A Study of Growth and Enzymatic Characteristics of Endodontic, Salivary and Fecal Isolates of Streptococcus Faecalis

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A STUDY OF GROWTH AND ENZYMATIC CHARACTERISTICS
OF ENDODONTIC, SALIVARY AND FECAL ISOLATES OF
STREPTOCOCCUS FAECALIS

by

Roy M. Naito, D.D.S.

A Thesis Submitted to the Faculty of the Graduate School
of Loyola University in Partial Fulfillment of
the Requirements for the Degree of
Master of Science

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AUTOBIOGRAPHY

Roy Mitsuaki Naito was born and raised in Kohala, Hawaii, on June 16, 1934. He is a graduate of Kohala High School.

In 1952 he entered the University of Hawaii and received the degree of Bachelor of Arts in zoology in 1956. He attended the University of Oregon, Eugene, Oregon, in 1956-1957 to complete his predental requirements.

He entered the Baltimore College of Dental Surgery, Dental School, University of Maryland in the fall of 1957 and graduated with the degree of Doctor of Dental Surgery in June, 1961.

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He currently holds the rank of Major, United States Army.
DEDICATION

To my parents, Kenichi and Yuriko Naito, whose many sacrifices to support a belief in the importance of education have helped to make my hopes a reality.
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# TABLE OF CONTENTS

<table>
<thead>
<tr>
<th>Chapter</th>
<th>Title</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>I.</td>
<td>INTRODUCTION</td>
<td>1</td>
</tr>
<tr>
<td>II.</td>
<td>REVIEW OF THE LITERATURE</td>
<td>3</td>
</tr>
<tr>
<td></td>
<td>A. Taxonomy</td>
<td></td>
</tr>
<tr>
<td></td>
<td>B. Characteristics of the Enterococci</td>
<td></td>
</tr>
<tr>
<td></td>
<td>C. Incidence</td>
<td></td>
</tr>
<tr>
<td></td>
<td>D. Resistance to Drugs and Medicaments</td>
<td></td>
</tr>
<tr>
<td></td>
<td>E. Pathogenicity</td>
<td></td>
</tr>
<tr>
<td></td>
<td>F. Factors Associated with Virulence</td>
<td></td>
</tr>
<tr>
<td>III.</td>
<td>MATERIALS AND METHODS</td>
<td>24</td>
</tr>
<tr>
<td></td>
<td>A. Materials</td>
<td></td>
</tr>
<tr>
<td></td>
<td>B. Collection of Samples</td>
<td></td>
</tr>
<tr>
<td></td>
<td>C. Bacteriological Laboratory Tests</td>
<td></td>
</tr>
<tr>
<td>IV.</td>
<td>FINDINGS</td>
<td>33</td>
</tr>
<tr>
<td></td>
<td>A. Hemolytic and Proteolytic Activity</td>
<td></td>
</tr>
<tr>
<td></td>
<td>B. Penicillin Sensitivity</td>
<td></td>
</tr>
<tr>
<td></td>
<td>C. Deoxyribonuclease</td>
<td></td>
</tr>
<tr>
<td></td>
<td>D. Hyaluronidase</td>
<td></td>
</tr>
<tr>
<td>V.</td>
<td>DISCUSSION</td>
<td>41</td>
</tr>
<tr>
<td>VI.</td>
<td>SUMMARY AND CONCLUSIONS</td>
<td>49</td>
</tr>
<tr>
<td></td>
<td>A. Summary</td>
<td></td>
</tr>
<tr>
<td></td>
<td>B. Conclusions</td>
<td></td>
</tr>
<tr>
<td>VII.</td>
<td>BIBLIOGRAPHY</td>
<td>52</td>
</tr>
</tbody>
</table>
LIST OF TABLES AND FIGURES

Tab. 1 Hemolytic and Proteolytic Activity .... 34
Tab. 2 Penicillin Sensitivity .................. 36
Tab. 3 Concentration and Percentage Light Transmission of Residual Hyaluronate Standard .................. 38
Fig. 1 Relationship Between Light Transmission and Turbidity Developed by Varying Concentrations of Hyaluronic Acid .................. 39
Tab. 4 Results of Test Reactions ............... 40
CHAPTER I

INTRODUCTION

The streptococci belonging to the enterococcus group consist of *Streptococcus faecalis* and its variants, *Streptococcus faecalis* var. *liquefaciens* and *Streptococcus faecalis* var. *zymogenes*; and *Streptococcus faecium* and its variant *Streptococcus durans*. They belong to Lancefield's group D and are often referred to as the Enterococci. These organisms are the predominant streptococci in the intestinal tract of man and some warm-blooded animals and are commonly found in fecal samples.

The enterococci have attracted widespread interest from the Public Health consideration as a possible index of fecal contamination of water, as a record of sanitary history of food products and as a potential pathogen when introduced into the tissues of the body.

The enterococci are characterized by their hardy growth characteristics and are known to survive far from their normal environment. They are resistant to many antibiotics and medicaments and can become a problem in medicine. They have been isolated from the oral cavity of man and have been reported by several investigators in the root canals of pati-
ents undergoing endodontic therapy. *Streptococcus faecalis* is the species most often encountered. Due to their growth characteristics and resistance to many drugs, they can become difficult to eradicate and thus become a problem in conservative endodontic therapy.

The pathogenicity of the enterococci is not clearly defined. They are considered to be of low virulence compared to other microorganisms that are classified as pathogens. In endodontic therapy it is often difficult to distinguish between the frank pathogen and the contaminant from saliva and those organisms that tend to persist in the root canal after several treatments must be considered to constitute the main threat as actual or potential pathogen.

A comparative study of some growth and enzymatic properties of *Streptococcus faecalis* isolated from root canals, saliva samples and fecal samples from patients undergoing endodontic therapy would provide some insight into the similarities or differences of the organisms obtained from the various sources. Some of the factors associated with the virulence of these organisms to be investigated include hemolysis of blood agar, proteolytic activity, penicillin sensitivity, and the production of the enzymes deoxyribonuclease and hyaluronidase.
CHAPTER II

REVIEW OF THE LITERATURE

A. Taxonomy

The first definitive scheme in the classification of the streptococci dates back to 1906 when Andrews and Horder differentiated seven groups of streptococci primarily on morphology, fermentative ability and growth characteristics in milk. The predominant streptococcus, isolated from human feces, was termed *Streptococcus faecalis* and was characterized by the active fermentation of several carbohydrates and abundant growth at 20°C (Diebel, 1964).

Crla-Jensen, in 1919, (Diebel, Lake and Niven, 1963) investigated the fermentative and heat resistant characteristics of fecal streptococci and proposed the separation of these streptococci into three species, *Streptococcus faecium*, *Streptococcus glycerinaceus* and *Streptococcus liquefaciens*.

In 1937, Sherman classified the facultative streptococci into four general groups: the pyogenic, the viridans, the lactic and the enterococcus group. The arrangement of the species into four groups was based on a series of common physiologic characteristics of the respective groups. The most important characteristic was the temperature limits of growth. The term enterococcus was employed in recognition
of Thiercelin who first used the term in 1899 in describing a streptococcus of fecal origin. Sherman's enterococcus group was characterized by growth at 10° C and 45° C, tolerance to sodium chloride, resistance to heat (60° C for 30 minutes), and its initiation of growth at pH 9.6. At first, four species were included in the enterococcus group. In a later study, Sherman (1938) observed the close relationship of Streptococcus faecalis, Streptococcus liquefaciens and Streptococcus zymogenes and a varietal status was accorded the latter two organisms since they differed only in their proteolytic and hemolytic activities. Streptococcus durans possessed a number of differing physiologic properties and it was considered that this organism was distinctive at a species level.

Skadhauge in 1950 (Diebel, Lake and Niven, 1963) observed that the enterococci could be separated into two groups on their ability to grow on an agar medium containing 0.04% potassium tellurite. The organisms that were able to grow on this medium included Streptococcus faecalis var. zymogenes and liquefaciens and some Streptococcus faecalis (Crla-Jensen's Streptococcus glycerinaceus and Streptococcus liquefaciens). Those organisms which failed to grow on the 0.04% potassium tellurite medium were Streptococcus faecium and Streptococcus durans.
In 1955, Shattock (Diebel, Lake and Niven, 1963) studied the physiological properties of 350 strains of fecal streptococci and claimed that *S. faecium* was distinctive at a species level.

Diebel, Lake and Niven (1963) in a study of the growth and fermentative activities of the enterococcus group proposed that *S. faecalis* and *S. faecium* be designated as two distinct species. *S. liquefaciens* and *S. zymogenes* differ from *S. faecalis* in their proteolytic and hemolytic activities and should be accorded a varietal status. *S. durans* differs from *S. faecium* in its inability to ferment arabinose and mannitol and should be considered as a variant. They recommended that the term "enterococcus" be used only when referring to *S. faecalis* and *S. faecium* and their varieties.

**B. Characteristics of the Enterococci**

The enterococci present many interesting characteristics and are endowed by unique physiological properties. In this group of streptococci there is a merging of the hemolytic and non-hemolytic strains in what appears to be an otherwise physiologically homogeneous group. The only known proteolytic streptococci is also found in this group. Motility in the streptococci has also been associated with the enterococcus group (Sherman, 1937).

Sero logically, all streptococci in Sherman's enterococcus
group contain the group D antigen. In addition to the enterococci, *Streptococcus bovis* and *Streptococcus equinus* also possess the group D antigen.

Breed, Murray and Smith (1957) in *Bergey's Manual of Determinative Bacteriology* present the following characteristics of the genus *Streptococci*:

"The cells are spheroid or ovoid, rarely elongated into rods, and occur in pairs or short chains. They are gram positive. Capsules are not discernible but may become conspicuous with some species under certain conditions. They are non-motile except a few strains in the enterococcus group. No pigments are produced with the exception of an occasional strain in Lancefield's group B and D, which may produce brick-red or yellow pigments under appropriate environmental conditions. A fermentable carbohydrate or polyhydroxy alcohol is necessary for satisfactory growth in artificial media. Growth in broth culture is variable in character. Growth on agar surface is usually scanty and the colonies are small, usually less than one millimeter in diameter. They are facultative with respect to oxygen requirement. Proteolytic strains are found only in the enterococcus group. All streptococci are fastidious with respect to their nutritional requirements. The streptococci are commonly found wherever organic matter containing sugars is accumulated. They occur regularly in the mouth and intestinal tract of man and other animals, in dairy and other food products and in fermenting plant juices."

The enterococcus group is characterized by Breed, Murray and Smith (1957) in accordance with Sherman's classification in the following manner:

Growth at 10° C and 45° C. Growth in 6.5 percent sodium chloride broth, at pH 9.6 and in 0.1 percent methylene blue milk. Tyrosine not decarboxylated. Lancefield Group D.
1. Not Beta hemolytic
   a. Gelatin not liquefied. *Streptococcus faecalis*.

2. Beta hemolytic
   a. Mannitol and sorbitol fermented. Litmus reduced before curdling milk. May or may not liquefy gelatin. *Streptococcus faecalis* var. *zymogenes*.

C. Incidence

Fecal Samples

The enterococci occur as part of the normal bacterial flora of the intestinal tract of man and some animals. Studies on the animal hosts reveal that variations in the incidence of enterococci in the intestinal flora occur with diet, age, geographical location and species of the animal. Seasonal changes have been noted as well as changes in the predominant species at different intervals. *Streptococcus bovis* and *Streptococcus equinus* are found in larger numbers than the enterococci in cattle, swine, horses and sheep. (Diebel, 1964).

Kenner, Clark and Kabler (1960) found the enterococci to be the predominant streptococci in human feces. Geographic variations in the incidence of enterococci species are found
in the human host and Shattock (1962) suggested the possible influence of diet on these variations (Diebel, 1964).

In the United States, Bartley and Slanetz (1960) observed a greater frequency of *S. faecalis* over *S. faecium*. Cooper and Ramadan (1955) in Great Britain, found *S. faecalis* in 69 percent of the cases.

Kjellander (1960) found enterococci of various types in 68 of 73 healthy individuals in Denmark. In 34 of the 68 samples (50 percent) it was *S. faecalis* and its variants. In France, Buttiaux (1958) reported a greater frequency of *S. faecium* (Diebel, 1964). Guthof (1957), in Germany observed a greater incidence of *S. faecium* in the adults. *S. faecalis* was found in larger numbers in children but the number decreased with increasing age (Diebel, 1964).

**Oral Cavity**

The enterococci have been demonstrated in the oral cavity in 4 - 34 percent of the patients studied. They have been isolated from saliva samples, deep carious lesions, subgingival scrapings, interproximal spaces and the tonsillar area by various investigators.

Williams, Forbes, Blau and Eickenberg (1950) reported the presence of enterococci in saliva samples in 45 of 206 (21.8%) patients. *S. faecalis* was the enterococci most commonly found (37 cases). *S. faecalis* var. *liquefaciens* was observed in 5
cases while *S. faecalis* var. *zymogenes* was found in 3 cases. They also found that when enterococci were present in the saliva there was likely to be a higher lactobacillus and lower yeast count.

Morris (1954) reported that enterococci were more common in saliva samples of patients with active caries as compared to saliva samples from patients clinically free of caries. Hugh, Klopp and Rysihenkov (1959) observed enterococci in 4 percent of 297 normal adults. They found enterococci in the saliva samples of 25 percent of the patients with ulcerated malignant neoplasms of the oral cavity and in 16 percent of the patients with neoplasms in other areas of the body. *S. faecalis* was the organism most frequently observed. The presence of enterococci in the oral cavity was not found to be directly correlated with achlorhydria, hypochlorhydria or the state of oral hygiene. Bahn, Shklair, Mazarella and Calandra (1960) in a study of the incidence of Group D streptococci found enterococci to be present in 70 of 303 (23.1%) dental clinic patients. In contrast, of 443 Naval recruits tested, only 3.6 percent demonstrated enterococci in the saliva. During an upper respiratory virus infection, 34.1 percent of 64 Naval recruits tested demonstrated group D streptococci in saliva samples. No correlation was made between the presence of enterococci and oral disease.
Burnett and Scherp (1951) investigated the bacterial flora of the deep advancing dentinal lesion and found that 90 percent of the bacteria consisted of gram positive cocci. Five of 8 strains studied in detail were found to be enterococci.

MacDonald, Socransky and Sawyer (1960) observed that the bacterial count of the periodontal flora of the rice rat increased with an increase in the severity of periodontal disease. The higher bacterial counts were attributed to an increased number of enterococci. Gibbons, Kapsimalis, Socransky, Sawyer and MacDonald (1964) found that 25 percent of the gram positive facultative cocci isolated from the periodontal pocket were enterococci. Rosan and Williams (1964) investigated the presence of hyaluronidase producing microorganisms from subgingival scrapings. They were able to recover enterococci which were found to be the organisms which produced hyaluronidase. Smith and Bodily (1968) were unable to isolate enterococci from subgingival scrapings and salivary samples of 24 normal and periodontally involved patients.

Engstrom (1964) recovered enterococci from the interproximal spaces in 8 of 26 cases (30.8%) and from the tonsillar area in 5 of 26 (19.2%) patients undergoing endodontic treatment.

**Root Canals**

Enterococci have been isolated from the root canals in
8 - 31 percent of the positive cultures. They have been recovered from non-vital teeth with intact pulp chambers, retreatments of root filled teeth and from teeth open to salivary contamination. Variations in the incidence of the enterococci may be due in part as to whether the cultures were taken on initial entry into the tooth or during the course of treatment.

Williams, Forbes, Blau and Eickenberg (1950) reported 14.2 percent of positive cultures from non-vital teeth to be enterococci. Engstrom and Frostell (1961) isolated enterococci in pure cultures from 2 of 21 (9.5%) non-vital teeth with intact pulp cavities on initial culture. Of these, one strain was *S. faecalis* var. *liquefaciens* and one strain was *S. faecalis* var. *zymogenes*.

Winkler and Van Amerongen (1959) in an analysis of the frequency of organisms isolated from 1,141 positive root canal cultures from vital and necrotic pulps found *S. faecalis* in 285 (20.0%) of the samples. Of these, 240 were observed in pure cultures and 45 were in mixed cultures. Culture samples used in this study included initial and treatment cultures. Streptococci were found to be the predominant bacterial type recovered from positive cultures. *S. faecalis* and *S. mitis* were the microorganisms most frequently encountered.
Melville and Slack (1961) reported an incidence of 8 percent of 695 microorganisms from 392 root canals to be fecal streptococci. Culture samples were taken during the course of treatment.

Engstrom (1964) studied the incidence and growth characteristics of enterococci isolated from infected root canals, tonsils and interproximal spaces to determine the source of the enterococci found in the root canals. Growth was obtained in 134 of 223 (60.1%) initial samples from teeth with non-vital pulps or previously root-filled teeth. Enterococci were isolated from 20 of the 134 (14.9%) initial positive cultures. In 17 cases S. faecalis was the organism isolated while in the other 3 cases it was S. faecalis var. liquefaciens. In 12 of 18 cases enterococci were isolated from the interproximal spaces while in 7 of 18 cases enterococci were also found in the tonsillar area. In all except one case the same bacterial type was demonstrated from the interproximal spaces, tonsils and root canals of the same patient.

Fox and Isenberg (1967) isolated enterococci in 84 of 381 (22%) positive root canal cultures. Vital and non-vital teeth were included in this study and cultures were taken before and after each treatment session. Five percent sodium hypochlorite solution was used to irrigate the root canal during treatment. Eugenol was used as an intracanal medi-
cament between appointments in vital teeth while PBSC, a polyantibiotic-antifungal suspension, was used as an intracanal medicament between appointments in non-vital teeth.

Myers and associates (1969) observed that 25.9 percent of the cases that demonstrated one negative culture after debridement of the root canal were found to be positive when another culture was taken immediately prior to filling the root canal. *S. faecalis* was isolated in 20.8 percent of the cases of a culture reversal.

Goldman and Pearson (1969) studied the post debridement bacterial flora of the root canal by taking a culture immediately following debridement of the root canal with files and irrigation with sodium hypochlorite. Enterococci were found in 36 percent of the vital cases and 27 percent of the non-vital teeth. They noted that the enterococci were the most persistent organisms encountered.

D. Resistance to Drugs and Medications

Resistance to Antibiotics

The resistance of some strains of enterococci to penicillin has been noted by numerous investigators. Fleming (1929), in his original paper on the inhibitory effect of penicillin on many species of bacteria, observed that enterococci were resistant to the drug.
Robbins and Thompsett (1949) observed that penicillin arrested all but 10 percent of the cases of subacute bacterial endocarditis. The majority of the cases failing to respond to treatment were caused by the enterococci belonging to group D, primarily *S. faecalis* and *S. faecalis* var. *zymogenes*. These organisms were found to be highly resistant to penicillin and also resistant to streptomycin.

Tulacek and Tilden (1947) studied the action of antibiotics on microorganisms isolated from the root canal and noted the high resistance of *S. faecalis* and *S. faecalis* var. *liquefaciens* to penicillin. They observed that one strain of *S. faecalis* was found to be four times as resistant to penicillin and streptomycin as any other streptococci. *S. faecalis* var. *liquefaciens* was found to be moderately sensitive to streptomycin.

Grossman and Christian (1952) used a serial dilution study to determine the bactericidal effect of antibiotics on microorganisms isolated from root canals. A high concentration of penicillin, 400u/cc., was required to exert a bactericidal effect on *S. faecalis*. They also found streptomycin to be practically ineffective against *S. faecalis*.

Engstrom (1964) studied the resistance patterns of 68 strains of enterococci from initial root canal samples, interproximal spaces and tonsils. He found all strains to
be resistant to sulphonamides, polymyxin and kanamycin. All strains were sensitive to erythromycin and chloramphenicol. Three percent of the strains were found to be sensitive to penicillin, 38 percent fairly sensitive, 53 percent slightly sensitive and 6 percent were resistant.

Fox and Isenberg (1967) found 4 of 84 enterococci strains isolated from root canals to be resistant to penicillin. They also noted the high resistance of the enterococci to streptomycin (40 cases). These findings are in close agreement to that of Goldman and Pearson (1969) who found that 5 percent of the non-hemolytic enterococci were resistant to penicillin and 41 percent were resistant to streptomycin.

**Resistance to Root Canal Medicaments**

The resistance of the enterococci to non-specific root canal medicaments can best be observed by the persistence of these organisms in the root canal after thorough debridement and the placement of intracanal medicaments.

Engstrom and Frostell (1964) reported enterococci in pure culture in 13 of 36 cases on the third culture appointment following thorough debridement and medication of the root canal. Enterococci were also recovered in pure cultures in 14 of 84 cases immediately prior to filling the root canal after a negative culture had been obtained at the previous appointment. A solution of 5 percent iodine in 10 percent
Potassium iodide was sealed into the root canals as an intracanal medicament between appointments.

Goldman and Pearson (1969) used sodium hypochlorite as an irrigating solution during debridement of the root canal. Camphorated paramonochlorophenol, a commonly used intracanal medicament, was sealed into the root canal between appointments. They found the enterococci to be the most persistent microorganism in the root canal. These organisms were found after three and four treatments following thorough debridement of the root canal.

Vikari (1969) investigated the minimal lethal dose of various medicaments against stock cultures of microorganisms commonly found in the root canal. A 1:9 dilution of one percent aqueous parachlorophenol was required to exert a bactericidal effect on S. faecalis. The minimal lethal dose endpoint for camphorated paramonochlorophenol was a 1:7 dilution. S. faecalis was found to be the most resistant test strain of the microorganisms studied. These findings were in close agreement to that of Harrison (1969) who found a 1:8 dilution of one percent aqueous parachlorophenol was required to exert a bactericidal effect on test strains of S. faecalis.

E. Pathogenicity

The enterococci do not have a clearly defined pathogenicity.
They may cause disease when introduced into the tissues, blood stream, urinary tract or meninges (Jawetz, Melnick, Adelberg, 1968).

Evans and Chinn (1947) studied the virulence of the enterococci by injecting mice intraperitoneally with broth cultures of the organisms. Two strains killed mice in $10^{-2}$ dilution, 22 killed mice in $10^{-1}$ dilution while 9 strains failed to kill in $10^{-1}$ dilution. The virulence of the enterococci for mice is low compared with the virulence of the other strains of streptococci of groups A and C.

MacDonald, Socransky and Sawyer (1960) noted that the bacterial count of the periodontal flora of the rice rat increased with the severity of periodontal disease. The higher bacterial counts were largely attributed to an increase in the numbers of enterococci. Cultures of enterococci obtained from the periodontal flora were injected subcutaneously into the groin of the rice rat. One animal died during the first 24 hours and had inflammation and adhesions at the site of infection. All but one of the 15 rice rats injected with viable cultures developed local abscesses which contained a small amount of thick exudate from which enterococci were recovered.

F. Factors Associated with Virulence
Hemolysis of Blood Agar

Many streptococci are able to hemolyze red blood cells in vitro in varying degrees. Beta hemolysis results from the complete disruption of the red blood cell and the release of hemoglobin. Incomplete hemolysis of the erythrocytes with the formation of green pigment is called alpha hemolysis. No disruption of the red blood cells is called gamma hemolysis. This characteristic is dependent on the ability of the microorganisms to produce hemolysins and is often used in the classification of the streptococci. The majority of the invasive beta hemolytic streptococci pathogenic for man fall into group A (Jawetz, Melnick and Adelberg, 1968).

The enterococci cause variable hemolysis. *S. faecalis* var. *zymogenes* causes a strong beta hemolysis and this characteristic is used to differentiate this organism from other strains of *S. faecalis*. Diebel, Lake and Niven (1963) observed that all strains of *S. faecalis* produced either weak greening in sheep's blood agar or they produced no change at all. In contrast, all strains of *S. faecium* produced a strong alpha hemolysis on sheep's blood agar.

Proteolytic Activity

The test for proteolytic activity of the members of the genus *Streptococcus* involves inoculation of the organisms in a broth media containing 5 percent gelatin. Those cul-
tures that form a gel when chilled in an ice water bath after incubation are considered to be non-proteolytic. Proteolytic activity by the members of the genus *Streptococcus* by this method has been limited to the enterococcus group. *S. faecalis* var. *liquefaciens* hydrolyzes gelatin and this characteristic has been used to differentiate it as a variant of *S. faecalis*. Some strains of *S. faecalis* var. *zymogenes* also demonstrate proteolytic activity.

Diebel (1963) observed that anaerobic conditions enhanced the proteolytic activity of the more virulent group A streptococci. Unlike the group A streptococci, *S. faecalis* var. *liquefaciens* will actively hydrolyze gelatin under aerobic conditions.

**Deoxyribonuclease**

Deoxyribonuclease is an enzyme which depolymerizes deoxyribonucleoprotein and deoxyribonucleic acid. Furulent exudates owe their viscosity largely to deoxyribonucleoprotein (Jawetz, Melnick and Adelberg 1968). The enzyme is produced by certain microorganisms and is effective in hydrolyzing the nucleic acids and nucleoproteins released by necrotic cells. The products of hydrolysis are in turn utilized by the microorganisms (Dubos and Hirsch, 1965).

McCarty (1948) demonstrated the production of deoxyribonuclease as well as ribonuclease in all strains of group A
streptococci tested. Brown (1950) observed that deoxyribonuclease production was commonly found among the group A, B and C streptococci. He tested 12 strains of *S. faecalis*, 3 strains of *S. faecalis* var. *liquefaciens* and 23 strains of *S. faecalis* var. *zymogenes* and found none to produce deoxyribonuclease.

Smith and Bodily (1968) reported desxyribonuclease production in 5 of 16 strains of enterococci of fecal origin.

**Hyaluronidase**

The enzyme hyaluronidase is involved in the hydrolysis of hyaluronic acid, an important component of the ground substance of connective tissue. This enzyme thus aids in spreading infecting microorganisms (Jawetz, Melnick and Adelberg, 1968). Hyaluronidase production by the streptococci has been associated with the members of group A. It has also been detected by certain strains of group B, C and G streptococci (Dubos and Hirsch, 1965).

Most of the studies on hyaluronidase production by streptococci has been associated with the hemolytic streptococci, primarily those belonging to group A. Hale (1938) observed that hyaluronidase produced by the streptococci was more sensitive to pH than the enzyme produced by staphylococci and *Clostridium*. Streptococcal hyaluronidase was rapidly inactivated at pH 4.6 but was stable at pH 7.0. Meyer,
Chaffee, Hobby and Dawson (1941) found that not all strains of hemolytic streptococci produced hyaluronidase and that the production and activity of the enzyme was variable. Rogers (1945) studied conditions controlling the production of the enzyme by organisms grown in simplified media and found that hyaluronidase production by group A and C streptococcci and *Clostridium welchii* was an adaptive response and produced the enzyme in proportion to the amount of hyaluronic acid in the buffered growth medium. Staphylococcci and *Clostridium septicum* did not respond to the inclusion of hyaluronate in the growth medium.

Pike (1948) observed that some strains of group A streptococcci did not produce significant amounts of the enzyme in 24 hours but on prolonged incubation for 7 days sufficient hyaluronidase was produced to completely hydrolyze the hyaluronic acid present in the culture medium.

Lisanti (1950) used the viscosimetric technique and demonstrated hyaluronidase activity in unstimulated saliva in 48 of 64 patients. Mahler and Lisanti (1952) found *Streptococcus mitis* to be the predominant hyaluronidase producers isolated from unstimulated saliva. Cannulated saliva showed no hyaluronidase activity. Not all of the organisms produced the enzyme. The enzyme producing strains of *S. mitis* were recovered most easily from patients with perio-
dental disease and upper respiratory infections.

Schultz-Haudt and Scherp (1955) observed that hyaluronidase was produced by 26 of 56 strains of *S. mitis* and 7 of 15 strains of *S. Salivarius* isolated from patients with marginal gingivitis. Hyaluronidase production in these isolates was tested by growing the microorganisms in a broth free of the substrate. Ten strains that did not produce the enzyme constitutively were then grown in a broth medium with hyaluronic acid. It was found that the 10 strains that did not produce the enzyme constitutively did not produce the enzyme adaptively.

Smith, Thomassen and Sweet (1958) studied the relationship between infection and pathology in the pulp canal and periapical region of 96 cases of intact, devitalized teeth. Culture samples were taken on initial entry into these teeth. The microorganisms were grown in a broth free of hyaluronic acid. Supernates of centrifuged broth cultures were added to an aqueous solution containing a known concentration of hyaluronic acid. After incubation turbidities were developed and read on a Coleman spectrophotometer. Twenty-five of 95 microorganisms isolated produced hyaluronidase. No attempt was made to identify the microorganisms.

Rosan and Williams (1964) investigated cultures of streptococci obtained from subgingival scrapings of patients
with periodontal disease for hyaluronidase production. The enterococci were the only streptococci found to produce the enzyme. All 15 strains producing hyaluronidase were identified as *S. faecalis*. Seven strains identified as *S. faecalis* did not produce hyaluronidase. The procedure used in this study was to inoculate cultures of the organism into a broth containing a known concentration of hyaluronate. It was observed that the substrate was hydrolyzed on prolonged incubation. The amount of hyaluronidase produced by the strains of *S. faecalis* was less than that produced by some strains of group A streptococci. It was observed that microorganisms incubated in a broth medium free of the substrate did not produce the enzyme.
CHAPTER III

MATERIALS AND METHODS

A. Materials

The materials used in this study consisted of 130 positive root canal cultures taken during the course of endodontic treatment at the Endodontic Clinic, Loyola University Dental School. All samples were obtained from patients who were not undergoing antibiotic therapy.

The following media were used and prepared according to manufacturer's instructions*: thioglycollate broth Brewer modified, SF broth and agar (prepared by adding 1.5% agar to SF broth), brain heart infusion broth and agar, trypticase soy broth and agar, nutrient gelatin and DNase test agar. A 6.5% sodium chloride broth was prepared by adding 6.5% sodium chloride to a base of trypticase soy broth. Blood agar was prepared by aseptically adding 5% sterile, defibrinated sheep's blood to trypticase soy agar.

Hyaluronidase activity was determined by using the purified sodium salt of hyaluronic acid from human umbilical cord** in brain heart infusion broth. Penicillin sensitivity

* Baltimore Biological Laboratories; Cockeysville, Maryland
** Sigma Chemical Company; St. Louis, Missouri
was tested by using 2 and 10 units of penicillin G, dispens-o-disc*.

All media used in this study were freshly prepared to avoid contamination or chemical changes. Bacterial cultures were used only after a new 24 hour broth incubation period to assure viable cultures.

The equipment and media used were sterilized before use in a steam autoclave for 20 minutes at 121°C temperature and 15 pounds per square inch pressure. Indicator tapes were used to assure absolute sterility.

A Coleman spectrophotometer** was used to accomplish the turbidimetric analysis of residual hyaluronate.

B. Collection of Samples

Endodontic culture specimen were collected by using an aseptic technique. The tooth undergoing treatment was first cleaned by using pumice on a polishing cup. A rubber dam was used to isolate the tooth and the field of operation was disinfected with Bactine*** on a sterile cotton applicator. After removal of the temporary seal the operating field was again disinfected with Bactine. A sterile paper point was intro-

*Difco Laboratories; Detroit, Michigan

**Coleman, Junior II Spectrophotometer, Model 6/35, The Coleman Co.; Maywood, Illinois

***Miles Laboratories, Inc.; Elkhart, Indiana
duced into the root canal for 30 seconds and transferred to 12 ml of thioglycollate broth in a screw capped tube.

All root canal cultures were incubated aerobically at 37° C and observed daily for seven days. At the first sign of turbidity or growth one loopful of the endodontic culture was streaked on to SF agar. The plates were then incubated aerobically at 37° C for 24 to 48 hours. If growth occurred on the SF agar, gram stains were made of the endodontic culture to look for pure cultures of streptococci. SF agar is a selective medium for \textit{S. faecalis}. The sodium azide inhibits growth of organisms other than \textit{S. faecalis} and its variants and these microorganisms grow as small, bright yellow colonies with reduction of the brom cresol purple indicator.

Colonies growing on the SF agar from a pure culture of \textit{S. faecalis} from the root canal were then transferred to a brain heart infusion agar slant. The brain heart infusion agar slant was then incubated at 37° C for 48 hours and stored at 8° C until a sufficient number of samples were obtained to conduct bacteriological tests.

Saliva samples were obtained from those patients with pure cultures of \textit{S. faecalis} in the root canal. Five milliliters of unstimulated saliva were collected in a sterile test tube. One-half milliliter was then inoculated into the SF broth and incubated at 37° C for 48 hours. Growth was
indicated by reduction of the brom cresol purple indicator. One loopful of SF broth was then streaked on to SF agar to isolate individual colonies.

Fecal samples were obtained whenever possible. A small amount of feces was placed in the SF broth and incubated at 37°C for 48 hours. One loopful of SF broth was then streaked on to SF agar to isolate individual colonies.

Colonies of *S. faecalis* were then transferred from the SF agar to brain heart infusion agar slants, incubated at 37°C for 48 hours, and stored at 8°C until a sufficient number of samples were collected to conduct bacteriological tests.

**C. Bacteriological Laboratory Tests**

Bacteriological laboratory tests to confirm the presence of *S. faecalis* and its variants included growth in 6.5% sodium chloride and survival at 60°C for 30 minutes. Growth in 6.5% sodium chloride was conducted by inoculating one loopful of microorganisms from a 24 hour broth culture in a trypticase soy broth with 6.5% sodium chloride. The tubes were then incubated at 37°C for 48 hours and growth was indicated by turbidity or sedimentation. Survival at 60°C for 30 minutes was conducted by placing a 48 hour broth culture of *S. faecalis* in trypticase soy broth into a 60°C water bath for 30 minutes. One loopful was then streaked
on to brain heart infusion agar and the plates were incubated to verify survival of the microorganisms.

Identification of the variants of *S. faecalis* was determined by hemolytic and proteolytic activity. Blood agar plates, prepared by aseptically adding 5% sterile, defibrinated sheep's blood to trypticase soy agar, were used to demonstrate hemolytic activity. Type of hemolysis was noted at 24 and 48 hours of incubation. Proteolytic activity was observed on nutrient gelatin tubes. The tubes were inoculated and incubated aerobically at 37° C for 72 hours. The nutrient gelatin tubes were then immersed in an ice water bath for 30 minutes. Positive gelatinase activity was noted when the nutrient gelatin failed to resolidify.

The microorganisms were then classified in the following manner:

1. Not beta hemolytic
   a. Gelatin not liquefied. *S. faecalis*
   b. Gelatin liquefied. *S. faecalis* var. *liquefaciens*.

2. Beta hemolytic
   a. May or may not liquefy gelatin. *S. faecalis* var. *zymogenes*.

Sensitivity to penicillin was observed by "mopping" a brain heart infusion agar plate with a viable, 24 hour old broth culture of the microorganism and placing 2 and 10 unit
discs of penicillin G on the surface of the agar. The plates were then incubated at 37° C and zones of inhibition around the antibiotic discs were noted at 24 and 48 hours. Sensitivity of the microorganisms to penicillin was rated in the following manner:

1. Sensitive- zones of inhibition on high and low concentrations.
2. Slightly sensitive- zone of inhibition on high concentration only.
3. Resistant- no zone of inhibition.

The test for deoxyribonuclease activity was conducted by inoculating plates of DNase test agar with a one inch surface streak of viable 24 hour broth culture of the microorganism. The plates were then incubated at 37° C for 48 hours and developed by flooding with 1 N hydrochloric acid. Deoxyribonuclease positive cultures were indicated by a clear zone around the streak. The precipitation of deoxyribonucleic acid by 1 N hydrochloric acid results in a cloudy area around the streak in those organisms that do not produce the enzyme deoxyribonuclease.

The turbidimetric assay method used to determine the presence of hyaluronidase was modified from that described by Tolksdorf and colleagues (1949). The method was first described by Kass and Seastone (1944) and is based on the
observation that pure hyaluronate at pH of 4.2 gives a fairly stable colloidal suspension with dilute serum, while depolymerized hyaluronate remains clear. The method is reproducible to about 10 percent (Meyer, 1947).

The sodium salt of hyaluronic acid was weighed in an analytical balance* and dissolved in the previously autoclaved brain heart infusion broth so that a concentration of 0.4 mg of hyaluronate in 1 ml of brain heart infusion broth was obtained. The broth was then filtered through a millipore filter** and kept at a temperature of 5°C during the experimental procedure.

Two buffer solutions were prepared as described by Tolksdorff et al. (1949). One-tenth molar acetate buffer pH 6.0 was prepared by combining 3 ml of 0.5 M acetic acid, 97 ml of 0.5 M sodium acetate and 4.384 Gm of sodium chloride and diluting to 500 ml with water. This gave a concentration of 0.15 M sodium chloride. One-half molar acetate buffer pH 4.2 was prepared by combining 130 ml of 0.5 M sodium acetate with 370 ml of 0.5 M acetic acid. The pH of the buffer solutions were checked before use and adjusted when necessary.

Acidified 10% horse serum, pH 3.1 was prepared by add-

*Analytical Balance, Model No. 100 (200 Gm capacity) Voland Corp.; New Rochelle, New York

**Millipore Corporation; Bedford, Mass.
ing 1 N hydrochloric acid to normal horse serum* until the pH was brought to 3.1 and brought to volume with water. The acidified horse serum was prepared immediately prior to use.

One-tenth milliliter of viable, 24 hour broth cultures of enterococci obtained from the various sources was inoculated into 5 ml of brain heart infusion broth, and incubated at 37° C. One milliliter samples were removed daily over a period of 4 days.

The broth culture samples removed daily were centrifuged at 2400 revolutions per minute. One-half milliliter of supernates of centrifuged broth cultures were removed and added to tubes with 0.5 ml of brain heart infusion broth containing 0.2 mg of sodium hyaluronate. Control tubes containing 0.2 mg, 0.15 mg, 0.10 mg, 0.05 mg and no hyaluronic acid were set up and brought to 1.0 ml with uninoculated, incubated brain heart infusion broth. One milliliter of 0.1 M acetate buffer pH 6.0 was added to each tube. All tubes were then incubated at 37° C for 45 minutes. During incubation the hyaluronidase, if present, would hydrolyze the substrate.

After incubation the tubes were placed in a 60° C water bath for 10 minutes to inactivate the enzymatic process.

*Grand Island Biological Co.; Grand Island, New York.
The tubes were then allowed to cool to room temperature. Turbidities were developed by adding 6 ml of 0.5 M acetate buffer pH 4.2 and 2 ml of acidified 10% horse serum pH 3.1 to each tube. The tubes were allowed to stand for 15 minutes before readings were taken.

The turbidities were developed in proportion to the amount of hyaluronic acid remaining in each tube and were read by means of a Coleman spectrophotometer. Standards were established by using control tubes with varying concentrations of hyaluronic acid. The 100% light transmission reading was set by using the control tube with no hyaluronic acid at the wavelength of 550 m\(\text{m}\). Spectrophotometer readings were made with control tubes containing 0.2 mg, 0.15 mg, 0.10 mg and 0.05 mg of hyaluronic acid. The residual hyaluronic acid concentration of the samples were then determined by spectrophotometer readings and comparison to the standards established by the controls.
CHAPTER IV

FINDINGS

Enterococci were isolated in pure cultures from 22 of 130 positive root canal cultures (16.9%) obtained during the course of endodontic treatment. Of the 22 patients, isolates of \textit{S. faecalis} and its variants were found in 13 saliva samples (59.1%). Fecal samples were obtained from 5 of these patients. A total of 40 samples were used for this study.

A. Hemolytic and Proteolytic Activity.

Hemolytic and proteolytic activity were used to differentiate the variants of \textit{S. faecalis}. All 40 samples demonstrated gamma hemolysis on 5% sheep's blood agar. Isolates with gamma hemolysis and no gelatinase activity were classified as \textit{S. faecalis}. Isolates demonstrating gamma hemolysis and positive gelatinase activity were identified as \textit{S. faecalis} var. \textit{liquefaciens}.

A total of 27 strains were found to be \textit{S. faecalis}. Of these, 16 were isolated from root canals, 9 from saliva samples and 2 strains from fecal samples. Thirteen of the 40 strains isolated were identified as \textit{S. faecalis} var. \textit{liquefaciens}. Of these isolates, 6 were found in the root canals, 4 were from saliva samples and 3 were from fecal samples. There were no strains of \textit{S. faecalis} var. \textit{zymogenes} among
<table>
<thead>
<tr>
<th></th>
<th>Total</th>
<th>Endodontic</th>
<th>Salivary</th>
<th>Fecal</th>
</tr>
</thead>
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<tr>
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<td></td>
<td></td>
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<td>0</td>
</tr>
<tr>
<td>Gamma Hemolysis</td>
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<tr>
<td><strong>Proteolytic Activity</strong></td>
<td></td>
<td></td>
<td></td>
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</tr>
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<td>Gelatinase Positive</td>
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<td>6</td>
<td>4</td>
<td>3</td>
</tr>
<tr>
<td><strong>S. faecalis</strong></td>
<td>27</td>
<td>16</td>
<td>9</td>
<td>2</td>
</tr>
<tr>
<td><strong>S. faecalis var. liquefaciens</strong></td>
<td>13</td>
<td>6</td>
<td>4</td>
<td>3</td>
</tr>
</tbody>
</table>
the isolates.

The same bacterial type in respect to hemolytic and proteolytic activity was found in the root canal and saliva in 11 of 13 cases (84.6%). In 4 of 5 cases, the same bacterial type was isolated from the root canal, saliva and fecal samples.

B. Penicillin Sensitivity

A high degree of resistance to penicillin was noted among the strains of _S. faecalis_ and _S. faecalis_ var. _liquefaciens_ isolated from the various sources. Thirteen of 40 strains (32.5%) were found to be resistant to penicillin, 23 strains were slightly sensitive and 4 strains were sensitive to penicillin. Eight strains of _S. faecalis_ were found to be resistant to penicillin, 16 strains were slightly sensitive and 3 strains were sensitive to penicillin. Five strains of _S. faecalis_ var. _liquefaciens_ were found to be resistant to penicillin, 7 strains were slightly sensitive while 1 strain was found to be sensitive.

Isolates of _S. faecalis_ and _S. faecalis_ var. _liquefaciens_ from saliva and fecal samples showed a greater number of resistant strains as compared to isolates of _S. faecalis_ and _S. faecalis_ var. _liquefaciens_ from the root canals.

C. Deoxyribonuclease Activity

None of the isolates of _S. faecalis_ or _S. faecalis_ var.
### TABLE 2

**PENICILLIN SENSITIVITY**

<table>
<thead>
<tr>
<th>Penicillin Sensitivity</th>
<th>Total</th>
<th>Endodontic</th>
<th>Salivary</th>
<th>Fecal</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sensitive</td>
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<td>3</td>
<td>1</td>
<td>0</td>
</tr>
<tr>
<td>Slightly Sensitive</td>
<td>23</td>
<td>15</td>
<td>6</td>
<td>2</td>
</tr>
<tr>
<td>Resistant</td>
<td>13</td>
<td>4</td>
<td>6</td>
<td>3</td>
</tr>
</tbody>
</table>

**S. faecalis**

<table>
<thead>
<tr>
<th>Penicillin Sensitivity</th>
<th>Total</th>
<th>Endodontic</th>
<th>Salivary</th>
<th>Fecal</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sensitive</td>
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<td>2</td>
<td>1</td>
<td>0</td>
</tr>
<tr>
<td>Slightly Sensitive</td>
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<td>10</td>
<td>5</td>
<td>1</td>
</tr>
<tr>
<td>Resistant</td>
<td>8</td>
<td>4</td>
<td>3</td>
<td>1</td>
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</tbody>
</table>

**S. faecalis var. liquefaciens**

<table>
<thead>
<tr>
<th>Penicillin Sensitivity</th>
<th>Total</th>
<th>Endodontic</th>
<th>Salivary</th>
<th>Fecal</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sensitive</td>
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<td>1</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Slightly Sensitive</td>
<td>7</td>
<td>5</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>Resistant</td>
<td>5</td>
<td>0</td>
<td>3</td>
<td>2</td>
</tr>
</tbody>
</table>
Liquefaciens from endodontic, salivary and fecal samples were found to produce the enzyme deoxyribonuclease. Two strains of Staphylococcus aureus were used as controls and these microorganisms demonstrated hydrolysis of deoxyribonucleic acid on the DNase test agar.

D. Hyaluronidase Activity

None of the isolates of S. faecalis or S. faecalis var. liquefaciens from endodontic, salivary and fecal samples produced any measurable amount of the enzyme hyaluronidase when grown in a brain heart infusion broth medium free of the substrate. Two strains of Staphylococcus aureus were used as controls and both microorganisms produced a varying amount of hyaluronidase over the four day test period.
TABLE 3

CONCENTRATION AND PERCENTAGE LIGHT TRANSMISSION
OF RESIDUAL HYALURONATE STANDARDS

<table>
<thead>
<tr>
<th>Concentration mg/ml</th>
<th>0.4 mg/ml BHI Hyaluronate</th>
<th>BHI Broth</th>
<th>Percent Light Transmission</th>
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</thead>
<tbody>
<tr>
<td>0.2</td>
<td>0.5 ml</td>
<td>0.5 ml</td>
<td>11</td>
</tr>
<tr>
<td>0.15</td>
<td>0.375 ml</td>
<td>0.625 ml</td>
<td>22</td>
</tr>
<tr>
<td>0.10</td>
<td>0.25 ml</td>
<td>0.75 ml</td>
<td>37</td>
</tr>
<tr>
<td>0.05</td>
<td>0.125 ml</td>
<td>0.875 ml</td>
<td>63</td>
</tr>
<tr>
<td>0</td>
<td>---</td>
<td>1.0 ml</td>
<td>100</td>
</tr>
</tbody>
</table>
FIGURE 1

RELATIONSHIP BETWEEN LIGHT TRANSMISSION AND TURBIDITY DEVELOPED BY VARYING CONCENTRATIONS OF HYALURONIC ACID

CONCENTRATION OF HYALURONIC ACID (mg/mL)

PERCENT LIGHT TRANSMISSION AT 550 nm
<table>
<thead>
<tr>
<th></th>
<th>Total</th>
<th>Endodontic</th>
<th>Salivary</th>
<th>Fecal</th>
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<td><strong>Hemolysis</strong></td>
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<td>0</td>
</tr>
<tr>
<td>Gamma</td>
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<td>5</td>
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<tr>
<td><strong>Proteolysis</strong></td>
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<tr>
<td>Gelatinase Negative</td>
<td>27</td>
<td>16</td>
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<td>2</td>
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<tr>
<td>Gelatinase Positive</td>
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<td>6</td>
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<td>3</td>
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<td><strong>Penicillin Sensitivity</strong></td>
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<td></td>
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<tr>
<td>Sensitive</td>
<td>4</td>
<td>3</td>
<td>1</td>
<td>0</td>
</tr>
<tr>
<td>Slightly Sensitive</td>
<td>23</td>
<td>15</td>
<td>6</td>
<td>2</td>
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<tr>
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<td>6</td>
<td>3</td>
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<td><strong>Deoxyribonuclease</strong></td>
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<td><strong>Hyaluronidase</strong></td>
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<td>Negative</td>
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<td>Positive</td>
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</table>
CHAPTER V

DISCUSSION

This investigation represents the results of 130 positive root canal cultures over a period of 6 months at the Endodontic Clinic of Loyola University Dental School. Irrigating solutions used during the course of endodontic treatment included hydrogen peroxide, 5% sodium hypochlorite and saline. Camphorated paramonochlorophenol was the intracanal medication most often used. The cultures were taken by graduate and undergraduate students and the procedures followed were specified in the clinical endodontic syllabus. All culture samples were inoculated in thioglycollate medium which promoted excellent growth of _S. faecalis_ and its variants.

Enterococci were isolated in pure cultures in 22 of 130 patients (16.9%) during the course of endodontic treatment. Each incidence of pure culture of enterococci was counted once although it was observed that treatment was often prolonged and 3 or 4 culture and medication appointments were often necessary before a negative culture was obtained.

The high incidence and persistence of enterococci in root canals were reported by other investigators. Winkler and Van Amerongen (1959), Myers, et al. (1969) and Goldman and Pearson
(1969) reported incidence of 20 - 31%. They also reported that it was not uncommon to find enterococci in pure or mixed cultures in 3 - 4 consecutive cultures.

The persistence of these microorganisms and their resistance to medications commonly used in endodontic therapy can create a problem in conservative endodontic therapy and often results in prolonged treatment. Hedman (1951) obtained initial cultures of the root canal and periapical area of non-vital teeth with periapical radiolucency. He found that 68.5% had viable bacteria in the pulp canal and periapical area. When a negative culture was obtained from the root canal a negative culture was also obtained from the periapical tissues. It was also observed that all cases that had streptococci in the root canal had streptococci in the periapical tissues. No attempt was made to determine the type of streptococci. The persistence of the enterococci in the root canal then becomes significant since it gives the microorganism an opportunity to become established in the periapical tissues.

The enterococci occur as part of the normal flora of the oral cavity in 4 - 34 percent of the patients. Williams, Forbes, Blau and Eickenberg (1950) observed that when enterococci were found in saliva there was likely to be a higher lactobacillus and lower yeast count. Morris (1954) found
enterococci more common in the saliva of patients with active caries as compared to patients free of caries. This finding supports that of Williams, et al. (1950) since a high lactobacillus count is directly correlated with open carious lesions. Burnett and Scherp (1951) identified enterococci in deep advancing carious dentin.

Eighty percent of all endodontic cases are due to carious exposure of the dental pulp (Sommer, Ostrander and Crowley, 1966). It becomes apparent that the enterococci can become established in the root canal from the carious exposure of the dental pulp or as a contaminant from saliva during endodontic treatment. This and the persistence of these microorganisms may account for the high incidence of enterococci in the root canal.

Enterococci were isolated from saliva samples of 13 of 22 patients (59.1%) demonstrating \textit{S. faecalis} or its variant in the root canal. In 11 of the 13 cases where enterococci were isolated from the root canal and saliva, the same bacterial type was recovered from both sources. Engstrom (1964) isolated enterococci from the interproximal spaces of 30.8% of the patients with enterococci in initial root canal cultures. In all except one case the same bacterial type was recovered from the root canal, interproximal spaces and tonsillar area. From these results it appears that there is a
close correlation between the occurrence of enterococci in the saliva and in the root canal.

In 4 of 5 cases the same bacterial type was isolated from the root canal, saliva and fecal samples. Fecal samples were obtained from 5 of 22 patients in this study. \textit{S. faecalis} or \textit{S. faecalis var. liquefaciens} was isolated from all 5 fecal samples.

All 40 strains of enterococci isolated from the various sources demonstrated gamma hemolysis on 5\% sheep's blood agar. Hemolytic activity among the enterococci is a variable characteristic. Diebel, Lake and Niven (1963) observed that all strains of \textit{S. faecalis} produced either weak greening in 5\% sheep's blood agar or they produced no change at all. All 40 strains tested produced no change at all. None of the strains produced beta hemolysis on sheep's blood. Beta hemolysis among the variants of \textit{S. faecalis} is characteristic of \textit{S. faecalis} var. \textit{zymogenes}.

Hemolytic activity is dependent on the ability of the microorganisms to produce hemolysins to break down the red blood cells and can vary with the type of blood agar used. Evans and Chinn (1947) found that \textit{S. faecalis} var. \textit{zymogenes} produced beta hemolysis on sheep's blood agar and alpha hemolysis on rabbit's blood.

Twenty-seven strains produced no gelatinase and were
classified as \textit{S. faecalis}. Thirteen strains demonstrated gelatinase activity and these microorganisms were identified as \textit{S. faecalis} var. \textit{liquefaciens}.

A high incidence of \textit{S. faecalis} var. \textit{liquefaciens} was noted from all three sources. \textit{S. faecalis} var. \textit{liquefaciens} was found in 6 of 22 cases from the root canal. Engstrom (1964) reported 3 of 20 strains of enterococci from the root canal to be \textit{S. faecalis} var. \textit{liquefaciens}. The occurrence of this microorganism in this study was not associated with any clinical entity and no explanation can be given for the high incidence.

A high degree of resistance to penicillin was noted among the strains of \textit{S. faecalis} and \textit{S. faecalis} var. \textit{liquefaciens} isolated from the various sources. Thirteen of the 40 strains (32.5\%) were found to be resistant to penicillin.

Four of 22 strains (18.2\%) from the root canals were resistant to penicillin. This finding was much higher than those of Fox and Isenberg (1967), Goldman and Pearson (1969) and Engstrom (1964) who found that 4.8 - 6.0\% of the enterococci isolated from root canals were resistant to penicillin.

Six of 13 isolates of saliva samples and 3 of 5 isolates of fecal samples were found to be resistant to penicillin. The incidence of resistant strains of enterococci from saliva and fecal samples was higher than that of isolates from the
root canals.

The high incidence of strains of enterococci resistant to penicillin can present a problem in chemotherapy in acute endodontic episodes. In cases where enterococci are suspected it becomes important that a culture and sensitivity test be conducted to determine the antibiotic of choice.

The enzyme deoxyribonuclease is effective in hydrolyzing deoxyribonucleoprotein and deoxyribonucleic acid. Purulent exudates owe their viscosity to the deoxyribonucleoprotein released by necrotic cells. Those microorganisms that produce the enzyme are able to hydrolyze the nucleoproteins and nucleic acids and utilize the products of hydrolysis. This ability to produce the enzyme increases the ability of the microorganism to survive in such an environment.

None of the 40 isolates of S. faecalis or S. faecalis var. liquefaciens from endodontic, salivary and fecal samples produced the enzyme deoxyribonuclease when tested on DNase test agar. This finding is in agreement with that of Brown (1950) who tested 12 strains of S. faecalis, 3 strains of S. faecalis var. liquefaciens and 23 strains of S. faecalis var. zymogenes and found none produced deoxyribonuclease.

In contrast, Smith and Bodily (1968) reported 5 of 16 gamma hemolytic enterococci of fecal origin produced the enzyme deoxyribonuclease.
Hyaluronidase is involved in the hydrolysis of hyaluronic acid, an important component of the ground substance of connective tissue. This enzyme thus aids in spreading infecting microorganisms. The ability to produce this enzyme is of special interest in endodontic therapy since those microorganisms capable of producing the enzyme would be able to establish itself in the periapical tissues.

None of the 40 strains of *S. faecalis* and *S. faecalis* var. *liquefaciens* isolated from endodontic, salivary and fecal samples produced the enzyme hyaluronidase in a test period of four consecutive days. The microorganisms were grown in a brain heart infusion broth free of hyaluronic acid. The results indicated that these microorganisms did not constitutively produce the enzyme hyaluronidase. A test for the adaptive production of hyaluronidase was not conducted at this time.

Rosan and Williams (1964) observed that 15 of 22 strains of *S. faecalis* isolated from subgingival scrapings produced small amounts of hyaluronidase adaptively and hydrolyzed the substrate on prolonged incubation. It was also observed that these microorganisms did not produce the enzyme when grown in a broth free of the substrate.

Other tests for the virulence and pathogenicity of the enterococci would be to inject viable broth cultures of the
organism intraperitoneally into test animals to determine the minimal lethal dose and into the subcutaneous tissue of test animals.

The results of this study indicate that the enterococci are of low virulence compared to other streptococci.
CHAPTER VI

SUMMARY AND CONCLUSIONS

A. Summary

Positive root canal cultures were screened for pure cultures of enterococci. Gram stains, growth on selective medium and the heat and salt resistant characteristics were used to identify the microorganisms as *S. faecalis* or its variant. Saliva and fecal samples were collected from these patients to isolate enterococci from these sources.

Bacteriological laboratory tests were conducted on the isolates of enterococci from endodontic, salivary and fecal samples to observe the similarities or differences in reactions. Tests conducted to study some factors associated with virulence included hemolytic and proteolytic activity, penicillin sensitivity, and the production of the enzymes deoxyribonuclease and hyaluronidase.

B. Conclusions

Enterococci were isolated in pure cultures in 22 of 130 (16.9%) positive root canal cultures. They were also isolated from saliva samples of 13 of these patients with positive root canal cultures (59.1%). *S. faecalis* or its variant was recovered from all 5 fecal samples.

All of the 40 strains produced gamma hemolysis on sheep's
blood agar. Thirteen strains demonstrated gelatinase activity. Twenty-seven strains were identified as *S. faecalis*, and 13 were classified as its variant, *S. faecalis* var. *liquefaciens*. *S. faecalis* var. *zymogenes* was not found among the isolates.

The same bacterial type was isolated from the root canal and saliva in 11 of 13 cases (84.6%). In 4 of 5 cases the same bacterial type was recovered from endodontic, salivary and fecal samples.

A high degree of resistance to penicillin was observed among the strains of *S. faecalis* and *S. faecalis* var. *liquefaciens* isolated from the various sources. Thirteen of the 40 strains (32.5%) were resistant to penicillin.

None of the strains of *S. faecalis* or *S. faecalis* var. *liquefaciens* produced the enzyme deoxyribonuclease.

None of the strains of *S. faecalis* or *S. faecalis* var. *liquefaciens* produced the enzyme hyaluronidase when grown in a broth free of the substrate.

Isolates of *S. faecalis* and *S. faecalis* var. *liquefaciens* from endodontic, salivary and fecal samples demonstrated similar characteristics. A slightly greater incidence of strains resistant to penicillin was observed among the salivary and fecal enterococci.

A high incidence of *S. faecalis* var. *liquefaciens* was
observed among the isolates from all three sources.
CHAPTER VII

BIBLIOGRAPHY


Fleming, Alexander: On the Antibacterial Action of Cultures of a Penicillium, with Special Reference to Their Use in the Isolation of B. Influenzae, British Journal of Experimental Pathology, 10:226-236. 1929.


APPROVAL SHEET

The thesis submitted by Dr. Roy M. Naito has been read and approved by members of the Department of Coral Biology.

The final copies have been examined by the director of the thesis and the signature which appears below verifies the fact that any necessary changes have been incorporated, and that the thesis is now given final approval with reference to content, form and mechanical accuracy.

The thesis is, therefore, accepted in partial fulfillment of the requirements for the degree of Master of Science.

May 13, 1970

Date

Signature of Advisor