Curriculum Vitae

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EDUCATION

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Baltimore College of Dental Surgery, Dental School, University of Maryland

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Doctor of Dental Surgery, May 2009

Baltimore College of Dental Surgery, Dental School, University of Maryland

Baltimore, MD

Bachelor of Arts Major: Economics, May 2005

University of Maryland College Park

College Park, Maryland

PROFESSIONAL LICENSURE

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Maryland State Dental License

Active, #14592, Expires 6-30-2012

Controlled Substance Registration Certificate

Drug Enforcement Agency (DEA)

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Controlled Dangerous Substances (CDS)

State of Maryland

Active, #, Expires 7-31-2012

PROFESSIONAL EXPERIENCE

Associate Dentist

Dental Specialists Integrated, Hagerstown and Frederick MD

June 2011- Present

Associate Dentist

Dr. K Michael Murphy and Associates, Baltimore MD

June 2011- Present

Associate Dentist

Hillandale Dental Partners, Silver Spring MD

August 2010- August 2011

RESEARCH EXPERIENCE

Biomedical Sciences Clerkship – University of Maryland Dental School

Selected to participate in a research project cooperating with faculty from the department of endodontics.

June 2008-May 2009

Research Volunteer - Department of Endodontics, University of Maryland Dental School

Assisted endodontic residents with their research projects. Responsibilities included lab work such as PCR as well as interpretation and analysis of the data.

January 2008- June 2008

Summer Intern – National Institute for Dental and Craniofacial Research (NIDCR), National Institutes of Health (NIH), Bethesda, Maryland

For three consecutive summers, performed experiments in conjunction with a post-doc researcher in the cell biology section. Responsibilities included microbiological, cell, and animal studies.

Summers 2001-2003

PROFESSIONAL AFFILIATIONS

American Association of Endodontics	2009- present
Edward C. Penick Endodontic Study Club	2009- present
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GRANTS AND FUNDING

American Association of Endodontists Foundation (2009-2011): The effects of microbial and clinical factors on long term healing of endodontic infections.

$9,900

PUBLICATIONS AND PRESENTATIONS

A Orgel, P. Chand., ML Hicks, R Nandakumar, SM Barbuto, BJ Paster, AF Fouad (2012). *Effects of treatment, microbial, and radiographic factors on endodontic outcomes.* American Association of Endodontists. Boston, Massachussets.

A Orgel, P. Chand., ML Hicks, R Nandakumar, SM Barbuto, BJ Paster, AF Fouad (2011). *Effects of treatment factors and residual microbial DNA on endodontic treatment outcomes at 2-years*. American Association of Endodontists. San Antonio, Texas.

A Orgel**,** P. Chand., ML Hicks, R Nandakumar, SM Barbuto, BJ Paster, AF Fouad (2010). *Effects of treatment factors and residual microbial DNA on endodontic treatment outcomes at 1-year***.** American Association of Endodontists. San Diego, California.

A Orgel, P. Chand., ML Hicks, R Nandakumar, SM Barbuto, BJ Paster, AF Fouad (2009). Effects *of residual microbial presence and diversity on endodontic outcomes*. International Association of Dental Research. Miami, Florida, IADR.

Elkin M, **Orgel A**, Kleinman HK. *An angiogenic switch in breast cancer involves estrogen and soluble vascular endothelial growth factor receptor 1*. J Natl Cancer Inst. 2004 Jun 2;96(11):875-8.

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Elkin M, Cohen I, Zcharia E, **Orgel A**, Guatta-Rangini Z, Peretz T, Vlodavsky I, Kleinman HK. *Regulation of heparanase gene expression by estrogen in breast cancer*. Cancer Res. 2003 Dec 15;63(24):8821-6.

Abstract

Title of thesis: Evaluation of Factors Related to Long-term Healing in Endodontic Treatment

Adam Orgel, Master of Science, 2012

Thesis Directed by: Dr. Ashraf F. Fouad, Professor and Chairman, Director of Postgraduate Endodontics, University of Maryland School of Dentistry

Introduction: This study sought to determine the effects of residual root canal bacteria and treatment factors on endodontic treatment outcomes up to 6 years post-operatively using traditional radiography (PA radiographs) and limited cone beam computed tomography (CBCT).

Methods: Root canal samples were obtained from 50 patients with pulp necrosis and a periapical lesion, following two-visit treatment using contemporary chemomechanical preparation techniques. PCR, with broad range 16S rDNA bacterial primers, was performed followed by cloning and sequencing on pre-obturation specimens. Periapical and CBCT radiographs were taken at 10 months to 6 years post-treatment. Images were scored by two blinded, calibrated endodontists using the conventional periapical index (PAI) and a CBCT index. Statistical analysis was performed using bivariate and multivariate regression analysis for treatment factors. Kaplan-Meier survival analysis and Pearson's regression analysis was used for year-over-year changes.

Results: 41 patients were included in the final data analysis. Recall decreased from 98% at 10-17 months to 51% at 33+ months. The percentage of patients healed at 10-17 months, 18-32 months and 33+ months and overall was 65%, 63%, and 66% respectively. Factors influencing outcomes were presence of bacteria at time of obturation (56% when present, 81% absent), primary versus persistent disease (71% vs. 39%), tooth type (85% anterior,23% molar), obturation to the radiographic apex (100% to the apex, 56% not to the apex), and larger master apical file sizes (73%MAF$\geq$45 vs. 36% MAF<45).. Short-term outcomes had a strong positive relationship with final outcome (Pearson's, r^2=0.56, p=<0.01).

Conclusions: Bacterial DNA presence at the time of obturation adversely affects short-term and intermediate-term endodontic treatment outcomes, but this effect is not found in the long-term. Various patient factors such as tooth type and treatment factors such as master apical file size influence outcomes. Short-term treatment outcomes are good predictors of long-term outcomes.

Evaluation of factors related to long-term healing in endodontic treatment

by
Adam Orgel

Thesis submitted to the faculty of the Graduate School
of the University of Maryland Baltimore in partial fulfillment
of the requirements for the degree of
Master of Science
2012

Dedication

I would like to thank my family, friends, co-residents and faculty for their support and encouragement throughout the duration of my academic pursuits.

This thesis is dedicated to all of you.

Acknowledgements

I am forever grateful to Dr. Ashraf Fouad, my program chair and department head for his support and guidance throughout both dental school and endodontic residency. His mentorship has been pivotal in helping me to achieve my professional goals. I will always owe him a debt of gratitude.

A special thank you to Dr. Hegang Chen for his invaluable help in completion of statistical analysis performed in this thesis.

Thank you Dr. Chand, Hicks, and Tolba for your efforts in helping me to complete my thesis.

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List of Abbreviations

CBCT- Cone Beam Computed Tomography

CHX- Chlorhexidine

EDTA- Ethylenediaminetetraacetic Acid

NaOCl- Sodium Hypochlorite

PAI- Periapical Index

PA- Periapical Radiograph

Introduction

Etiology of Endodontic Infections

It is well established that the vast majority of endodontic diseases are infectious in nature[1]. While in a normal, healthy state the dental pulp is a sterile tissue, it is often through the infectious process, frequently a result of dental caries, that the pulp becomes inflamed and eventually necrosis [2]. As the infection progresses to the periapical tissues, bone loss becomes evident radiographically[3-4]. Though this etiology is known to be primarily bacterial in nature, the specific species involved have not yet been fully elucidated. In the past, examination of the bacteria responsible for the progression of endodontic disease has centered on the concept of identifying the one or few cultivable bacterial phylotypes which are responsible for a certain disease or symptom [5-6]. In fact, as more knowledge is gained, the polymicrobial nature of endodontic infections gains increasing credibility [7-8]. With over 700 types of bacteria known to inhabit the oral cavity [9], variations between populations indicates a complexity not established by previous research. As understanding of biofilms and the nature of bacterial flora evolves it is becoming evident that there is not 'one' species to blame, but many [10]. Further, one species which in and of itself is innocuous alone may become pathogenic in combination with one or more other species. Being able to understand the profile of more robust and pathogenic biofilms seems to be more promising than to identify the one main offender in endodontic infections. This identification serves as the first step towards designing specific treatment modalities aimed at eliminating the infection and promoting an environment conducive to healing.

Microbiologic Goals of Endodontic Treatment

The focus of modern endodontic treatment is the complete elimination of the bacteria within the root canal in order to regain a sterile environment [11]. While this is a noble goal, anatomic variations such as isthmuses, fins and the apical delta, as well as operator and instrument limitations often prevent this from occurring [12-14]. Complete healing does not occur in 100% of cases [12]; often times the periapical disease persists. This event is referred to as a "non-healed" lesion or sometimes as "failed" treatment. Many studies have sought to identify the factors associated with diminished healing rates [12, 15-18]. While the main focus of some of these studies has been on the type of cultivable residual bacteria in the canal system, other factors certainly moderate the healing process. These may range from treatment factors such as over/underfilling and poor obturation quality, as well as patient factors such as diminished immune response. Understanding the effects of variations in treatment on outcomes is key to creating a set treatment protocol to optimize long-term outcomes.

Determination of Periapical Health Status

Prior to analysis of the data an accurate and reproducible determination of the status of the periapical health must be established. Ultimately, it is the histology of the infected tissues which is the gold standard in treatment outcome. Unfortunately, due to the invasive nature of making this determination, histology is not an acceptable method for clinical investigation of non-surgical cases. Therefore many previous studies have sought to establish their own unique criteria for healing based on secondary evidence such as radiography and presence of symptoms while others have followed criteria set by

other studies [12]. A popular method for making this determination has been the Periapical Index (PAI)[19]. This index is based on histology and its correlation to radiographic presentation. The PAI is scored on an ordinal scale from 1-5 with increases in PAI corresponding to increasing periapical inflammation. One shortcoming of the PAI or any other method is they are only as good as the technology they are based on [20]. Recent advances in radiology have brought a more accurate technology for imaging the hard tissues of the mouth; Cone Beam Computed Tomography (CBCT). Studies have shown CBCT to be superior to traditional x-rays in its ability to detect disease (sensitivity) and to show the true representation of its extent [21]. CBCT is quickly becoming the gold standard of disease detection in endodontic practice [22]. A new Periapical Index for CBCT (CBCTPAI) has been developed recently [23]. The use of this technology minimizes uncertainty and error for any study it is used in.

Importance of Host Factors and Novel Analysis

The focus of many previous studies has been solely on the etiologic factor for endodontic disease, namely bacteria. One important factor these studies often overlook is the importance of host response on this pathogenic process. The differences in disease manifestation may be explained in part by patient variation. For example, the overall medical health such as diabetes has shown to be a modifying factor in endodontic disease in humans [24]. Smoking has long been implicated as a modifying factor in periodontal disease [25]. Similarly, it is shown that smokers experience increased prevalence of apical periodontitis compared to non-smokers [26]. Other factors must be accounted for as well such as age, gender, and race. While other studies have not found statistically significant differences in healing based on demographic data these studies have only

looked at these factors independently of each other. Their effects may be insubstantial alone, but in concert are very significant. This study seeks a novel approach to analysis of the factors of healing: cluster analysis. Cluster analysis is often employed in longitudinal studies as well as when study subjects do not represent independent observations [27-28]. This analytic method creates profiles based on set criteria and analyzes significance by comparing these profiles rather than a single factor in and of itself. For example, it is plausible that short obturation and bacterial presence not to be independent of one another as uninstrumented canal spaces may harbor bacteria. Furthermore, specific microbes within the biofilm of an infection may exhibit symbiotic relationships, and as such the observation of combinations of bacteria within the biofilm is not an independent event. Therefore cluster analysis provides a way to understand the complexities of the interplay between the bacteria present, the variations in treatment, and the differences of patient demographics.

Specific Aims

Aim 1

to determine the relationship between residual pre-obturation bacterial phylotypes, singly and in combinations, and long-term healing of endodontic lesions as determined by traditional radiography and by CBCT, and

Aim 2

to determine the relationship between a panel of treatment and patient factors and long-term treatment outcomes using cluster analysis.

Aim 3

to determine the relationship between short-term and long-term endodontic treatment outcomes.

Hypothesis

Hypothesis 1:

Ho1: There is no difference in the pre-obturation, residual microbiological composition between healed and non-healed endodontically treated teeth.

Ha1: Pre-obturation samples of residual root canal bacteria in non-healed cases show more detectable bacteria and in specific combinations as compared to healed cases.

Hypothesis 2:

Ho2: Specific patient and treatment factors do not alter the healing rates in healed versus non healed endodontically treated teeth.

Ha2: Differences in patient and treatment factors affect the outcome of root canal therapy.

Hypothesis 3:

Ho3: There is no correlation between short-term endodontic outcomes and long-term endodontic outcomes.

Ha3: There is a positive outcome between sort- and long-term endodontic treatment outcomes.

Materials and Methods

Patient Selection

Patients who consented to participate in the study and who had not been treated with antibiotics in the preceding month were included. All teeth involved had a negative pulp test result (or had previous endodontic treatment that was completed over 1 year before) and presented with a primary or persistent periradicular lesion at least 3mm in diameter. The age of patients included in this study was in the range of 19-94 years with an average age of 51 years. A total of 50 patients were enrolled in the study. Of the cases 41 that returned for one-year recall 30 were primary infections and 11 were persistent cases.

Inclusion Criteria

- No previous treatment on the tooth in question.
- Patients had pulp necrosis as a result of caries or trauma.
- Seemingly adequate previous endodontic treatment and restoration over 2 year before that is not healing and for which non-surgical retreatment is indicated.
- Previous treatment over 6 months with clinical signs and symptoms of disease.
- Periapical lesions at least 3 mm in diameter

Exclusion Criteria

- Systemic debilitating disease such as diabetes mellitus, liver disease, chronic infections, rheumatoid arthritis or any other systemic disease that compromise the immune system.

- Patients taking chronic systemic steroids or chemotherapeutic agents.
- Patients who have been on antibiotics in the preceding month, or who require prophylactic antibiotic before dental treatment.
- Patients who have active chronic or aggressive marginal periodontitis, patients with probing of more than 5mm of the tooth involved were also excluded.
- Women who were pregnant at the time of initial treatment.
- Teeth which are difficult to isolate adequately.
- Children less than 18 years of age and teeth with immature apex.

Treatment Protocol and Sample Collection

Treatment was performed by two endodontics who are on the faculty at the University of Maryland, and six postgraduate Endodontics residents in their senior year. All providers were calibrated on the technique for treatment and sampling.

Following isolation with rubber dam, the field was disinfected with a sequence of 30% H_2O_2, then 5% tincture of iodine, then 2.5% sodium hypochlorite, and then the halogens were inactivated with 5% sodium thiosulphate. If caries was present, it was removed and the sequence was repeated. At this point a surface specimen was taken. Access preparation was performed without water coolant. A microbiological specimen was then obtained by introducing sterile paper points into the canal. Paper pointes were stored in storage medium in sterile DNA and RNA free vials at -70^{o} C. Root canals were instrumented in the following manner: following working length determination, straight-line access was done using Gates Glidden burs #2-4, rotary instrumentation was performed used a combination of Profile instruments and Light Speed/LSX. The latter

was used until about 10-12 pecks were needed to bring the instrument to the desired working length of instrumenting to the radiographic apex. Irrigation throughout the instrumentation procedure was with 2.5% sodium hypochlorite. At the end of the instrumentation phase, 17% EDTA was used in conjunction with the NaOCl to remove the smear layer. Finally the canal(s) were dried and a final irrigation with 2% Chlorhexidine was done. The canals were then dried and a second specimen was obtained. Calcium hydroxide paste was then placed in the canal, and the tooth was then sealed with a combination of Cavit and Fuji IX temporary filling. At the second appointment, the medicament was removed, the irrigation sequence was repeated in the same way, and a specimen was obtained prior to obturation. The last irrigant to be used was 2% chlorhexidine. In order to prevent this irrigant from having a carry-over effect, the PCR buffer that was used to transport and store the specimen had among its ingredients L-alpha lecithin in Tween, which is a known inactivator of Chlorhexidine [29]. Canals were obturated using Resilon master cones corresponding to the master apical file coated in Epiphany sealer (SybronEndo, Savannah, GA). Lateral condensation technique was used for each case with the emphasis on avoiding overfill which would interfere with reading the follow-up radiographs. Finally, a temporary restoration was placed consisting of 2 mm of Cavit (3M, Saint Paul, MN) and Fuji IX glass ionomer (GC America, Alsip, IL). Patients were referred to their primary care dentist for final restoration of the tooth.

DNA Extraction

DNA extraction was performed using a QIAamp DNA mini kit (Qiagen, Valencia, CA) according to the manufacturer's protocol. Briefly, buffers and lytic

enzymes are added to the samples. These stabilize nucleic acids, and enhance selective DNA adsorption to the QIAamp membrane; impurities pass through the membrane. Alcohol is added and the lysates are loaded onto the QIAamp spin column. Wash buffers are used to remove impurities and pure, ready-to-use DNA is then eluted in water or low-salt buffer.

Primer Selection

The oligonucleotide sequences of PCR primers and reaction parameters used were as described by Fouad et al [30-31]. PCR amplification mixture: 10µL extracted DNA from root canal samples, 5µL 10X PCR buffer, 0.5µL (2.5U) HotStar *Taq* DNA polymerase (Qiagen), 0.2mM concentrations each of the 4 deoxynucleotide triphosphates (Takara, Otsu, Shiga, Japan) and 0.5µM concentration of each (sense and antisense) primer to a total volume of 50µL. PCR conditions for broad range primers: denaturation at 94^{o}C/15s, annealing at 56^{o}C/15s and extension at 72^{o}C/45s and for *Enterococcus*-specific (*tuf*) primers: denaturation of 94^{o}C/15s, annealing at 55^{o}C/15s, extension at 72^{o}C/15s. Initial denaturation at 94^{o}C/15min, final extension at 72^{o}C/5min and 35cycles were uniform.

Construction of the 16S rRNA Clone Libraries

A 16S rRNA clone library was constructed for each sample as follows: 20 µl of the PCR product from amplified DNA of each sample was run on a 0.8% low melting agarose gel in Tris-Boric Acid-EDTA (TBE) buffer. The 16S rDNA band of the expected size was excised from the gel and purified by using spin columns QIAquick Gel Extraction Kit (QIAGEN, Valencia, CA) according to manufacturer's instructions. The

purified PCR product (3-4 µl) was cloned by a TA cloning method by using a TOPO TA Cloning Kit (Invitrogen, Carlsbad, CA) according to manufacturer's instructions. Successful cloning was demonstrated by bluish color colonies. Clones with the correct size inserts were analyzed by sequencing.

Sequencing of 16S rDNA Gene

Purified PCR products were sequenced with an ABI Prism cycle sequencing kit using BigDye Terminator cycle sequencing ready reaction kit according to manufacturer's instructions (PE Applied Biosystem, Foster City, CA). This used a 519R (TKA CCG CGG CTG CTG) primer. Sequencing reactions were run on ABI model 3100 DNA sequencer.

Data Analysis of Unrecognized Inserts

Sequences with the correct size insert of approximately 1500 bases were analyzed. Approximately 500 bases were obtained first to determine identity or approximate phylogenetic position. For identification of closest relatives, sequences of the unrecognized inserts were compared to the 16S rRNA gene sequences of over 10,000 microorganisms in database at the Forsyth Institute, Boston, and the 100,000 sequences in the Ribosomal Database Project (RDP) (http//rrna.uia.ac.be/rrna/ssu/forms/index) and GenBank by BLAST (http://www.ncbi.nih.nlm.gov).

Radiographic Collection

Polyvinyl siloxane (PVS) stents were fabricated for each patient to ensure all follow-up radiographs were taken from the same angle. Pre-operative periapical digital

radiographs were taken as well as follow-up radiographs at six month intervals for the first year after treatment. Digital radiographs were taken either using Schick technologies or Romexis (Planmeca). At long-term (33+ months) post-operative recall cone beam computed tomography (CBCT) scans were taken. CBCT scans were taken in the radiology department by a licensed radiology technician under the supervision of an oral maxillofacial radiologist.

Radiographic Interpretation

Periapical radiographs were interpreted for determination of periapical health status using the Periapical Index (PAI) as previously described. Radiographs were viewed and scored independently by two calibrated faculty endodontists at the University of Maryland. Calibration was achieved using a set of 100 sample radiographic images supplied by the author with a standardized score for each as well as a key for determination of score of 1-5. An increased score indicates increased periapical inflammation. Examiners could not score study images until they were determined to be sufficiently calibrated with a kappa statistic greater than k=0.61 indicating "substantial agreement" with the standardized images. Following independent scoring by the examiners, a collaborative meeting was arranged to arrive at consensus for images in which there was disagreement between the examiners. The consensus PAI score was the score used to determine the periapical health status with scores or 1 and 2 correlating with health and scores of 3 or greater with periapical inflammation.

Interpretation of Cone Beam Computed Tomography (CBCT) scans of the teeth were performed in accordance with criteria set forth by Estrela [23]. Scans were taken by

a licensed radiology technician working under the supervision of an Oral Maxillofacial Radiologist at the University of Maryland Dental School using a Planmeca ProMax (Planmeca, Helsinki, Finland). All scans were taken with a limited field to a single tooth as the area of interest to minimize the dosage of ionizing radiation. Images were viewed using Invivo5 software (Anatomage, San Jose, CA). The greatest diameter of any periapical radiolucency was measured and a corresponding CBCTPAI score was given based on the lesion size.

Patient and Treatment Variables

Multiple patient and treatment variables were collected for multivariate analysis. These included:

- Presence versus absence of bacteria at the time of obturation
- Bacterial phylotypes present at the time of obturation
- Gender
- Age
- Race
- Smoking status
- Initial versus final lesion size
- Pre-operative PAI versus Post-operative PAI
- Obturation length from the apex
- Obturation density
- Presence of symptoms peri-operatively
- Final restoration type

- Number of roots
- Master apical file size
- Pre-operative and one week post-operative visual analog pain scale (VAS)
- Presence of swelling
- Periapical diagnosis
- Body Mass Index (BMI)
- Restoration

Statistical Analysis

Hypothesis 1

Chi-square was used, unless, when computing chi-square the expected value was less than 5 in more than 20% of cells in a 2 x 2 table. In that case, Fisher's Exact Test was used.

Cluster analysis was performed to group like clusters of patient populations based on centered correlation and average linkage as well as Euclidian distance and average linkage. Chi-square was then used to test for differences between the groups.

Student's t-test was performed when analyzing differences between the means of two populations. (i.e. number of phylotypes present in healed vs. non-healed groups)

Hypothesis 2

Chi-square was used, unless, when computing chi-square the expected value was less than 5 in more than 20% of cells. In that case, Fisher's Exact Test was used.

Cox multivariate regression analysis was used to analyze the significance of treatment and patient factors on healing relative to each other.

Kaplan-Meier survival curves were used to determine the relationship of treatment and patient factors to healing over time.

Hypothesis 3

Chi-square test was used.

Pearson’s product-moment correlation coefficient was used

Results

Patient Population

In total, 50 patients met the inclusion and exclusion criteria and were enrolled in the study. Following treatment, for various reasons (Figure 1), 9 patients were excluded from the data analysis leaving 41 included patients. In total, 21 male and 29 females with ages ranging from 20-95 years were enrolled. Thirty-three cases were primary infections, whereas 16 were persistent. In one case, upon access, a cotton pellet was discovered which had been placed at the previous treatment approximately 15 years previously. The previous root canal treatment was not completed and therefore the case was classified neither as primary nor persistent. The goal of recall was to follow-up on patients at 1-, 2-, and 3-years post treatment, but because of scheduling conflicts, patients recall intervals ranged from 10-17 months, 18-32 months and 33+ months; here on out referred to as short- intermediate- and long-term . Of the included 41 patients, the number presenting for recall was 40 (98%), 32 (78%), and 21 (51%) patients respectively. Common causes for failure of patients to return for recall include patient moving to a different city and per the patient's request.

Bacteria were identified in 47 specimens collected after access and prior to instrumentation and irrigation procedures. After chemomechanical treatment of the root canal system 25 (53%) samples remained PCR positive for bacteria. From these specimens 127 unique bacterial phylotypes were identified (Appendix 1). Samples from which bacteria were identified contained on average 9.48 different bacterial phylotypes. Most phylotypes were identified solely in one sample. The most prevalent phylotypes

were *Enterococcus casseliflavus* Oral Taxon 801; AF039899 (52%), *Exiguobacterium aurantiacum;* DQ019166 (44%), *Lactococcus lactis* Oral Taxon 804; M58837 (40%), and *Propionibacterium acnes* (28%).

Based on the bacterial profile, cases were classified into three distinct clusters using two different clustering methods. Method one used centered correlation and average linkage and method two used euclidian distance and average distance (Figures 2a and 2b). Main clusters and subclusters were analyzed for significance at each recall respectively.

Periapical Index calibration exercise resulted in kappa values of 0.62 and 0.73 indicating “substantial agreement” for both examiners rendering them able to score the study images.

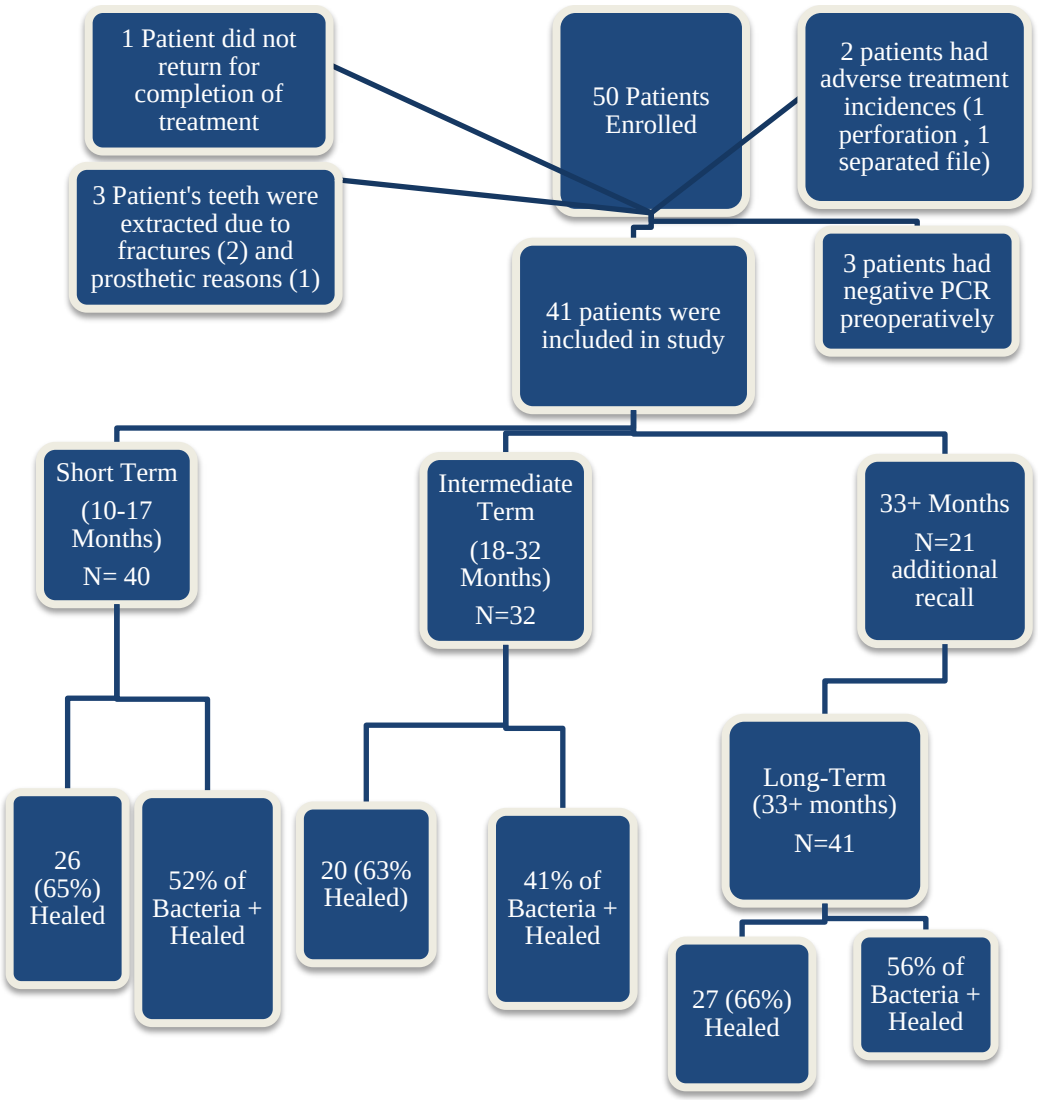
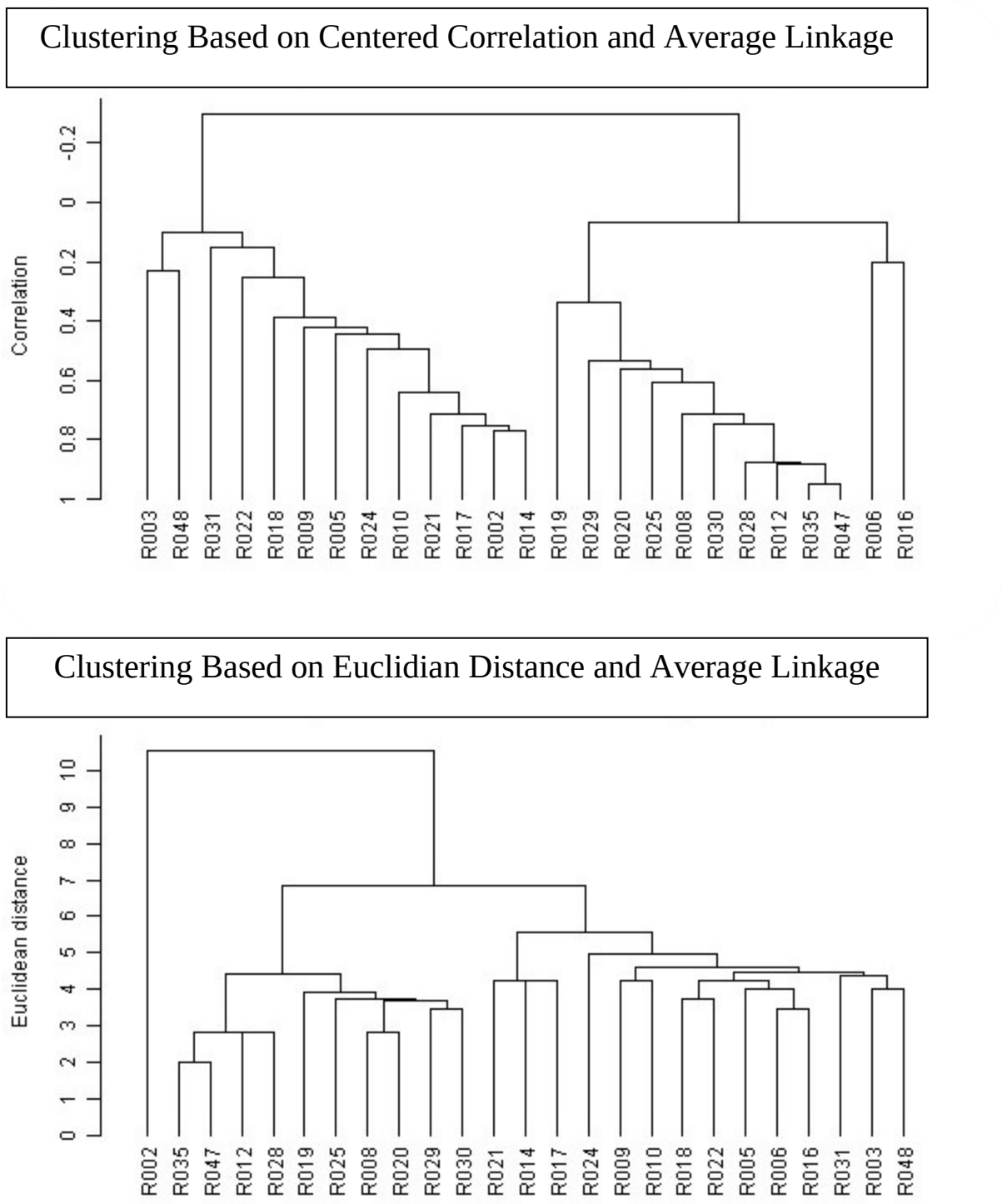


Figure 1: Flow chart of patient recall.



Figures 2a and 2b. Clustering based on centered correlation and average linkage

Clustering based on euclidian distance and average linkage.

Short-term Recall

Of the 41included participants, 40 (98%) returned for 10-17 month recall. One patient was lost to follow-up because of an inability to contact the patient. Overall, 26 (65%) cases were healed (PAI <3). Following instrumentation and irrigation protocol bacteria was detected in 25/40 (62%) one-year specimens, 13 of which healed. In comparison, in cases in which no bacteria was identified, 13/15 (87%) cases healed (Fisher's exact test, p=0.11).

Significantly fewer number of different phylotypes were detected in healed cases (3.6 ± 5.7) than non-healed cases (8 ± 5.3) (unpaired t-test, p=0.015) (Figure 3). Two phylotypes were significantly associated with non-healed cases at one year. *Enterococcus casseliflavus* Oral Taxon 801; AF039899 was present in 13 (32%) cases. These teeth healed 46% when *Enterococcus casseliflavus* Oral Taxon 801; AF039899 was present whereas 79% healed when this phylotype was absent (Chi-square, p=0.038). *Lactococcus lactis* Oral Taxon 804; M58837 was present in 10 (24%) and was also associated with poorer outcomes. When present 40% healed versus 77% when absent (Fisher's exact test, p=0.048). *Exiguobacterium aurantiacum*, present in 11 (27%) of cases, showed a trend towards reducing healing. When present 45% healed versus 77% when absent (Fisher's exact test, p=0.057). No other phylotypes either alone, or in combination were associated with a reduction in healing at in the short-term.

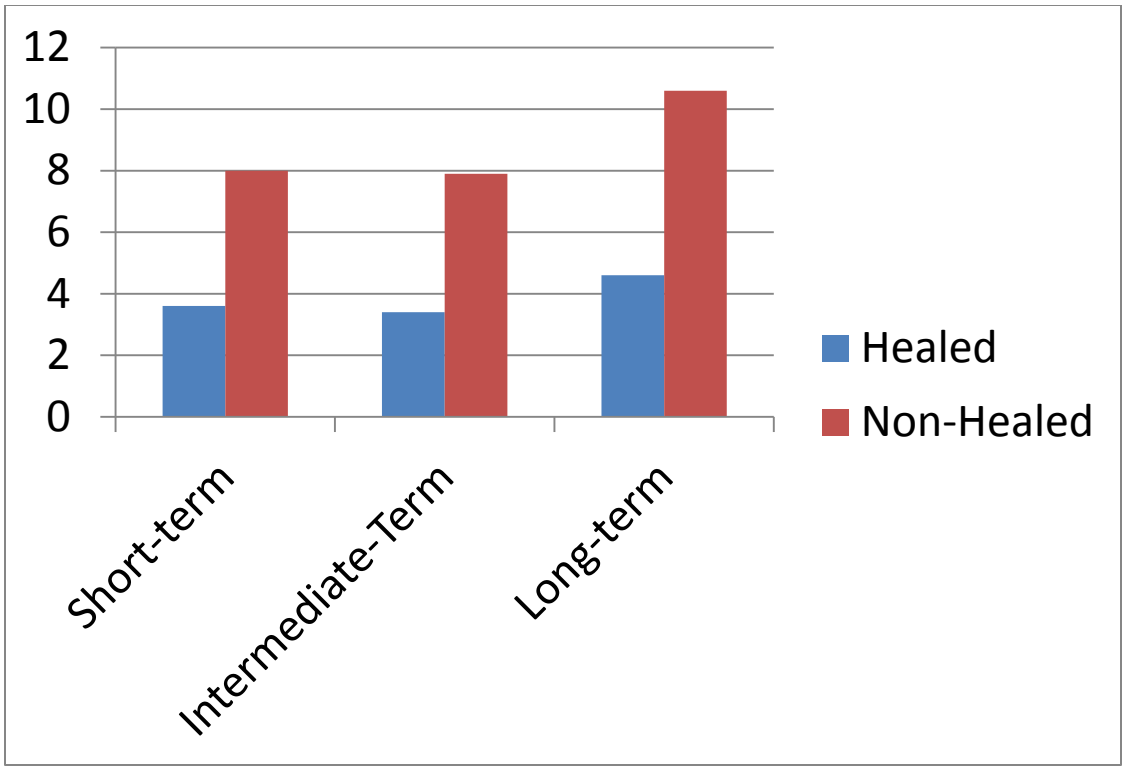


Figure 3. Effect of mean number of phylotypes per specimen on healing.

All (Student's t-test, $p<0.05$)

Based on the determined clusters (figures 2a and 2b), statistical analysis was performed and there was no statistically significant difference in the healing between groups. Clustering by average linkage, cluster 1 had 7/13 (54%) healed and cluster 2 had 7/12 (58%) healed (Chi square, $p= 0.82$) and method two used euclidian distance finding in cluster 1 6/10 (60%) healed versus 7/14 (50%) in cluster 2 (Fisher's exact test, $p= 0.70$). Further grouping the clusters did not result in any statistically significant differences between clusters.

Patient and treatment factors were analyzed for association with healing by bivariate analysis (Appendix 2). Molar teeth were less likely to heal than premolars and anterior teeth; 46% vs. 78% (Chi square, p=0.02). An association between obturation length and healing was found. Cases obturated to the radiographic apex were more likely to heal, 7/7 (100%), compared to 17/34 (50%) obturated either short or long. (Fisher's Exact, p=0.05). Cases obturated precisely to the radiographic apex were also less likely to have detectable bacteria (Fisher's Exact, p=0.003) than under/overfilled cases.

Cox multivariate regression model was performed for multivariate analysis of the data (Appendix 3). The more roots a tooth had, the less likely the case was to have healed outcome. Single rooted teeth healed 14/20 (70%) cases, teeth with 2 roots healed in 9/13 (69%) of cases, and three-rooted teeth healed in 1/7 (14%) of cases (p=0.008). Primary cases were more likely to be healed than persistent cases. Primary treatment resulted in 19/27(70%) cases healed versus 7/13(54%) persistent cases healed at one year (p=0.025). There was a trend towards healing when teeth were obturated to the radiographic apex versus short or long obturation. 7/7 (100%) cases obturated to the radiographic apex healed versus 20/33 (61%) cases obturated short or long (p=0.067). No other variables were found to be significant predictors of healing using multivariate analysis.

Intermediate-term Recall

Of the 41 patients eligible for recall during the second year, 32 (78%) returned for follow-up between 18 and 32 months. The most common reasons for patient drop-out were an inability to contact the patient (3) and the patient had moved out of the state (2).

Overall, 20 (63%) cases were healed (PAI <3). Following instrumentation and irrigation protocol bacteria was detected in 17/32 (53%) of these specimens, 7 (41%) of which healed. In comparison, in cases in which no bacteria was identified, 13/15 (87%) cases healed (Fisher's exact, p=0.01).

Significantly fewer number of different phylotypes were detected in healed cases (3.4 $\pm$ 4.9) than non-healed cases (7.9$\pm$ 5.3) (unpaired t-test, p=0.01) (Figure 3). Two phylotypes were significantly associated with non-healed cases. When *Enterococcus casseliflavus* Oral Taxon 801; AF039899 was present healed outcomes were 2/8 (25%) whereas 18/24 (75%) teeth healed when this phylotype was absent (Fishers exact, p=0.018). *Exiguobacterium aurantiacum* was also associated with poorer outcomes. When present 2/8 (25%) healed versus 18/26 (69%) healed when absent (Fisher's exact test, p=0.035). *Lactococcus lactis* Oral Taxon 804; M58837 showed a trend towards reducing healing. When present 3/7 (43%) healed versus 17/25 (68%) when absent (Fisher's exact test, p=0.28). No other phylotypes either alone, or in combination were associated with a reduction in healing at 18-32 months.

Similar to the 10-17 month recall data statistical analysis on the basis of clustering was performed and there was no statistically significant difference in the healing between groups. Clustering by average linkage, Cluster 1 had 3/10 (30%) healed and Cluster 2 had 4/7 (57%) healed (Fishers exact, p= 0.35) and method two used euclidian distance finding in Cluster 1 4/8 (50%) healed versus 4/9 (44%) in Cluster 2 (Fisher's exact test, p= 1.00). Further grouping the clusters did not result in any statistically significant differences between clusters.

Patient and treatment factors were analyzed for association with healing by bivariate analysis (Appendix 2). The association between molar teeth and reduced healing outcomes endured; 20% (molars) vs. 82% (others) (Fisher's exact test, p=0.002). Once again, the association between obturation length and healing was found. Cases obturated to the radiographic apex were more likely to heal, 6/6 (100%), compared to 14/26(54%) obturated either short or long. (Fisher's Exact, p=.0.06). Cases obturated precisely to the radiographic apex were also less likely to have detectable bacteria; 0/6 (0%) compared to 16/25 (64%) respectively (Fisher's Exact, p=0.001).

Cox multivariate regression model was not performed for multivariate analysis due to the amount of missing data from the drop-outs.

Long-term recall

Of the 41 patients eligible for recall during the third year, 21 (51%) returned for follow-up. 15 had CBCT images of the study tooth taken. 6 Patients did not receive a CBCT either due to clinical determination of non-healing or by declining to have the CBCT taken. Patients did not return for various reasons including inability to contact patient or patient moved (13), age (1), and cancer recurrence (2). Overall, 16/21 (76%) cases were healed using periapical radiographs and clinical signs and symptoms (PAI <3). Of the 15 teeth imaged with CBCT, 6/15 (40%) were healed (Chi-square, p=0.03). CBCT was more sensitive at determining disease. Comparing cases imaged with both PA and CBCT the two imaging modalities agreed on outcome 8/15 (53%) and disagreed 7/15 (47%). In each instance when the modalities did not concur PAI resulted in healing whereas CBCT resulted in non-healed lesion (Pearson's, r^2=0.32) (Student's t-test,

p=0.12). There were no statistically significant findings using CBCT PAI due to small sample size. Following instrumentation and irrigation protocol bacteria was detected in 8/15 (53%) of these specimens, 2 (25%) of which healed. In comparison, in cases in which no bacteria was identified, 4/7 (57%) cases healed (Fisher's exact, p=0.06).

Significantly fewer number of different phylotypes were detected in healed cases (4.6 $\pm$ 5.4) than non-healed cases (10.6 $\pm$ 1.5) (unpaired t-test, p=0.01) (Figure 3). No specific phylotype, either alone, or in combination, were associated with outcomes at one 33+ months.

Using cluster analysis by centered correlation and average linkage, Cluster 1 had 3/8 (38%) healed and Cluster 2 had 5/5 (100%) healed (Fishers exact, p= 0.04). Clustering by euclidian distance and average linkage, in Cluster 1: 5/5 (100%) healed versus 3/7 (43%) in Cluster 2 (Fisher's exact test, p= 0.07). Further grouping the clusters did not result in any statistically significant differences between clusters.

Patient and treatment factors were analyzed for association with healing by bivariate analysis (Appendix 2). Single canal teeth trended towards being more likely to heal, 12/13 (92%) teeth, than those with 2 canals, 3/6 (50%), or 3 canals, 1/2 (50%) (Chi-square, p=0.09). Primary cases were more likely to heal than persistent cases 87% vs. 40% (Fisher's exact, p=0.06). No other significant trends between treatment and patient factors and outcomes were noted.

Cox multivariate regression model was not performed for multivariate analysis due to the amount of missing data from the drop-outs.

Combined Data for Long-term Assessment of Outcomes

Adjusting for excluded patients, recall data was available for 41 patients at various times of follow-up. Of these patients, 27 (65.8%) were assessed as healed and 14 (34.2%) as non-healed. Two teeth were extracted resulting in 39/41 (95%) teeth remaining. Root end surgery was performed on 4 (9.7%) patients. 35/41 Patients had no additional procedures performed to the study tooth resulting in 85% survival. Outcomes at 10-17 months were good predictors of final outcomes. Short-term outcomes and long-term outcomes were equivalent in 26/40 (65%) cases for which there was data (Pearson's, r2= 0.56, $p<0.01$).

Teeth from which bacteria could be identified healed in 14/25 (56%) of cases compared to 13/16 (81%) of cases without detectable bacteria (Fisher's exact, p=0.09). Significantly fewer number of different phylotypes were detected in healed cases (4.3 $\pm$ 4.8) than non-healed cases (8.5$\pm$ 5.9) (unpaired t-test, p=0.01). Two phylotypes were significantly associated with non-healed cases. When *Enterococcus casseliflavus* Oral Taxon 801; AF039899 was present healed outcomes were 6/13 (46%) whereas 21/28 (75%) teeth healed when this phylotype was absent (Chi-square, p=0.035). *Exiguobacterium aurantiacum* was also associated with poorer outcomes. When present 5/11 (45%) healed versus 22/30 (73%) healed when absent (Fisher's exact, p=0.047). No other phylotypes either alone, or in combination were associated with a reduction in healing at 18-32 months.

Using cluster analysis by centered correlation and average linkage, Cluster 1 had 5/12 (42%) healed and Cluster 2 had 8/12 (67%) healed (Fishers exact, p= 0.23).

Clustering by euclidian distance and average linkage, in Cluster 1: 8/10 (80%) healed versus 6/14 (42%) in Cluster 2 (Fisher's exact test, p= 0.10). Further grouping the clusters did not result in any statistically significant differences between clusters.

Patient and treatment factors were analyzed for association with healing by bivariate analysis (Appendix 2). Anterior teeth were more likely to heal than posterior teeth; 86% vs. 52% (Chi square, p=0.002). Primary treatment cases healed 71% versus 39% of persistent treatment cases (Fisher's exact, p=0.07). An association between obturation length and healing was found. Cases obturated to the radiographic apex were more likely to heal, 7/7 (100%), compared to 19/34 (56%) obturated either short or long. (Fisher's exact, 0.07). Larger master apical preparations (MAF$\geq$45) led to increased healing than smaller preparations (MAF<45); 73% vs. 36% (Fisher's exact, p=0.03).

Kaplan-Meier estimate of survival of teeth was calculated (Appendix 3) using the assumption that (1) a tooth, once deemed healed could not revert to non-healed status, and (2) the last observation of non-healed status was the decisive observation in the status of the tooth. Using these assumptions and controlling for drop-out statistical analysis resulted in the following findings: A trend towards reduction in healing outcomes with bacteria present compared with bacteria absent (Log-rank, p=0.08) (Figure 4a). A reduction in healing outcomes in multi-rooted teeth over time was shown (p=0.002) (Figure 4b). A trend towards reduction in healing outcomes with persistent compared with primary treatment (p=0.08) (Figure 4c). Obturation to the radiographic apex increased healing rates (p=0.002) (Figure 4d). Lastly, a master apical file size of $\geq$45 had a strong trended towards improving healing outcomes compared to smaller final

instrument size (p=0.054). No other studied variables were found to be statistically significantly associated with outcomes using the Kaplan-Meier test.

Cox regression model was performed for multivariate analysis of the data (Appendix 3). The variables entered into the regression model include in the following order: bacteria presence, number of roots, obturation length from the radiographic apex, primary/persistent disease, and master apical file size. Other variables were not input into the analysis because their effects were not significant in the bivariate analysis and thus would not be expected to become significant in a more stringent test. The effect of neither bacteria, nor MAF size on outcomes was statistically significant using multivariate analysis. The more roots a tooth had, the less likely the case was to have healed outcome. Single rooted teeth healed 19/23 (83%) cases, teeth with 2 roots healed in 6/14 (43%) of cases, and three-rooted teeth healed in 2/6 (33%) of cases (p=0.05). Primary cases were more likely to be healed than persistent cases. Primary treatment resulted in 20/28 (71%) cases healed versus 5/13(39%) persistent cases healed at one year (p=0.03). There was greater healing when teeth were obturated to the radiographic apex versus short or long obturation. 7/7 (100%) cases obturated to the radiographic apex healed versus 19/34 (56%) cases obturated short or long (p=0.02). No other variables were found to be significant predictors of healing using multivariate analysis.

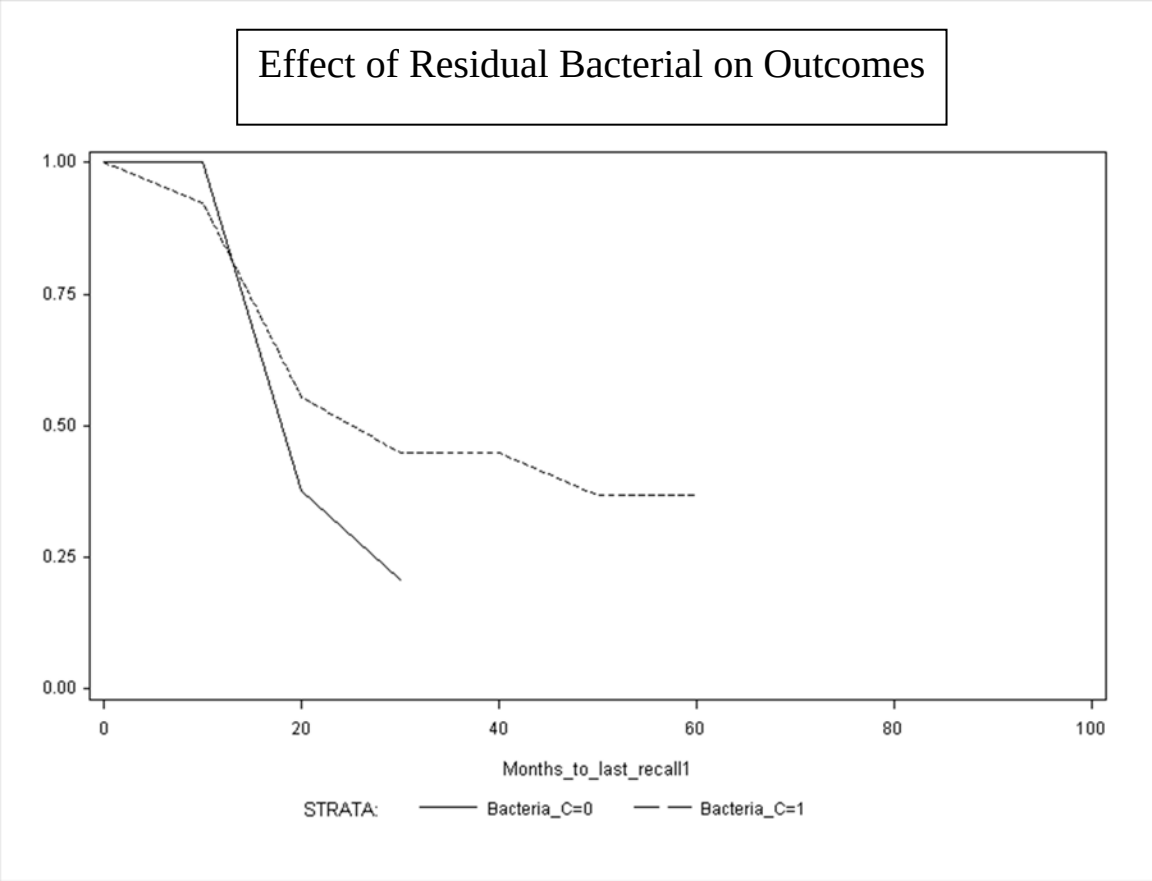


Figure 4a. Effect of Residual Bacterial on Outcomes

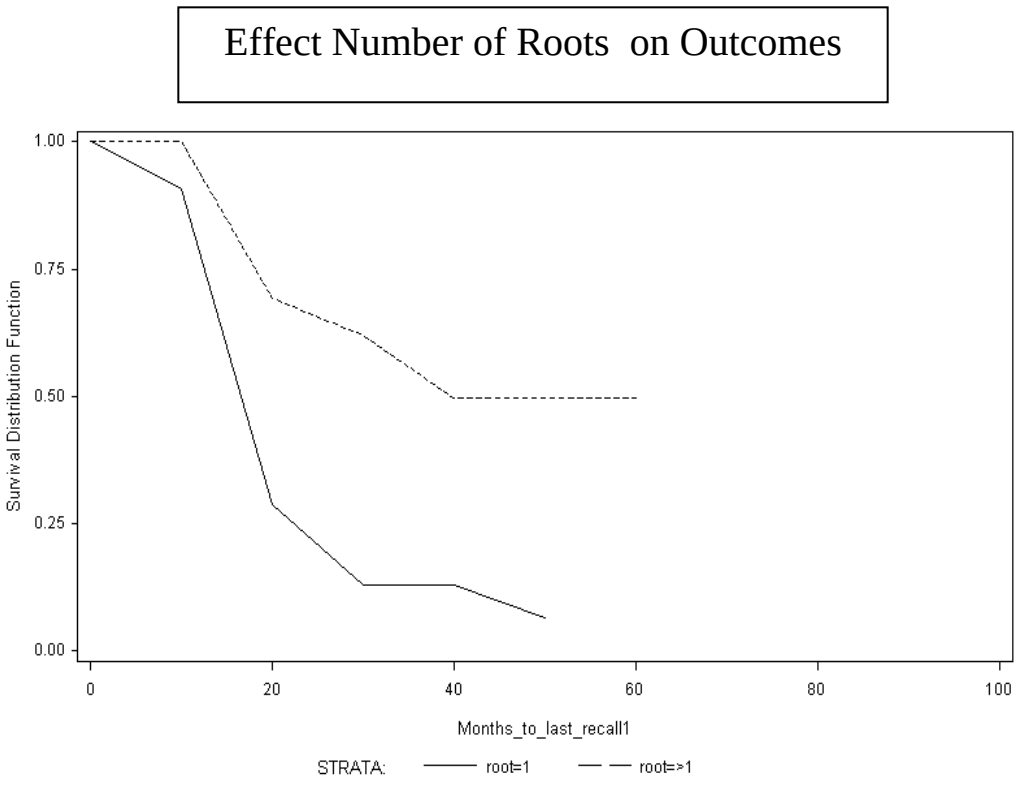


Figure 4b. Effect of Number of Roots on Outcomes

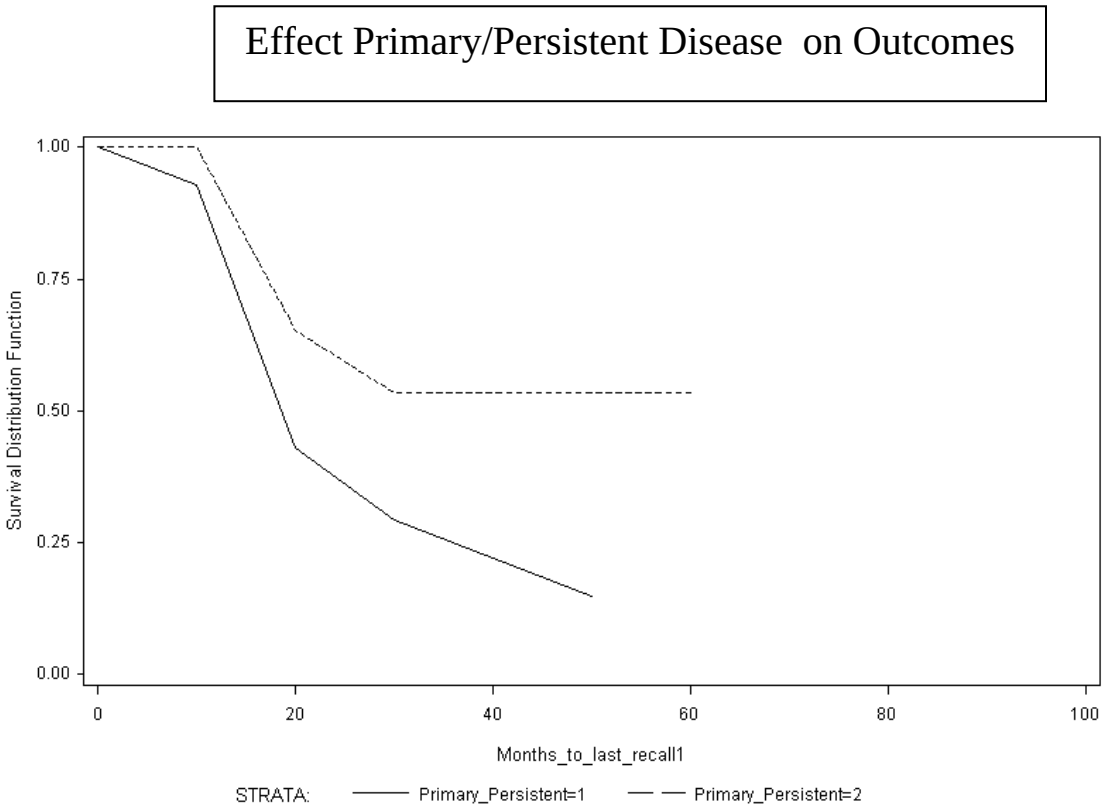


Figure 4c. Effect of Primay/Persistent Disease on Outcomes

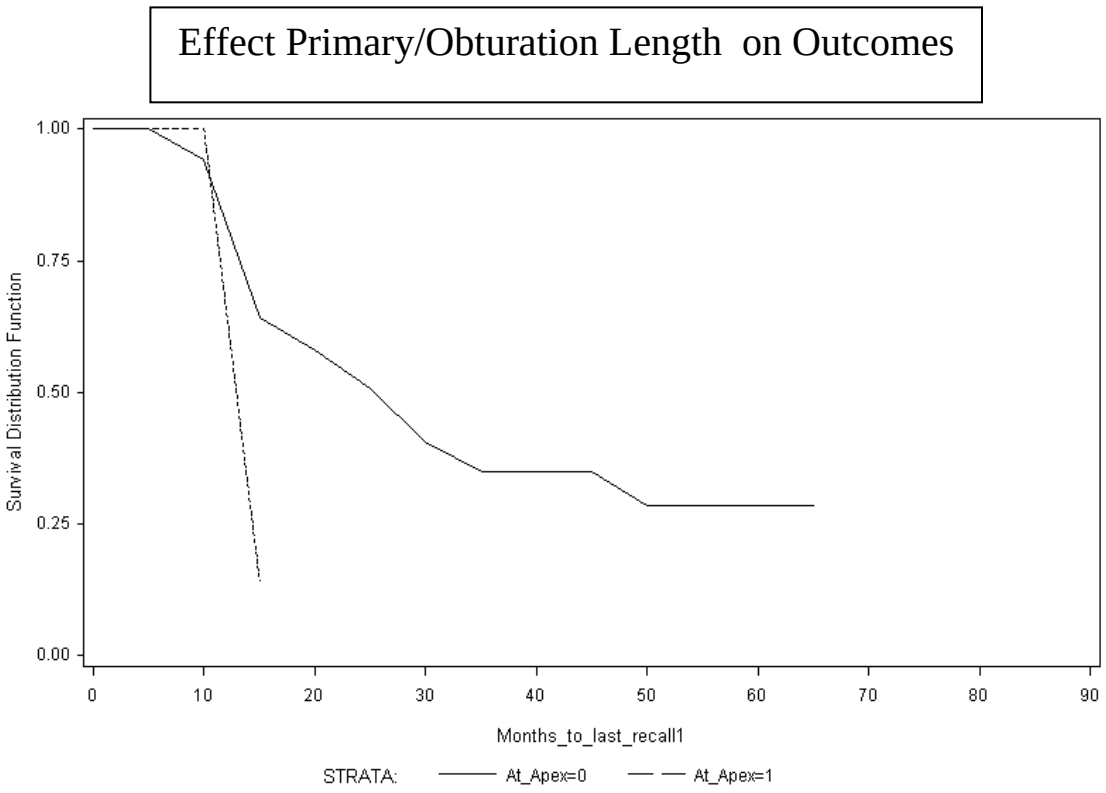


Figure 4d. Effect of Obturation Length on Outcomes

Discussion

Study Design

This prospective cohort study design assessed the effect of specific bacteria phylotypes as well as patient and treatment factors on endodontic outcomes. It is the highest level of evidence achievable for this study model. A randomized control trial could not be performed due to violation of ethical principles that would be undertaken in inoculating patients with bacteria. According to Cotton et al., [32] prospective cohort designs are considered stronger than their retrospective counterparts because they permit "blinded randomized treatment allocation, a priori standardization of techniques and sampling methods, and the simultaneous study of multiple variables." While retrospective studies are frequently used to study the success versus failure of RCT, this type of study does not allow for analysis of bacteria which are present at the time of obturation, only those which are present after the case has been determined to have failed. Prospective design is not without drawbacks. There is a higher cost associated with obtaining a large enough sample size to see significant trends in the data. Further, due to the nature of the healing process and the length of time it can take to see healing radiographically, 2-4 years according to Orstavik [33], longer follow-ups are necessary to ensure accuracy of the treatment outcome evaluation. Loss to follow-up becomes a major factor and is a function of time to follow-up. In the present study recall ranged from 97% in the short-term to 50% in the long-term despite repeated contact by phone and written letter. Patients were also enticed to participate with financial compensation for their time. Some of the reasons for loss to follow-up included patients moving to another city, recurrence of cancer, and drop-out due to age. Furthermore, a small number of patients

were able to be contacted, but declined to participate further for personal reasons. The Toronto studies by comparison reported a pooled recall of 41%.

Patient Population

All patients recruited for the study were referred to the post-graduate endodontic clinic at the University of Maryland Dental School in Baltimore, Maryland. The majority came from the undergraduate clinic. Additionally, some patients were referred from private dentists. Overwhelmingly, the patients were Caucasian and African Americans of lower socioeconomic status who lived in an urban or suburban environment. Thus the sample cohort may not be generalized to the population as a whole.

Treatment Procedures and Assessment of Healing

All treatment procedures were performed by faculty endodontists or endodontic residents working under the supervision of a faculty endodontist. All operators were calibrated to the established treatment protocol prior to treatment.

Two faculty endodontists who were not involved in the treatment procedure volunteered to assess outcomes. Outcomes were assessed on the basis of clinical signs and symptoms, as well as radiographic analysis. Outcomes were dichotomized to healed and non-healed with no "healing" group due to the small sample size and no standardized method of determining "healing."

The Periapical Index (PAI) was chosen as the best measure of periapical healing. Also, PAI was chosen due to its previously tested ability to accurately reflect periapical changes and ease of use. Alternative methods such as digital subtraction radiography

and expert opinion are available. Expert opinion has been used in previous studies, but does not provide a standard comparison and is too subjective to ensure consistent results. This method relies on experienced observers, who may also bring years of bias to their interpretation. PAI was deemed the most appropriate for this study due to the standardization of scorers along strict guidelines. Digital subtraction is an alternative method which uses superimposed radiographs computer software to calculate the difference in radiodensity between the two images. This method is not without its own drawbacks, namely cost of software, determination of area to analyze and the need to standardize radiographs which may be difficult and time consuming. Digital subtraction may be better suited to larger studies where economies of scale can be recognized and efficiency of the computer program compensates for the learning curve involved with computer software.

Assessment of Results

Bacteria and their virulence factors have been shown to be the main cause of primary and persistent endodontic disease. Previously reported studies have shown bacterial presence at the time of obturation to adversely affect endodontic outcomes [13, 34]. This finding has been corroborated in the present study. Endodontic therapy of the root canal system addresses this problem from a biologic perspective with treatments aimed at eliminating all bacteria from the root canal system. Shortcomings in achieving this goal are evidenced by previously published outcome studies and of the 25 specimens in this study from which bacteria were not eliminated. Canal anatomy, instrument limitations, and limitations in chemotherapeutic agents render this goal practically impossible.

The findings of this study may provide the clinician with treatment objectives focused on reducing bacteria and improving outcomes. Obturation to the radiographic apex was shown to both reduce bacteria and improve outcomes as compared to obturating short or long. Sjogren has previously shown the best outcomes are achieved when obturating within a specific range, namely 1-2mm from the apex, as compared to shorter fill or overfill [35]. However, his study included teeth with vital pulps which may skew the findings by masking the effects of necrotic pulps with a lesion. Theoretically, because vital pulps have sterile canal space apically, a fill short of the apex in these cases would not produce more non-healed lesions as there is no lesion to start and healthy, uninflammed pulp tissue would remain at the apex. In a similar finding, Chugal et al. examined outcomes, but categorized treatment by both pulpal and apical diagnosis and noted more "success" when obturation was closer to the radiographic apex in teeth with "disease" and "chronic apical periodontitis." [17] In teeth with a normal periapex, the finding was the converse; obturation slightly further from the apex resulted in better outcomes. The present finding suggests it is better to obturate at the radiographic apex than short or beyond. From a biologic perspective this is accomplishing both maximum intracanal bacterial reduction with minimal periapical inflammation caused by a foreign body (gutta percha and sealer).

With such biodiversity in the oral cavity the search is on for specific phylotypes responsible for various disease manifestations (apical periodontitis, abscess formation, etc.) as well as novel techniques and materials aimed at eliminating more bacteria. In the past, culture techniques have been used to identify these phylotypes with the intention of directing treatment modalities against the more virulent bacteria. For example,

Enterococcus faecalis has been shown to be associated with persistent endodontic infections [36]. Calcium hydroxide, which has been shown to reduce cultivable bacteria, is not as effective against *E. faecalis* while chlorhexidine has been [37]. This finding substantiates the use of chlorhexidine as a final rinse as was used in this study.

In the past, the idea of one bacteria/one disease was accepted (e.g. Koch's postulates). Current knowledge of infectious disease points to bacterial biofilms and multiple bacteria as the causative factors for endodontic infections [10]. In this study 125 different phylotypes were identified from the canals of teeth with bacteria remaining after disinfection. In teeth which were non-healed the mean number of unique phylotypes were identified was greater than in healed teeth. This strengthens the argument that more bacterial diversity is associated with disease. [38-39] Because of the diverse bacteria that were identified, a novel type of analysis, clustering, was used to look for patterns in the data. Cluster analysis was used to group patients based on similarities in bacterial profiles. Due to the wide diversity and the relatively small sample size no statistical differences between the groups was found. However, this methodology may prove insightful in future studies with larger sample.

Amongst the 125 various phylotypes identified from the canals of infected teeth in this study, two specific phylotypes were shown to be associated with reduced healing outcomes over the duration of the study; *Enterococcus casseliflavus* Oral Taxon 801; AF039899, and *Exiguobacterium aurantiacum*

Enterococci are of great significance in endodontic infections. They are gram-positive lactic acid bacteria that inhabit the gastrointestinal tract [40]. *Enterococcus*

casseliflavus is of interest as it is a motile bacterium which is closely related to *E. faecalis* and has commonly been associated with primary and persistent endodontic infections. *Enterococci* spp. are particularly important in medicine and dentistry as they have been shown to harbor antibiotic resistance genes which have been found in endodontic infections. [41-42] Exiguobacterium spp. are alkaliphilic, halotolerant, non-spore-forming Gram-positive bacilli. [43] *Exiguobacterium aurantiacum* is a facultative anaerobe first isolated from potato-processing effluent by Gee et al. [44] A report in 2003 found *Exiguobacterium aurantiacum* in a patient with periodontitis [45]. Using the same bacterial specimens as the current study, Li et al. reported its presence in endodontically infected teeth by [46]. Together, these two phylotypes inhabit the oral cavity and infect the root canal space. Future studies on the virulence of these organisms are warranted and strategies aimed at their elimination may lead to improved endodontic outcomes.

An important question which plagues clinicians is when to intervene when presented with an endodontically treated tooth. This question has powerful clinical relevance for the general practitioner and the restorative dentist and is particularly important in the realm of treatment planning. Furthermore, as the patient population has become more informed about treatment options and the effects of dental diseases on systemic health, the demand to know whether a particular treatment is working becomes paramount. Orstavik has shown that is can take between 2 and 4 years to determine the status of a treated tooth [33]. Another study, by Molven, showed late periapical changes-up to 27 years! [47] In this study we have addressed this question by correlating outcomes at the final recall with outcomes at the first recall. A correlation coefficient of 0.56 indicates a strong relationship between short-term and long-term healing. While not

definitive, this knowledge may be used to make treatment planning decisions sooner than was previously thought.

This study introduced a CBCT component at the time of final patient evaluation. CBCT will likely be the standard of care in the near future as ionizing radiation levels fall with improving technology. It is quickly becoming adopted in the endodontic community because of its superior image quality and ability to detect periapical disease [48]. Whereas the periapical radiograph (PA) component of this study used a series of standardized images, in comparison, a shortcoming of the CBCT component was that no image was taken at the time of treatment to serve as a baseline comparison for healing. However, the findings using CBCT were striking in comparison to PAs. While there was a positive correlation between the two technologies, CBCT was more likely to detect the presence of a lesion than PA recategorizing a previously "healed" tooth as "non-healed." When analyzing the data with CBCT all of the patient and treatment factors previously found to be significant were found not to be. However, a large amount of data could not be obtained due to loss to follow-up. The detection of disease with CBCT at late recall argues strongly for the need to correlate late findings with early finding in order to make clinical decisions.

Conclusions

Healing of endodontic lesions at 33+ months was 66% and 40% using PA (N=41) and CBCT (N=15) respectively, though survival equaled 85% (N=41). The presence of bacterial DNA at the time of obturation significantly reduces endodontic treatment outcomes in the short- and intermediate-terms, but this association diminishes in the

long-term. Specific bacterial phylotypes, *Enterococcus casseliflavus* Oral Taxon 801; AF039899, *Exiguobacterium aurantiacum* were associated with reduced long-term healing. Various patient factors such as tooth type and treatment factors such as master apical file size also influenced outcomes.

Short-term treatment outcomes are good predictors of long-term outcomes. However, CBCT imaging of teeth at 33+ months shows lesions which are not evident on traditional periapical radiographs to still be present. Future studies using CBCT are merited.

Appendix 1: Identified Phylotypes

Enterococcus casseliflavus Oral Taxon 801; AF039899

Exiguobacterium aurantiacum

Lactococcus lactis

Enterococcus sp. AF317351

Propionibacterium acnes

Enterococcus italicus/saccharominimus

Acinetobacter junii

Bacillus sp. DQ128250

Ralstonia detusculanense

Ralstonia insidiosa DQ278863

Micrococcus sp. B5W22-1, EF114312

Paenibacillus sp. VI003

Paracoccus carotinifaciens

Acinetobacter johnsonii

Acinetobacter sp. DQ336974

Bacillus sp. DQ129913

Burkholderia sp. Y14146

Micrococcus luteus

Mogibacterium timidum

Acidovorax temperans

Actinobacterium sp. AY770699

Actinomyces sp. 152R-3

Agrobacterium albertimagni

Agrobacterium tumefaciens

Bacillus sp. UY017

Bacillus sp. UY025

Bacteroidetes sp. X083

Burkholderia fungorum

Chryseobacterium sp. R-25053

Dialister invisus

Dietzia maris

Enterococcus sp. AJ576427

Flavobacteriaceae genomosp. C1 AY278614

Hydrogenophilus denitrificans

Novosphingobium aromaticivorans

Paracoccus homiensis

Paracoccus sp. AJ619068

Parvimonas micra

Prevotella oris

Pseudomonas stutzeri

Pseudoramibacter alactolyticus

Ralstonia insidiosa

Rhodobacter capsulate

Roseomonas mucosa

Rothia dentocariosa

Sphingomonas sp. SI022

Streptococcus mitis/pneumonia

Streptococcus sp. DQ444181

Acinetobacter lwoffi

Acinetobacter sp. AB195775

Actinomyces sp. AG004

Actinomyces sp. CCUG 34286

Actinomyces sp. Hal-1065; AF385521

Actinomyces sp. VT011

Actinomyces viscosus CCUG 35332

Agrobacterium larrymorrei Z30542

Alishewanella fetalis

Alpha proteobacterium DQ068912

Anoxybacillus kestanbolinensis; AY248710

Aquabacterium sp. clone YJQ-2 AY569280

Bacillus litoralis (+ others)

Bacillus sp. C20 AY504446

Bacteroidetes sp. AY563471

Bacteroidetes sp. DQ088221

Bosea sp. YS005

Bosea thiooxidans

Brevibacterium casei

Brevibacterium casei

Burkholderia ferrariae DQ514537

Burkholderia sp. clone DQ068912

Burkholderia sp. LMG 22932 AY949192

Burkholderia verschuerenii AY277699

Cellulosimicrobium cellulans AY665978 and others

Clostridium algidixylanolyticum AF092549

Clostridium clones AY570631; AB205620

Clostridium intestinale X76740

Comamonadaceae clone HrhB10; AM159227

Corynebacterium callunae

Corynebacterium simulans/xerosis

Corynebacterium sp. 9N-22 AB213381

Deinococcus proteolyticus

Dialister pneumosintes

Diaphorobacter nitroreducens

Dietzia natronolimnaea/maris

Erythromicrobiaum ramosum AF465837

Eubacterium infirmum

Eubacterium minutum

Fusobacterium sp. oral clone CY024

Gamma proteobacterium AF327558

Gemella haemolysans

Herbaspirillum huttiense

Klebsiella granulomatis DQ122317

Klebsiella oxytoca/Pantoea agglomerans/Enterobacter cancerogenus

Kluyvera ascorbata AF176566

Kocuria rhizophila

Lactobacillus iners

Lactobacter (Bacillus) thermoamylovorans AB190092

Leptothrix sp. AW043

Marimonas sp. JL-55 AY745826

Microbacterium flavescens

Microbacterium hominis

Microbacterium testaceum DQ888848

Micrococcus sp. CU207; EF522134

Moraxella osloensis

Nicotiana tabacum chloroplast Z00044

Novosphingobium aromaticivorans

Olsenella genomosp. C1 AY278623

Oribacterium sp. AO068; AF287771

Paenibacillus sp. CA007

Paenibacillus sp. GP26-03 AM162316

Pantoea agglomerans DQ518610

Paracoccus aminophilus

Paracoccus carotinifaciens

Paracoccus marcusii

Prevotella oralis

Prevotella sp. HF050

Propionibacterium sp. strain FMA5

Pseudomonas anguilliseptica

Pseudomonas otitidis

Pseudomonas saccharophila

Pseudomonas sp. cloRDC+8; AY834319

Pseudomonas sp. iMT19

Ralstonia taiwanensis

Rhodobacter gluconicum AB077986

Rhodobacter sp. SV019

Rhodobacter sp. UF020

Rubrobacter xylanophilus

Serratia marcescens

Sinorhizobium sp. L1 AJ879127

Sphingomonas sp. AJ006013

Sphingomonas sp. TZ019

Staphylococcus aureus

Stenotrophomonas

maltophilia

Streptococcus anginosus

Streptococcus constellatus

Streptococcus gordonii

Streptococcus salivarius

Streptococcus sp. BM035

Streptococcus sp. FN051

Tannerella forsythia

Triticum aestivum mitochondrial DNA

Veillonella atypical

Veillonella parvula/dispar

Williamsia maris AB010909

Appendix 2: Bivariate Analyses

Descriptor			Short-term		
		N	Healed	%	p-value
Sex	Male	17	13	76.5%	0.31
	Female	23	13	56.5%	
Age	25-99	25-99			
	≥50	24	19	79.2%	0.086
	<50	16	8	50.0%	
Race	African American	15	11	73.3%	0.79
	Caucasian	22	15	68.2%	
	Other	3	1	33.3%	
Smoking	Smoker	8	4	50.0%	1
	Non-smoker	29	21	72.4%	
Tooth Type	Anterior	14	9	64.3%	0.02
	Pre-molar	13	12	92.3%	
	Molar	13	6	46.2%	
Primary/Persistent	Primary	27	19	70.4%	0.29
	Persistent	13	7	53.8%	
Symptoms	Symptomatic	13	10	76.9%	1
	Asymptomaic	27	17	63.0%	
Bacteria	Bacteria +	25	14	56.0%	0.11
	Bacteria -	15	13	86.7%	
Periapical Diagnosis	Asymptomatic apical periodontitis	7	3	42.9%	0.39
	Symptomatic apicay periodontitis	29	22	75.9%	
	Chronic apical abscess	2	1	50.0%	
	Acute apical abscess	2	1	50.0%	
Obturation Length	At Apex	7	7	100.0%	0.05
	Short/Long	33	20	60.6%	
Final Restoration	Full Coverage	18	11	61.1%	0.9
	Amalgam/Composite	17	12	70.6%	
	Temporary	5	2	40.0%	
Master Apical File	≥45	30	19	63.3%	0.036
	<45	10	6	60.0%	

Descriptor		Intermediate-term			
		N	Healed	%	p-value
Sex	Male	12	10	83.3%	0.32
	Female	20	10	50.0%	
Age	23-89				
	≥50	22	16	72.7%	0.12
	<50	10	4	40.0%	
Race	African American	14	9	64.3%	0.73
	Caucasian	17	10	58.8%	
	Other	1	1	100.0%	
Smoking	Smoker	9	5	55.6%	1
	Non-smoker	21	14	66.7%	
Tooth Type	Anterior	9	7	77.8%	0.02
	Pre-molar	13	11	84.6%	
	Molar	10	2	20.0%	
Primary/Persistent	Primary	23	16	69.6%	0.21
	Persistent	8	3	37.5%	
Symptoms	Symptomatic	12	7	58.3%	0.2
	Asymptomaic	20	13	65.0%	
Bacteria	Bacteria +	17	7	41.2%	0.01
	Bacteria -	15	13	86.7%	
Periapical Diagnosis	Asymptomatic apical p	4	2	50.0%	0.53
	Symptomatic apicay pe	23	14	60.9%	
	Chronic apical abscess	3	3	100.0%	
	Acute apical abscess	2	1	50.0%	
Obturation Length	At Apex	6	6	100.0%	0.06
	Short/Long	26	14	53.8%	
Final Restoration	Full Coverage	16	10	62.5%	0.71
	Amalgam/Composite	12	7	58.3%	
	Temporary	4	3	75.0%	
Master Apical File	≥45	23	16	69.6%	0.28
	<45	8	4	50.0%	

Descriptor			33+ Months		
		N	Healed	%	p-value
Sex	Male	11	10	90.9%	0.31
	Female	10	6	60.0%	
Age	30-79				
	≥50	16	13	81.3%	0.55
	<50	5	3	60.0%	
Race	African American	10	9	90.0%	0.12
	Caucasian	9	5	55.6%	
	Other	2	2	100.0%	
Smoking	Smoker	7	6	85.7%	1
	Non-smoker	14	10	71.4%	
Tooth Type	Anterior	8	6	75.0%	0.09
	Pre-molar	9	8	88.9%	
	Molar	4	1	25.0%	
Primary/Persistent	Primary	15	13	86.7%	0.06
	Persistent	5	2	40.0%	
Symptoms	Symptomatic	11	8	72.7%	0.61
	Asymptomaic	5	7	140.0%	
Bacteria	Bacteria +	13	8	61.5%	0.54
	Bacteria -	8	8	100.0%	
Periapical Diagnosis	Asymptomatic apical periodontitis	2	1	50.0%	0.54
	Symptomatic apicay periodontitis	15	11	73.3%	
	Chronic apical abscess	3	3	100.0%	
	Acute apical abscess	1	1	100.0%	
Obturation Length	At Apex	3	3	100.0%	0.56
	Short/Long	18	13	72.2%	
Final Restoration	Full Coverage	9	8	88.9%	0.15
	Amalgam/Composite	9	5	55.6%	
	Temporary	3	3	100.0%	
Master Apical File	≥45	15	13	86.7%	0.11
	<45	6	3	50.0%	
BMI	25+	4	4	100.0%	0.53
	<25	15	10	66.7%	

Descriptor		33+ Months CBCT			
		N	Healed	%	p-value
Sex	Male	7	3	42.9%	0.6
	Female	8	2	25.0%	
Age	30-79				
	≥50	11	4	36.4%	1
	<50	4	1	25.0%	
Race	African American	6	2	33.3%	1
	Caucasian	7	3	42.9%	
	Other	2	0	0.0%	
Smoking	Smoker	1	1	100.0%	0.33
	Non-smoker	14	4	28.6%	
Tooth Type	Anterior	5	2	40.0%	0.61
	Pre-molar	7	3	42.9%	
	Molar	3	0	0.0%	
Primary/Persistent	Primary	11	4	36.4%	1
	Persistent	4	1	25.0%	
Symptoms	Symptomatic	7	3	42.9%	1
	Asymptomaic	8	2	25.0%	
Bacteria	Bacteria +	8	1	12.5%	0.11
	Bacteria -	7	4	57.1%	
Periapical Diagnosis	Asymptomatic apical p	2	0	0.0%	0.54
	Symptomatic apicay pe	10	2	20.0%	
	Chronic apical abscess	3	3	100.0%	
	Acute apical abscess	0	0	0.0%	
Obturation Length	At Apex	3	1	33.3%	1
	Short/Long	12	4	33.3%	
Final Restoration	Full Coverage	8	3	37.5%	0.79
	Amalgam/Composite	5	1	20.0%	
	Temporary	2	1	50.0%	
Master Apical File	≥45	12	4	33.3%	1
	<45	3	1	33.3%	
BMI	25+	5	2	40.0%	1
	<25	10	3	30.0%	

Descriptor		Long-Term Outcome			
		N	Healed	%	p-value
Sex	Male	18	14	77.8%	0.2
	Female	23	13	56.5%	
Age	25-99	25-99			
		55.6			
	≥50	25	19	76.0%	0.086
	<50	16	8	50.0%	
Race	African American	16	12	75.0%	0.51
	Caucasian	22	13	59.1%	
	Other	3	2	66.7%	
Smoking	Smoker	10	8	80.0%	1
	Non-smoker	22	11	50.0%	
Tooth Type	Anterior	14	12	85.7%	0.002
	Pre-molar	14	11	78.6%	
	Molar	13	3	23.1%	
Primary/Persistent	Primary	28	20	71.4%	0.07
	Persistent	13	5	38.5%	
Symptoms	Symptomatic	16	11	68.8%	0.63
	Asymptomaic	25	15	60.0%	
Bacteria	Bacteria +	25	14	56.0%	0.09
	Bacteria -	16	13	81.3%	
Periapical Diagnosis	Asymptomatic apical pe	7	4	57.1%	0.55
	Symptomatic apicay per	29	18	62.1%	
	Chronic apical abscess	3	3	100.0%	
	Acute apical abscess	2	1	50.0%	
Obturation Length	At Apex	7	7	100.0%	0.07
	Short/Long	34	19	55.9%	
Final Restoration	Full Coverage	19	12	63.2%	0.22
	Amalgam/Composite	17	9	52.9%	
	Temporary	5	4	80.0%	
Master Apical File	≥45	30	22	73.3%	0.036
	<45	11	4	36.4%	

Appendix 3: Multivariate Analysis and Kaplan-Meier

Cox Multivariate Regression	P	Short-Term HazardRatio	95%	CI
Roots	0.008	0.29	0.11	0.73
Obturation Length From Apex	0.067	2.7	0.93	7.9
Primary/Persistent	0.025	0.31	0.12	0.87

Cox Multivariate Regression	P	Overall HazardRatio	95%	CI
Bacteria Presence	0.75	1.17	0.44	3.09
Roots	0.05	0.256	0.06	1
Obturation Length From Apex	0.025	3.58	1.17	10.9
Primary/Persistent	0.026	0.292	0.09	0.86
MAF≥45	0.85	0.884	0.24	3.23

Kaplan-Meier Analysis	Log-Rank, p=
Roots	0.002
Primary/Persistent	0.08
Symptoms	0.59
Bacteria Presence	0.08
Periapical Diagnosis	0.84
Obturation Length From Apex	0.002
Final Restoration	0.19
MAF≥45	0.054

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