogen. This same group (Wu *et al.*, 2000) also looked at the association between periodontal disease and cerebrovascular accidents (CVAs). While this relationship has not been well studied, the investigators found that

periodontitis is a significant risk factor for total CVA and, in particular, nonhemorrhagic stroke.

Beck and associates (1999) looked at five longitudinal studies that show oral conditions being associated with the onset of coronary heart disease while controlling for a variety of established coronary heart disease risk factors. Offenbacher and associates (1996) studied the relationship of periodontal disease and pre-term low birth weight. Their data indicates that periodontal disease represents a previously unrecognized and clinically significant risk factor for pre-term low birth weight.

Several bacterial species have been identified from the root canals of pathologically involved teeth (Fabricius *et al.*, 1982). Egress of these bacteria and their products into the periapical tissues can lead to the formation of antibodies (Torabinejad and Bakland, 1978). Kettering and Torabinejad (1984) studied the serum concentrations of circulating immune complexes, immunoglobulins G, M, and E, and the C3 complement component of 35 patients with acute apical abscesses. They demonstrated that acute periapical lesions may lead to measurable systemic immunological reactions, but the clinical significance of these changes remains unclear.

Other researchers have examined whether the oral cavity and dental plaque may serve as a reservoir for bacteria that may cause systemic disease. Nguyen and co-workers (1993) analyzed *Helicobacter pylori* in supragingival and subgingival plaque. Their detection of *H. Pylori* in dental plaque suggests that this colonization is not restricted to the gastric mucosa and that this ecological niche may serve as a possible sanctuary, which may be responsible for reinoculation of the stomach after topical anti-*H. pylori* therapies such as bismuth.

Recently, a possible link between chronic respiratory diseases and infections has been investigated. In a study of 189 elderly subjects it was found that predictors of respiration pneumonia included dependency upon feeding and oral care, as well as the number of decayed teeth (Langmore *et al.*, 1998).

Over the years, there have been many correlations between diabetes mellitus and oral disease. Patients with diabetes mellitus are more subject to chronic infections than non-diabetic patients and diabetes has been associated with increased prevalence, severity, and progression of oral conditions. The presence of certain pathogens may result in elevated cytokine levels in a host. A number of pro-inflammatory cytokines may exert cytotoxicity to islet cells leading to insulin-dependent diabetes mellitus (Rabinovitch, 1998).

There are a number of shared risk factors for oral and systemic diseases, and the limitations of many studies require careful interpretation. While these common risk factors may partly explain the reported associations, it is also possible that a causal relationship exists.

Infective Endocarditis

Infective endocarditis (IE) is a serious and often fatal systemic disease that has been associated with dental diseases and treatment (Beck *et al.*, 1996). The diagnosis is based on infection of the endocardial surface of the heart and indicates the presence of microorganisms in the lesion. The acute form has a fulminant course with high fever and leukocytosis, resulting in death in less than six week (Nord and Heimdahl, 1990). The conventional concept of endocarditis is that susceptibility is usually the result of abnormal (congenital or acquired) heart valves or endocardium and that bacteremia

causes the infection. Rheumatic heart disease, congenital cardiac defects and prosthetic valves are the most obvious predisposing factors (Dajani, 1985). These defects may cause an abnormal, high-velocity jet stream of blood that can damage the cardiac endothelium. The subsequent adhesion of platelets and fibrin form sterile vegetations on the cardiac valves. During a bacteremia, microorganisms may colonize these vegetations and cause infective endocarditis (Hienz *et al.*, 1996).

Each year, the incidence of IE is 11 to 50 cases per million of population. Before the advent of antibiotics, the mortality rate from this disease condition was nearly 100%. Today, the mortality rate for endocarditis associated with the most common microbial isolates is under 10%, but increases when heart colonization results from less common microorganisms (Uyemura, 1995).

Gram-positive cocci, mainly streptococci and staphylococci, continue to cause the majority of cases of IE. Among the streptococci implicated in this infection, the long-standing predominance of oral or viridans-group streptococci has progressively faded. At the same time, the number of cases caused by "enteric streptococci" (*Streptococcus bovis* and enterococci) has increased (Hoen, 2002). Most of these oral streptococci, along with *S. bovis* strains can be managed using penicillin (Francioli, 1995). However, despite advances in antibacterial therapy, the incidence of infections caused by multidrug-resistant Gram-positive organisms is increasing (Baquero, 1997).

While the mortality rate for endocarditis caused by alpha-hemolytic (viridans) streptococci has decreased, it is still 40% to 80% for endocarditis resulting from less common microorganisms (Uyemura 1995). Staphylococci and enterococci are two microbial isolates that may resist conventional treatment of endocarditis. Among the

staphylococci causing endocarditis, the increasing proportion of coagulase-negative and methicillin-resistant strains observed in recent years has changed the approach to choice of antibiotic therapy (Hoen 2002). *S. aureus* has frequently been implicated in cases where severe sepsis produced a high mortality rate (Casabe et at., 2003). Furthermore, for a growing population of patients who have HIV infection along with IE and a face high morbidity and mortality rate, *Staphylococcus aureus* is the most common etiological agent (Miro *et al.*, 2002). Enterococci are a long known cause of bacterial endocarditis and a more recently recognized cause of nosocomial infection and superinfection (Murray and Weinstock 1999). Strains of this problematic pathogen have become resistant to vancomycin and teicoplanin and highly resistant to penicillins and aminoglycosides (Baquero, 1997). This presents an evolving problem for both clinicians and investigators.

Bacteremias

It has been shown that bacteremia, the presence of bacteria in the bloodstream, can occur from many different dental and oral pathways. As far back as 1935, Okell and Camb showed transient streptococcal bacteremia after dental operations, and that in cases of a severe alveolar infection, 75% of the patients who underwent multiple extractions had positive blood cultures immediately after surgery. Palmer and Kempf (1939) demonstrated cases of bacteremia (and subsequent endocarditis) following tooth extraction and other dental treatments. In 1963, Bender and coworkers reported that exodontic, periodontic, and certain endodontic procedures were of major concern in the production of bacteremias. Another study showed a concentration of 3×10^3 bacteria/ml

of blood after instrumentation of the root canal of a tooth beyond the apex during endodontic therapy in a human patient (Baumgartner et al., 1976).

Traditionally, invasive procedures have been considered as the only significant cause of bacteremias. Bacteria, however, may enter the blood stream due to physiological functions that are independent of procedural manipulations. Circulating bacteria may be detected in 5 out of 30 (16.7%) blood samples drawn from patients four minutes after tooth brushing (Sconyers et al., 1973). Additionally, bacteremias occur in 38% of patients after normal mastication, and in 11% of patients with oral sepsis in the absence of any intervention (Guntheroth, 1984). Some authors believe that bacteremia of a random nature causes more infective endocarditis than invasive dental procedures. Chewing and various oral hygiene practices may be significant factors in at risk patients because cumulative exposures to bacteremias are significantly greater from everyday events than operative procedures (Roberts, 1999; Seymore *et al.*, 2000). This theory is supported by findings that show oral streptococci cause approximately 50% of all infective endocarditis cases (Kaye, 1986), whereas only 15% of patients diagnosed report dental or medical treatment within the previous three months (Cherubin, 1971).

If the only significant cause of bacteremias were invasive procedures, correct antibiotic prophylaxis should result in a decrease in the incidence of IE. However, although there has been an overall decrease in the morbidity and mortality rate, there has not been a decline in the incidence of IE since the pre-antibiotic era.

Furthermore, antibiotic prophylaxis is not without its own dangers. It has been concluded that if dental treatment was assumed to be routinely responsible for infective endocarditis and if all patients with mitral valve prolapse who are undergoing dental

procedures receive prophylactic penicillin, the risk of death would be greater from an anaphylactic reaction to penicillin than from endocarditis (Kaye 1986, Pallasch 1983, Pallasch 1986). This concern was supported by Seymore (2000) who calculated that patients receiving penicillin (amoxycillin) prophylaxis to prevent IE are five times more likely to die from an anaphylactic reaction to the drug than to die from contracting endocarditis.

Experimental Models

For this study to be clinically relevant, we selected a bacterial strain that is commonly found in dental infections, as well as being a common cause of infective endocarditis. Research has shown that *Streptococci* (including the species *faecalis*), is the largest single group of bacteria associated with infective endocarditis (Crawford and Russell 1986). *Enterococci*, one of the group D *Streptococci*, have been implicated in five to twenty percent of all cases of bacterial endocarditis (Wilkowske 1982, Kaye 1982, Megran 1992), and *Enterococcus faecalis* is the pathogen responsible for most enterococcal infections seen today (Tailor *et al.*, 1993). The role of bacteria in endodontic disease has been widely accepted (Kakehashi and associates,1965). *E. faecalis* has been discovered routinely in teeth with failed endodontic treatment (Sundqvist *et al.*, 1998), and has also been demonstrated in the canals of teeth undergoing endodontic therapy, despite the presence of calcium hydroxide (Dahlen 2000).

In today's research climate, it is difficult to conduct clinical studies using human subjects and pathogenic bacteria. For this reason, animal models have been designed to study infective endocarditis. Another benefit is that they allow research in the progression and dissemination of bacteria *in vivo*. As far back as 1885 Wyssokovitsch

developed a method of direct valve damage plus intravenous injection of bacteria (Gutschik 1984). This technique proved to be reliable and successful and there was no significant difference in susceptibility among the three commonly used laboratory animals (dogs, rabbits, and rats). The most successful animal model of endocarditis was described by Garrison and Freedman (1970) in rabbits, and modified by Durack (1972). In 1978, Santoro and Levison described a very popular and less expensive model in rats. All these models used a plastic-catheter induced non-bacterial thrombotic endocarditis (NBTE) to achieve left-sided infective endocarditis. Using a similar rabbit model, Bahn and associates (1978) created vegetative cardiac lesions and injected *Streptococci* into the soft tissues of the oral cavity, yielding a 94% incidence of infective endocarditis. Santoro's model (1978) involved passing polyethylene catheters, with the aid of a guide wire, into the left ventricle through the right carotid artery of Sprague-Dawley rats. One to two days later, a volume of *Streptococcus faecalis* was intravenously injected, consistently producing infective endocarditis.

In previous experiments, a wide range of bacterial concentrations has been used with the various animal models. In rabbits, the concentration of the inoculum necessary to cause infective endocarditis in 100% of the experimental animals (ID^{100}) has been reported to be 1×10^8 cfu (Gutschik and Christensen 1978). When enterococci is injected into rabbits, the amount of inoculum necessary to cause infective endocarditis in 50% of the experimental animals (ID^{50}) is reportedly $5 - 9 \times 10^6$ cfu (Durack *et al.*, 1977). For rats the amount of inoculum necessary to cause infective endocarditis in 90% of the test animals (ID^{90}) using enterococci in experimental models of antibiotic prophylaxis has

faecalis containing the *lux* gene and Cm^r cassette carried on a plasmid to cause infective endocarditis in a susceptible rat model.

The present study produced a damaged heart by using the rat model of Santoro and Levison (1978). Paired with low-light imaging and luminescent bacteria, this well-accepted model may provide an exceptional opportunity for the further study of the relationship between bacteremias, dentistry and infective endocarditis. The purpose of this study was to use this methodology to determine the minimum concentration of intravenously injected *Enterococcus faecalis* that is necessary to colonize a susceptible heart in the rat model.

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While previous studies have shown that presence of bacteria in systemic circulation can cause infective endocarditis, there is no information on the specific amount of bacteria necessary to cause this condition. The purpose of this study was to establish the minimum level of circulating bacteria that will cause colonization of damaged heart valves in rats. Fifty-two Sprague-Dawley rats were anesthetized and their heart valves were damaged using an established protocol. A clinical isolate of *Enterococcus faecalis* was transformed with a plasmid bearing the *luxF* and Chloramphenicol cassettes to label the bacteria with the light-emitting protein. After four weeks, the rats were re-anesthetized and intravenously injected with various concentrations of the labeled *E. faecalis* ranging from 1×10^2 to 1×10^8 cells in a 100 µl volume. The animals were sacrificed one week after infection and the hearts were excised and incubated overnight in brain heart infusion containing chloramphenicol. The presence or absence of luminescent bacteria was examined under a low light imager and recorded. All animals injected with 1×10^8 *E. faecalis* cells consistently demonstrated colonization of their heart valves. Variable incidence of colonization was also observed in animals injected with 1×10^3 to 1×10^7 cells. None of the rats injected with 5×10^2 or 1×10^2 bacterial cells demonstrated colonization of their heart valves. This study has established for the first time the lowest level of bacteria, which can cause colonization of damaged heart valves in rats.

INTRODUCTION

Despite many advances in medicine, infective endocarditis (IE) remains elusive to conventional preventive measures and treatment modalities. Gram-positive cocci, mainly streptococci and staphylococci, continue to cause the majority of cases of IE. Among the streptococci implicated in this infection, the long-standing predominance of oral or viridans-group streptococci has progressively faded. At the same time, the number of cases caused by "enteric streptococci" (*Streptococcus bovis* and enterococci) has increased. [1] Most of these oral streptococci, along with *S. bovis* strains can be managed using penicillin. Enterococci, however, are relatively resistant to penicillins and cephalosporins. Strains resistant to beta-lactams, glycopeptides and aminoglycosides have become more prevalent as well. [2]

Each year, the incidence of IE is 11 to 50 cases per million of population. Before the advent of antibiotics, the mortality rate from this disease condition was nearly 100%. Today, the mortality rate for endocarditis associated with the most common microbial isolates is under 10%, but increases when heart colonization results from less common microorganisms. [3]

Traditionally, invasive procedures have been considered as the only significant cause of bacteremias. Dental treatment has long been implicated as one pathway for introducing bacteria into the bloodstream. As far back as 1935, transient streptococcal bacteremia has been shown to occur after dental extractions. [4] Bacteremias, however, may result from physiological functions that are independent of procedural manipulations. Circulating bacteria may be detected in 5 out of 30 (16.7%) blood samples drawn from patients four minutes after tooth brushing. [5] Additionally,

bacteremias occur in 38% of patients after normal mastication, and in 11% of patients with oral sepsis in the absence of any intervention. [6] If the only significant cause of bacteremias were invasive procedures, correct antibiotic prophylaxis should result in a decrease in the incidence of IE. However, although there has been an overall decrease in the morbidity and mortality rate, there has not been a decline in the incidence of IE since the pre-antibiotic era. In fact, bacteremia of a random nature may be a more likely cause of IE than invasive procedures. [7]

A search of the literature revealed that while the occurrence of bacteremia has been extensively studied, little is known about the actual concentration of circulating bacteria that is necessary to colonize a susceptible heart. Many methodologies have been used to determine presence of bacteria in tissues, including Gram staining, observation of colony morphology and color, automated culture identification techniques, utilization of vital stains, polymerase chain reaction (PCR) and DNA hybridization, as well as labeling bacteria with light-emitting proteins. Bioluminescent technology, utilizing light-emitting proteins, relies on the use of fluorescent proteins and low light image analysis of luciferase-catalyzed luminescence. Luciferase catalyzes oxidation of reduced flavin mononucleotide (FMNH2) in the presence of decanal, to yield FMN, decanoic acid, water and a photon of light that can be measured at 490 nm. The emitted light can be captured by x-ray film, a photomultiplier tube or an image intensifier coupled to a video camera. [8] This technology makes possible identification and localization of the test bacteria used in the experiment.

The purpose of this study was to use this methodology to determine the minimum concentration of intravenously injected *Enterococcus faecalis* that is necessary to colonize a susceptible heart in the rat model.

MATERIALS AND METHODS

Bacterial strains. A clinical strain of *E. faecalis* (ATCC 4082) originally isolated from an endodontic infection was used in this study.

Plasmids. *E. faecalis* (ATCC 4082) were transformed with the plasmid pDC-luxF. The *luxF* gene was constructed by the fusion of *luxA* and *luxB* genes isolated from *Vibrio harveyi.* [9] In addition to this gene, this plasmid carries a chloramphenicol transacetylase expression cassette that allows selection of transformants.

Animals. Fifty-seven 300-gram male Sprague Dawley rats were used in this study. All animal experiments were carried out in accordance with a protocol approved by the Loma Linda University Institutional Animal Care and Use Committee. Animals infected with engineered bacteria were kept in the Loma Linda University animal care facility at biosafety level two.

DNA transformation of bacteria. For the transformation experiments, a pre-culture of *E. faecalis* in BYGT broth (brain heart infusion, yeast extract, glucose, Tris, pH 8) and 0.5% glycine was diluted 1:100 in pre-warmed BYGT media containing glycine. Cells were incubated at 37 °C for 90 minutes without agitation. The bacterial cells were then chilled on ice and harvested by centrifugation at 4 °C and washed twice with the electroporation solution (0.5 M sucrose, 10 mM $MgCl_2$, 10 mM $CaCl_2$, pH 4.0). The cells were then resuspended in 1/100 volume of electroporation solution and frozen

in liquid nitrogen in 100 µl aliquots or the competent cells were stored in –80 °C solution for future use.

For the electroporation experiments, 0.5 µg of DNA was added to 100 µl of bacterial cells. The cells were then electroporated using 2.5 kV, 25 µF capacitance, 200 Ω resistance yielding a time constant of 4.5 to 4.7 milliseconds. The cells were then incubated on ice for 2 minutes, and diluted in 1 ml BHI broth. After incubation in 37 °C for 120 minutes (in the absence of agitation), the cells were plated on THB plates containing 20 µg/ml chloramphenicol and incubated in 37 °C for 48 h.

Generation of rats with heart lesions. The rats were anesthetized via intraperitoneal injection with a combination of Ketamine HCl, 0.1mg/g (Abbott, Chicago, IL) and Xylazine, 0.01mg/g (AnaSed, Akorn Inc., Decatur, IL) mixed in the same syringe. Following a midline neck incision, the right carotid artery was exposed. The artery was carefully perforated with a 28.5 gauge sterile needle and a 3.5-cm long catheter was advanced towards the heart until resistance was met indicating intimate contact with the aortic valve. The catheter was then secured in place with 10-0 sutures (AROSurgical Instrument Corporation, Japan). The neck incision was closed with 4-0 silk sutures (American Cyanamide Company, Wayne, New Jersey).

Intravenous injection of bacteria. After four weeks, the rats were anesthetized via intraperitoneal injection with a combination of Ketamine and Xylazine. An incision was made to expose the right femoral vein. Bacteria in the mid-log phase in concentrations ranging from 1×10^8 to 1×10^2 (Figure 1) were intravenously injected in a 100 µl volume . The incisions were closed with 4-0 sutures.

Determination of bacterial concentration. Bacterial inoculum concentrations were determined using optical density measurement and confirmed with plating serial dilutions of the inoculum.

Low light imaging of the hearts. The animals were sacrificed one week after infection and the hearts were excised and placed in brain heart infusion media containing chloramphenicol and incubated overnight in a 37 °C incubator. The presence or absence of light emission was monitored under a low light imager (Argus 100, Hamamatsu, Hamamatsu, Japan) and recorded using the Imagepro software (Media Cybergenics, Silver Springs, MD).

RESULTS

Eight groups of catheterized Sprague-Dawley rats with simulated heart defects were used in this study (Table 1). The animals in group 1 served as the positive control. These animals were intravenously injected with a total amount of bacteria that has previously been shown to consistently cause IE. The remaining groups were intravenously injected with various concentrations of labeled *E. faecalis* ranging from 1 x 10^7 to 1 x 10^2. In group 1, all three of the animals (100%) injected with 1 x 10^8 *E. faecalis* cells demonstrated colonization of their hearts. Two of the three rats (66.7%) in group 2 that were injected with 1 x 10^7 bacteria developed heart colonization. Groups 3 and 4, injected respectively with 1 x 10^6 and 1 x 10^5 *E. faecalis*, each resulted in one animal (33.4%) with heart colonization. Group 5 was comprised of 12 rats, 5 of which developed bacterial heart colonization (41.7%) after being intravenously injected with 1 x 10^4 *E. faecalis*. In group 6, 2 of the 11 animals injected with 1 x 10^3 bacteria showed heart colonization (18.2%). Animals in groups 7 and 8 that had intravenous injections

respectively of 5 x 10^2 and 1 x 10^2 *E. faecalis* demonstrated no incidence of bacterial heart colonization.

DISCUSSION

The conventional concept of endocarditis is that susceptibility is usually the result of abnormal heart valves or endocardium and that bacteremia causes the infection. [10] Despite advances in antibacterial therapy, the incidence of infections caused by multidrug-resistant Gram-positive organisms is increasing. [11] While the mortality rate for endocarditis caused by alpha-hemolytic (viridans) streptococci has decreased, it is still 40% to 80% for endocarditis resulting from less common microorganisms. [3] Staphylococci and enterococci are two microbial isolates that may resist conventional treatment of endocarditis. Among the staphylococci causing endocarditis, the increasing proportion of coagulase-negative and methicillin-resistant strains observed in recent years has changed the approach to choice of antibiotic therapy. [1] *S. aureus* has frequently been implicated in cases where severe sepsis produced a high mortality rate. [12] Furthermore, a growing population of patients have HIV infection along with IE and a face high morbidity and mortality rate. For these patients, *Staphylococcus aureus* is the most common etiological agent. [13] Enterococci are a long known cause of bacterial endocarditis and a more recently recognized cause of nosocomial infection and superinfection. [14] Strains of this problematic pathogen have become resistant to vancomycin and teicoplanin and highly resistant to penicillins and aminoglycosides. [11] This presents an evolving problem for both clinicians and investigators. In today's research climate, it is difficult to conduct clinical studies using human subjects and

pathogenic bacteria. For this reason, animal models have been designed to study infective endocarditis.

A number of animal models have been utilized to simulate a heart that is susceptible to bacterial colonization. As far back as 1885 Wyssokovitsch developed a method of direct valve damage and intravenous injection of bacteria. [15] This technique proved to be reliable and successful and there was no significant difference in susceptibility among the three commonly used laboratory animals (dogs, rabbits, and rats). The most successful animal model of endocarditis was described by Garrison and Freedman [16] in rabbits, and modified by Durack. [17] In 1978, Santoro and Levison described a very popular and less expensive model in rats. [18] All of these models use a plastic-catheter induced non-bacterial thrombotic endocarditis (NBTE) to achieve left-sided infective endocarditis. Santoro and Levison's model involves passing polyethylene catheters, with the aid of a guide wire, into the left ventricle through the right carotid artery of Sprague-Dawley rats. The present study produced a damaged heart by using the well-accepted rat model of Santoro and Levison.

In previous experiments, a wide range of bacterial concentrations has been used with the various animal models. In rabbits, the concentration of the inoculum necessary to cause infective endocarditis in 100% of the experimental animals (ID^{100}) has been reported to be 1×10^8 cfu. [19] When enterococci is injected into rabbits, the amount of inoculum necessary to cause infective endocarditis in 50% of the experimental animals (ID^{50}) is reportedly $5 - 9 \times 10^6$ cfu. [20] For rats the amount of inoculum necessary to cause infective endocarditis in 90% of the test animals (ID^{90}) using enterococci in

experimental models of antibiotic prophylaxis has been 1×10^4 cfu. [21] In another rat model utilizing labeled *E. faecalis*, the ID^{100} was found to be 1×10^8. [22]

This study utilized an inoculum concentration of 1×10^8 injected intravenously into catheterized rats for its positive control group. The literature suggests that higher concentrations of intravenously injected bacteria should lead to colonization in a susceptible rat heart. Because the intent of this study was to demonstrate the minimum concentration that could result in colonization, a single animal showing heart colonization was considered reason to test the next lower bacterial concentration. This allowed us to conserve experimental animals by limiting the first four groups to three rats each.

Groups 5 through 8 contained more experimental animals. Previous studies have not focused on investigating the lower limit of bacterial inoculum concentration that can colonize a damaged heart. Therefore, it was reasonable to hypothesize that one of these groups may not readily demonstrate colonization and should be subject to a larger sample size. The negative control group was represented by five un-catheterized rats injected with a bacterial concentration of 1×10^8 *E. faecalis.* None of these animals showed evidence of heart colonization. Previous studies have shown that undamaged rat hearts are not subject to colonization regardless of the concentration of bacterial inoculum. [22]

A shortcoming in earlier animal models was the inability to prove that bacteria found in the heart lesions actually originated from the surgical (or inoculation) site. In the past, concerns over contamination have always haunted bacteriological studies. Recent advances in light emitting systems (containing the *lux* gene) give investigators a tool that reliably and safely labels bacteria. Current technology in the imaging of luciferase expression in living cells, tissues and organisms allows investigators to track

the dissemination of these labeled bacteria with certainty. Low light image analysis has allowed investigation of gene expression in bacteria, plant cells and mammalian cells. The major advantages of this technique include the system's sensitivity and safety for the investigator and the ability to study microorganisms in their natural environment through a real time measurement of gene expression. In vivo monitoring of bacterial infections [23] and optical measurement of neoplastic lesions [24] in living animals is possible using this technology. More recently, light-emitting bacteria and viruses have been used to identify solid tumors and metastases in live animals using low- light image analysis. [25]

Based on the results of this study, we have determined the minimum level of bacteria necessary to colonize a susceptible rat heart. The question of how much bacteria enters the bloodstream following various causative procedures and physiologic functions has not been well investigated and remains unanswered. One study showed a concentration of 3×10^3 bacteria/ml of blood after instrumentation of the root canal of a tooth beyond the apex during endodontic therapy in a human patient. [26] This is the equivalent to approximately 1×10^7 bacteria in the whole body, but proper antibiotic prophylaxis should protect the susceptible patient during these predictable bouts of bacteremia.

Findings show that oral streptococci cause approximately 50% of all infective endocarditis cases [27] whereas only 15% of patients diagnosed report dental or medical treatment within the previous three months. [28] An important observation of this study is the fact that very low numbers of bacteria are required in a bacteremia event to result in the colonization of a susceptible site. Therefore, colonization of susceptible sites may result from factors causing these low level bacteremias such as physiological function. In

fact, spontaneous bacteremias are more likely to cause IE in at risk patients than specific episodes of invasive treatment because cumulative exposures to bacteremias is significantly greater from everyday events than operative procedures. [7,29] Future studies need to explore the levels of bacteremia caused by different physiological functions or look at the effects of repetitive exposure to low levels of bacteremia. This model, paired with low-light imaging of luminescent bacteria may provide an exceptional opportunity for the further study of the relationship between bacteremias and infective endocarditis.

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Table 1. Catheterized rats with bacterial heart colonization after intra-venous injections with various amounts of *E. faecalis*.

Groups	1	2	3	4	5	6	7	8
Total Bacterial Injected	1×10^8	1×10^7	1×10^6	1×10^5	1×10^4	1×10^3	5×10^2	1×10^2
n =	3	3	3	3	12	11	9	8
Bacterial Heart Colonization	3	2	1	1	5	2	0	0

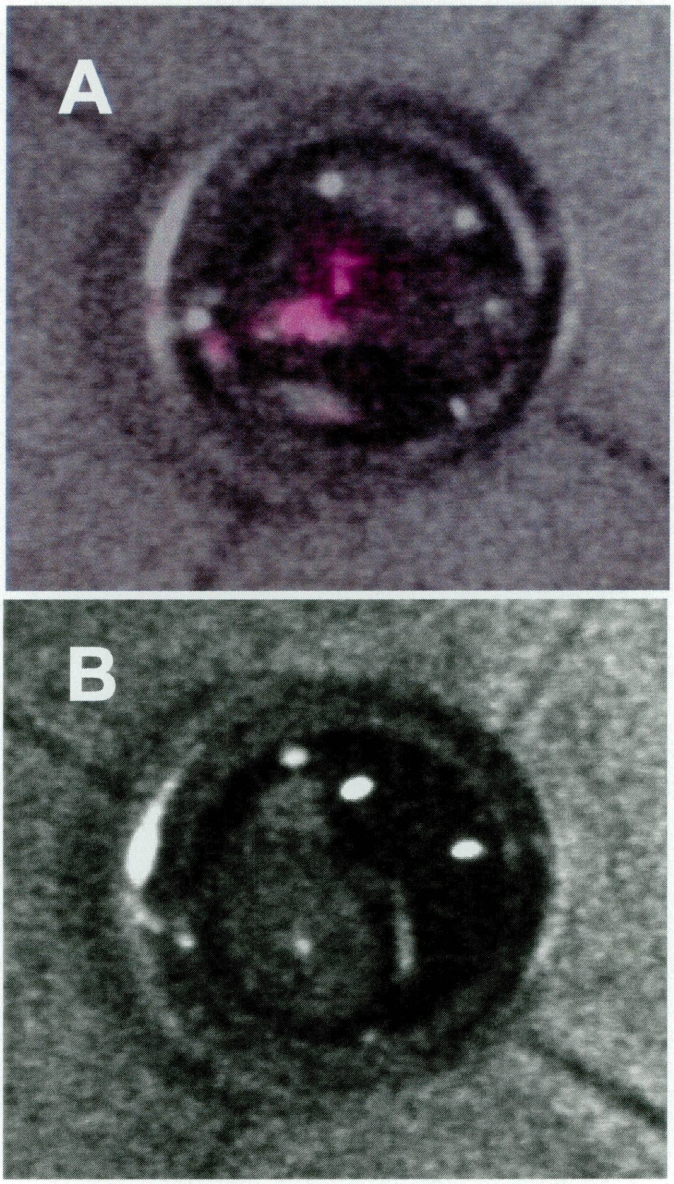

Figure 1. *A,* Excised catheterized heart clearly demonstrating light emission indicating the presence of luminescent bacteria colonizing the heart as visualized under the low light imager (photon collection for one minute). *B*, Excised non-catheterized heart demonstrating absence of any luminescent bacteria under the low light imager (photon collection for one minute).

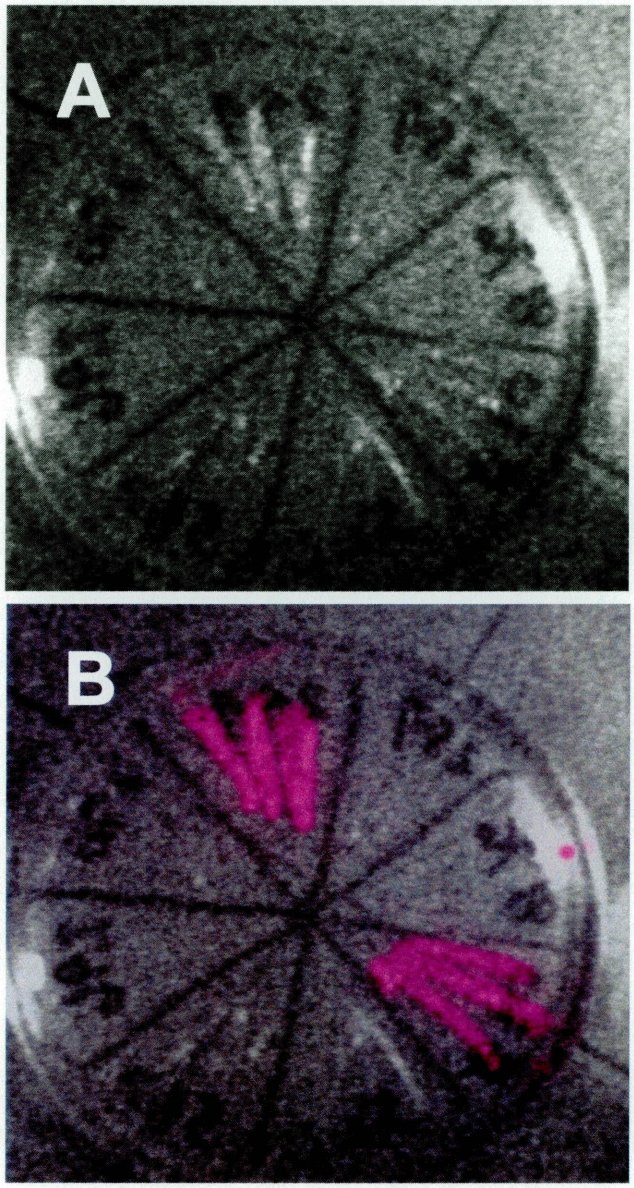

Figure 2. Localization of light emitting bacteria by superimposing the low light image over the photographic image. The cultured bacteria on selective media were incubated for 24 hours to visualize growth. *A,* Photographic image of cultured bacteria on selective media incubated for 24 hours. *B*, Superimposed image of collected photons over the photographic image.