EX-VIVO EVALUATION OF THE ANTIMICROBIAL ACTIVITY OF VARIOUS COMBINATIONS OF INTRACANAL MEDICAMENTS AND VEHICLES ON SELECTED PATHOGENS FROM DECIDUOUS MOLARS WITH NECROTIC PULP

Synopsis Submitted to
THE KLE ACADEMY OF HIGHER EDUCATION AND RESEARCH, BELAGAVI
(KLE DEEMED UNIVERSITY)
[Declared as Deemed-to-be-University u/s 3 of the UGC Act, 1956 vide Govt. of India Notification No.F.9-19/2000-U.3 (A)]
(Re-Accredited ‘A’ Grade by NAAC & Placed in ‘A’ Category by MHRD, GoI)

For the Award of the Degree of
Doctor of Philosophy
In the Faculty of Dentistry
(Pedodontics & Preventive Dentistry)

By
Dr Triveni Mohan Nalawade
(Registration No: KLEU/Ph.D/13-14/ DOUNI3008)

Under the Guidance of
Dr Kishore Bhat MD
Hon. Consultant Microbiologist,
KLE University,
Belagavi, Karnataka

Dr Alka D. Kale MDS, Ph.D
Dean, Faculty of Dentistry
KLE University
Belagavi, Karnataka.

2016
INTRODUCTION

1.1 Background:

Preservation of primary tooth in a disinfected state is the best space maintainer. Pulp therapy is the ideal treatment alternative but it has its own challenges. Pulp therapy in primary teeth is a subject of countless controversies, exhaustive literature and numerous techniques in an attempt to preserve the natural tooth. Some of the challenges involving pulp therapy in primary teeth is the complex root canal morphology with numerous ramifications and anatomical irregularities. The highly porous inter-radicular dentin at the pulpal floor allows pulpal infection to easily spread to the furcal region and can affect the developing permanent tooth bud.

Successful pulp therapy in primary teeth depends on reduction and elimination of root canal infection; although no techniques for root canal instrumentation have been followed. Necrotic primary teeth, especially of long-standing nature and symptomatic with periapical bone destruction tend to harbor a high bacterial load and more complex anaerobic bacterial flora. Intracanal medicaments are essential to reduce these virulent and pathogenic bacteria for successful pulp therapy. Success of pulpectomized teeth depends on adequate disinfection of root canal systems before obturation of the primary tooth. Hence the use of intracanal medicaments may be required to resolve infection and promote healing.

Literature related to the efficacy of intracanal medicaments in deciduous teeth is scanty as compared to many studies in the permanent teeth. Hence, an impending need is upon the pedodontic speciality to find a reasonable alternative, which can best adapt to primary tooth physiology with maximum clinical benefit and minimum side effects. Hence, in this study an attempt has been made to evaluate the antimicrobial
activity of innovative combinations of intracanal medicaments and vehicles on selected bacteria isolated from deciduous molars with necrotic pulp.

1.2 Literature Review:

Ideally an intracanal medicament should be able to neutralize the virulence of micro-organisms and pathogenic factors (such as proteins, enzymes, toxins, aggregation substances) and induce a host response that favours periapical tissue healing. The continuous presence, however, of positive microbial cultures after root canal shaping, sanitization and use of calcium hydroxide as interappointment, intracanal dressing justifies the investigations of antimicrobial substances.\(^8\)

Calcium hydroxide introduced by Hermann in 1920, is the gold standard, against which the efficacy of all other medicaments has been tested since many years. Though there is significant literature about the use of Calcium hydroxide and its effects, the need to find other alternatives is evident through the failure of the calcium hydroxide to sterilize organisms within the dentinal tubules in many cases.\(^9\) The failure of Calcium hydroxide against various intracanal microorganisms, researchers then turned their attention to antibiotics and natural alternatives.

The use of antibiotics as intracanal medicaments began as early as 1951 where Grossman used the polyantibiotic paste for the first time known as PBSC consisting of Penicillin, Bacitracin, Streptomycin and Caprylate Sodium.\(^10\) Later, other broad spectrum antibiotics were introduced singly or in combination to overcome the polymicrobial endodontic infections of pulpal and periradicular tissues. Ledermix paste was then introduced by Schroeder and Tridian in 1960 which was a combination of a Tetracycline based antibiotic – Demeclocycline hydrochloride and a Corticosteroid component – Triamcinolone acetonide.\(^11\) The most recent addition of
this list of intracanal medicaments is the introduction of Triple Antibiotic Paste by Sato et al in 1992 consisting of Ciprofloxacin, Metronidazole and Minocycline.\textsuperscript{12}

This Triple Antibiotic Paste (TAP) became the mainstay of all intracanal medicaments as compared to the gold standard; Calcium hydroxide due to its bactericidal action and excellent antimicrobial activity. It is extensively being used in the Regenerative Endodontics as an intracanal medicament to disinfect the canal, prior to inducing blood clot formation. However, researchers have tried to change this combination ever since with various antibiotic and vehicles combinations.

Then through numerous \textit{in vitro} studies, efficacy of Double Antibiotic Paste (DAP) was proved to be equally effective as TAP. A randomized, double blinded study registered at Clinical Trials registry NIH(USA) Identifier:NCT00881491 is evaluating the treatment outcomes in permanent teeth with necrotic pulp and immature root development that undergo a regenerative procedure using a triple antibiotic paste (Ciprofloxacin, Metronidazole, Minocycline) versus a double antibiotic paste (Ciprofloxacin, Metronidazole) compared to the commonly used mineral trioxide aggregate (MTA) apexification treatment since 2009 and has been completed in February 2016.\textsuperscript{13}

Though this overcame the use of Minocycline which has an detrimental effect of discolouring teeth\textsuperscript{14} but the use of antibiotics always had an inherent risk of development of antibiotic resistance. So, the use of Biocides and herbal alternatives began; as intracanal medicaments from these sources do not develop resistance.\textsuperscript{15,16} Also, the role of vehicles needs to be explored and used to its optimum benefit to have more effective, biocompatible and economical intracanal medicaments for thorough disinfection of root canals and thus, improving the success of pulp therapy and preserving the deciduous teeth.
1.3 Justification for the study:-

However, these antibiotics have their own advantageous contributions and side effects, with the eventual drug resistance as the most catastrophic consequence. Hence, an impending need is upon the fraternity of dentistry to find a reasonable alternative, which can best adapt to primary tooth physiology with maximum clinical benefit.

Selection of Antimicrobials:

1. Amoxicillin and clavulanic acid is one of the most effective antibiotic against oral microflora which consists of gram positive and gram negative obligate and facultative anaerobes including resistant Enterococci and β-lactamase producing bacteria.17

2. Metronidazole as infected root canals of teeth with abscess have predominant obligate anaerobes.12,18

3. Chlorhexidine(CHX) has a broad spectrum activity against a wide array of oral microbes, high substantivity and capacity of inhibiting dentin matrix metalloproteinases.15

4. Also, as per Principles of Antibiotic therapy; prudent combination of two bactericidal drugs and that too narrow spectrum drugs should be used to overcome the global menace of antibiotic resistance.19

Selection of Vehicles:

1. The vehicle Polyethylene glycol showed antimicrobial effect and allowed greater penetration of the association with different intracanal medicaments.20

2. The vehicle Propylene glycol delivered dye through the root canal system rapidly and more effectively indicating its potential use in delivering intracanal medicaments.21
3. Glycerine as it is readily available at all pharmacies.

Selection of Micro-organisms:

A) FOR SUSCEPTIBILITY TO ANTIMICROBIAL DRUG COMBINATIONS:

1. *Streptococcus spp*; one of the pre-dominant species almost making upto 85% of the cultivable microflora of infected root canals by microbial culture.\(^{22}\)

2. *Porphyromonas gingivalis*; as most commonly isolated obligate anaerobe and many studies have shown an association between black-pigmented bacteria and endodontic infections.\(^{23,24}\)

3. *Enterococcus faecalis*; is found in both primary and secondary endodontic infections in Deciduous teeth and is a virulent organism held responsible for failure of endodontic therapy.\(^{25,26}\)

B) FOR POLYMERASE CHAIN REACTION:

1. *Porphyromonas gingivalis*; has shown contrary findings in the two studies in deciduous teeth using polymerase chain reaction.\(^{27,28}\)

2. *Treponema denticola*; as they are difficult to grow and identify through microbial cultures.\(^{29}\)
AIMS AND OBJECTIVES

1.4 RESEARCH HYPOTHESIS

To test the equality of various combinations of intracanal medicaments and vehicles on selected pathogens from deciduous molars with necrotic pulp.

1.5 AIMS AND OBJECTIVES

Aim of the study:- To test the antimicrobial activity of various combinations of intracanal medicaments and vehicles on selected pathogens from deciduous molars with necrotic pulp.

Objectives of the study:-

PRIMARY OBJECTIVE

1. To evaluate and compare the antibacterial activity of:

   Group A: Ciprofloxacin and Metronidazole (Double Antibiotic Paste-DAP) with glycerine,
   Group B: Ciprofloxacin and Metronidazole (Double Antibiotic Paste-DAP) with polyethylene glycol,
   Group C: Ciprofloxacin and Metronidazole (Double Antibiotic Paste-DAP) with propylene glycol,
   Group D: Amoxicillin with clavulanate plus Metronidazole (Modified Double Antibiotic Paste-Modified DAP) with glycerine,
   Group E: Amoxicillin with clavulanate plus Metronidazole (Modified Double Antibiotic Paste-Modified DAP) with polyethylene glycol,
   Group F: Amoxicillin with clavulanate plus Metronidazole (Modified Double Antibiotic Paste-Modified DAP) with propylene glycol,
   Group G: Chlorhexidine gluconate with glycerine,
Aims and Objectives

Group H: Chlorhexidine gluconate with polyethylene glycol and
Group I: Chlorhexidine gluconate with propylene glycol on the selected
pathogens from infected root canals of deciduous molars.

SECONDARY OBJECTIVE

1. To identify selected micro-organisms from root canals of infected deciduous
   molars by means of microbial culture. (*Streptococcus* spp, *Enterococcus faecalis* and *Porphyromonas gingivalis*)

2. To detect difficult to grow microorganisms from root canals of infected
deciduous molars by means of Polymerase Chain Reaction. (*Enterococcus faecalis*, *Porphyromonas gingivalis* and *Treponema denticola*)
MATERIALS AND METHODS

The present research was conducted during the time period from May 2014 to July 2015 on 5-8 year old patients attending the Department of Pedodontics and Preventive Dentistry, KLE VK Institute of Dental Sciences, Belagavi. The present experimental study was conducted in 2 phases. The Phase 1 consisted of the procurement of materials required for the study followed by *in vitro* bactericidal activity of vehicles using broth dilution method against American Type Culture Strains (ATCC) strains. Later, *in vitro* antibacterial activity of innovative endodontic medicaments and different vehicle combinations using agar well diffusion against ATCC strains was done using pure and commercial drugs. The Phase 2 consisted of a pilot study before the recruitment of first subject followed by the main study which was conducted on children selected based on the inclusion and exclusion criteria.

The main study comprised of two parts pertaining to the primary and secondary objectives. The primary objective of the research was assessment of the antibacterial activity of the three intracanal medicaments, i.e. DAP, modified-DAP and Chlorhexidine with three vehicles, namely Glycerine, PEG and PG against three selected endodontic microorganisms using an *Ex-vivo* model. The selected endodontic pathogens (*Streptococcus* spp, *Enterococcus faecalis*, *Porphyromonas gingivalis*) were isolated from deciduous molars with necrotic pulp. The endodontic sample collection procedure, its culture, and identification followed by the *Ex-vivo* antibacterial activity assessment was standardized. The same endodontic sample was used for the secondary objective of research following standardization of DNA extraction procedure and PCR assay for detection of the specified organisms i.e. *Enterococcus faecalis*, *Porphyromonas gingivalis* and *Treponema denticola*. 
Phase 1: *In vitro* studies against ATCC strains:

**Procurement of**
- Drugs
- Vehicles
- Microbiology related materials

**A. Determination of**
**Bactericidal activity of**
1. Propylene glycol (PG)
2. Glycerine
3. Polyethylene glycol (PEG) 400
4. PEG 1000
5. PG + PEG 400

**B. Antibacterial activity of**
**endodontic medicaments and**
**vehicle combinations against**
**selected microorganisms using**
**pure drugs**
1. *Streptococcus mutans*
2. *Staphylococcus aureus*
3. *E. faecalis*
4. *P. gingivalis*

**C. In vitro antibacterial**
**activity of innovative**
**endodontic medicaments**
**and different vehicle**
**combinations using**
**commercial**
1. *Streptococcus mutans*
2. *Staphylococcus aureus*
3. *E. faecalis*
4. *E. coli*

Phase 2: Main study:

**A. Primary Objective:** *Ex-Vivo* evaluation of Antimicrobial activity of various combinations of intracanal medicaments and vehicles

**Intracanal Medicaments** + **Vehicles** → **Clinical Isolates of**

- **DAP (C+M)**
- **Modified DAP (A+M)**
- **CHX**
- **Glycerine**
- **PEG 400**
- **PG**

**Streptococcus spp**
**Porphyromonas gingivalis**
**Enterococcus faecalis**

**B. Secondary Objective (Polymerase Chain Reaction)**

- Detection of *E. faecalis* from endodontic samples by Conventional PCR
- Detection of *P. gingivalis* and *Treponema denticola* by Multiplex PCR
Study design: **Experimental study**

**Phase 1**

In vitro Determination of Bactericidal activity of vehicles against ATCC strains

In vitro Antibacterial activity of endodontic medicaments and vehicle combinations against ATCC strains using pure and commercial drugs

**Phase 2**

Pilot study

Main study

Outpatients with the inclusion criteria, parental consent and assent

Sample collection done and transported in RTF to BSRC

A. **Primary Objective**

<table>
<thead>
<tr>
<th>Step III</th>
<th>Microbiological culture</th>
</tr>
</thead>
<tbody>
<tr>
<td>Step IV</td>
<td>Antimicrobial activity using <em>ex-vivo</em> model against:</td>
</tr>
</tbody>
</table>

1) *Streptococcus* spp  
2) *Enterococcus faecalis*  
3) *Porphyromonas gingivalis*  

<table>
<thead>
<tr>
<th>Step V</th>
<th>Polymerase Chain Reaction</th>
</tr>
</thead>
<tbody>
<tr>
<td>Step VI</td>
<td>Gel Documentation using Agarose gel Electrophoresis:</td>
</tr>
</tbody>
</table>

1) *Enterococcus faecalis*  
2) *Porphyromonas gingivalis*  
3) *Treponema Denticola*  

B. **Secondary Objective**

Microbial Isolation and Identification

DNA Isolation
SCHEMATIC REPRESENTATION OF THE METHODOLOGY

<table>
<thead>
<tr>
<th>Phases of study</th>
<th>Study conducted</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Phase 1</strong></td>
<td><em>In vitro</em> bactericidal activity of propylene glycol, glycerine, polyethylene glycol 400, and polyethylene glycol 1000 against selected microorganisms. (November - December 2013)</td>
</tr>
<tr>
<td></td>
<td><em>In vitro</em> antibacterial activity of innovative endodontic medicaments and different vehicle combinations using pure and commercial drugs. (December 2013 - January 2014)</td>
</tr>
<tr>
<td><strong>Phase 2</strong></td>
<td>Pilot study was conducted for standardization of sample collection, culture, antimicrobial activity assessment and DNA isolation. (January - March 2014)</td>
</tr>
<tr>
<td></td>
<td><em>Ex-vivo</em> antimicrobial activity of various combinations of intracanal medicaments and vehicles on selected pathogens from deciduous molars with necrotic pulp. (May 2014 - June 2015)</td>
</tr>
<tr>
<td></td>
<td>Detection of <em>E. faecalis</em>, <em>P. gingivalis</em> and <em>Treponema denticola</em> from the same endodontic samples by PCR. (June 2015 - July 2015)</td>
</tr>
</tbody>
</table>

Sample Size Estimation:
Considering 95% confidence interval and 5% tolerable error and Power of the test at 80; sample size will be calculated using formula:

\[
n = \frac{Z^2 \times q}{0.01 \times p}
\]

\[
n = \frac{(1.96)^2 \times 0.01}{4 \times 20}
\]

\[
n = \frac{96}{0.01}\]

\[
n = 96
\]

Where, \(Z\) stands for Standard Normal Variate.
PROCEDURE:

Phase 1:

A. *In vitro* Determination of Bactericidal activity of vehicles against ATCC strains by broth dilution

This study was carried out in Dr Prabhakar Kore Basic Science Research Centre, KLE University, Belagavi. The susceptibility of these organisms to PEG 400, PEG 1000, Propylene glycol, glycerine, and combination of Propylene glycol with PEG 400 was assessed using Broth dilution assay as Minimum Inhibitory Concentration (MIC) determination can be readily converted to determine the Minimum Bactericidal Concentration (MBC). As there is no study in literature comparing the effect of vehicles only, hence bactericidal activity of commonly used vehicles for intracanal medicaments was determined to find out the vehicle with maximum bactericidal activity against *Streptococcus mutans* ATCC 25175, *Staphylococcus aureus* ATCC 12598, *Enterococcus faecalis* ATCC 35550 and *Eschericia coli* ATCC 25922 using Brain Heart Infusion (BHI) broth for MIC and BHI agar for MBC as culture media. Triplicates were performed for each of the microorganisms.

B. *In vitro* Antibacterial activity of endodontic medicaments and vehicle combinations against ATCC strains by agar well diffusion method using pure drugs

The endodontic medicaments evaluated were DAP i.e. Ciprofloxacin with Metronidazole, modified DAP i.e. Amoxycillin clavulanate with Metronidazole and 2% Chlorhexidine gluconate solution and their combination with Polyethylene glycol (PEG 400), Propylene glycol (PG), glycerine and combinations of PG with PEG. The antimicrobial activity was carried out against five commonly isolated microorganisms.
from root canals; *Streptococcus mutans* ATCC 25175, *Staphylococcus aureus* ATCC 12598, *Enterococcus faecalis* ATCC 35550, *Porphyromonas gingivalis* ATCC 33277 and *Eschericia coli* ATCC 25922 strains were utilized for this *in vitro* study. *Streptococcus mutans* is one of the most commonly isolated organism from root canals of infected teeth and considered to play a crucial role in pathogenesis of dental caries. Whereas *Staphylococcus aureus* and *Enterococcus faecalis* are known to be resistant species, and not being susceptible to the most common drug regimens. *Porphyromonas gingivalis* is predominant in necrotic root canals of teeth and related to the signs and symptoms of periapical disease. Gram negative obligate anaerobe are considered to be more resistant due to the outer membrane of their cell-wall structure and as in the case of *Porphyromonas gingivalis.*

Evaluation of MIC of individual drugs and their combinations with vehicles was performed using broth dilution method first for single drug namely 2% Chlorhexidine gluconate, Ciprofloxacin, Amoxycillin clavulanate, Metronidazole and their combinations i.e. Ciprofloxacin with Metronidazole(C+M) and Amoxycillin clavulanate with Metronidazole(A+M). The concentrations used were 0.5mg/ml of Ciprofloxacin, 0.5mg/ml of Amoxicillin clavulanate and 2mg/ml of Metronidazole for single drugs and 1mg/ml concentration for both the drug combinations i.e. C+M and A+M.

The antibacterial activity of endodontic medicaments and various vehicles was determined using agar well diffusion method in terms of zones of inhibition. The endodontic medicaments tested were DAP, modified DAP & 2% CHX with PEG, PG, glycerine and PEG+PG. BHI agar plates have been used for facultative organisms and Blood agar was used for obligate anaerobes i.e. *P. gingivalis* for agar well-diffusion method. The tests were repeated in triplicates.
C. *In vitro* Antibacterial activity of innovative endodontic medicaments and different vehicle combinations against ATCC strains by agar well diffusion method using commercial drugs

The selected microorganism i.e. *Streptococcus mutans* (ATCC 25175), *Staphylococcus aureus* (ATCC 12598), *Enterococcus facealis* (ATCC 35550) and control as *Escherichia coli* (ATCC 25922) were inoculated in BHI broth as per CLSI guidelines for antimicrobial susceptibility testing. Also control strain of *E. coli* (ATCC 25922) was kept for monitoring antibacterial susceptibility testing. The agar well diffusion method was carried out using BHI agar plates. Agar well diffusion assay using commercially available drugs was carried out to standardize the amount of drugs required to be placed as intracanal medicaments to simulate the clinical situation. It also aided in comparison of antimicrobial activity assessment by means of agar well diffusion method and the *ex-vivo* model.

**Preparations of Microbial Inocula:**

The turbidity of the direct colony suspension of each organism to be tested was adjusted to 0.5 McFarland standard, for *S. mutans, S. aureus, E. faecalis, E. coli* as per CLSI guidelines.\(^3\)

**Preparation of the various combinations of endodontic medicaments and vehicles:**

Commercially available antibiotics such as Ciprofloxacin, Metronidazole, and Amoxicillin Clavulanate and 2% Chlorhexidine gluconate solution were used in the study. The removal of enteric coating of the tablets was done with a surgical scalpel # 15 blade with Bard Parker handle No.3 was used. The drugs were powdered using sterile porcelain mortar and pestle. These powdered drugs were stored in air-tight containers and refrigerated till use. Each drug in concentration of 0.5 mg each of
Ciprofloxacin 400mg, Metronidazole 200mg and Amoxicillin clavulanate 325mg was taken using a weighing scale. To create a paste with proper thickness the DAP and modified DAP were mixed with respective four vehicles prior to placement in the wells made in BHI agar plates for agar well diffusion assay in 1:1:1 ratio.\textsuperscript{38,39,40}

50 µl of 2% Chlorhexidine gluconate solution was be taken with a micropipette.

These endodontic medicaments in the ratio of 1:1 were mixed with respect to one drop i.e. 10 µl of vehicles i.e. PEG 400, PG, glycerine and combinations of PG with PEG whereas 2% Chlorhexidine gluconate was mixed with 50 µl of each vehicle. The tests were repeated three times.

**Phase 2:**

**I. Approvals:**

The **Ethical Clearance Certificate** was obtained for the proposed activities as per the experimental protocol approved by **Institutional and PhD Human Ethical Committee**. The PhD Human Ethical Committee meeting held on 29\textsuperscript{th} April 2014, and the research protocol, Informed consent, Assent form and the Proforma were approved.

Before collection of the samples from the first subject, the pilot study was approved by the Institutional Research and Ethical Committee followed by the PhD Human Ethical Committee Ref. No. KLEU/Ethic/14-15/D-73 approval.

Informed written consent was obtained from all the parents of children participating in the study. Assent was obtained from all the children participating in the study and approved.
Source of Data:

The study was conducted on patients seeking treatment at Department of Pedodontology and Preventive Dentistry, KLE VK Institute of Dental Sciences, Belagavi.

Selection of Subjects:

One hundred eleven endodontic samples were taken from 37 teeth (28 mandibular molars and 9 maxillary molars: three root canals per tooth) from 35 (15 female and 20 male) 5-8 year old children residing in Belagavi city and attending the outpatient department of Pedodontology and Preventive Dentistry, Belagavi, India. Informed consent was taken from each parent and assent was taken from children too. A proforma was recorded for each patient comprising of case history recording, along with investigations & diagnosis. Patients willing to participate in the study and fulfilling the inclusion criteria were included in the study.

INCLUSION CRITERIA:

1. Patient aged 5-8 years.
2. Deciduous molar teeth with necrotic pulp, chronic abscess and/or sinus tract.
3. Deciduous molar teeth with no gingival recession and free of periodontal pockets more than 2 mm deep.
4. Deciduous molars with almost 2/3rd of the roots present and no previous pulp therapy.
5. Compliant patients.

EXCLUSION CRITERIA:

1. Teeth with more than two third of tooth structure lost or tooth which could not be isolated with rubber dam.
2. Patients with antibiotic usage or antimicrobial mouthwashes for systemic diseases in past 3 weeks.

3. Teeth previously treated by any form of pulp therapy.

4. Teeth with failed pulp therapy and need re-treatment due to developed signs or symptoms.

5. Patient with systemic diseases and any history of drug allergy.

6. Unco-operative patients and patients or their parents not willing to accept proposed treatment plan and/or participate in the study.

➢ Main Study:

A. Primary Objective – Microbial culture and Antimicrobial activity of various combinations of intracanal medicaments and vehicles using ex-vivo model:

Step I

Outpatients with the inclusion criteria reporting to the Department of Pedodontics and Preventive Dentistry at the KLE VK Institute of Dental Sciences were selected for the study, informed consent from parents and assent from children taken. Proforma was filled; and preoperative intraoral periapical radiographs was taken.

Step II

Initial antisepsis of the oral cavity was carried out with 0.12% CHX mouthwash or swabbed with 0.12% Chlorhexidine swabs.\textsuperscript{31,41}

- Local anaesthesia was administered using 2% lignocaine with 1: 80,000 adrenaline.
- Rubber dam isolation was done of the single tooth which was diagnosed to be necrotic after taking a pre-operative diagnostic radiograph.
- Antisepsis with 1% Chlorhexidine digluconate was done.\textsuperscript{31,41}
Materials and Methods

- Standard access cavity preparation was done using a sterile round bur.
- The patency of the canals were checked with a #15 K file up to the working length assessed from the pre-operative radiograph.
- The #15 K-file was used in a filing motion i.e. up and down motion to agitate the contents of the canal for 30 seconds after insertion to the depth of the working length followed by two paper points sequentially inserted for 30 seconds each to soak up the contents within the root canal.
- The handle of #15 K file was cut off after sample collection and transferred to the Eppendorf tube containing reduced transport fluid i.e. RTF (transport medium).42
- Absorbent points were taken into the canal till the apex and were held there till 30 seconds, each one following another to absorb all the fluid in the root canal.42
- If the canal was dry, a small amount of RTF was added drop by drop into the dry canal to avoid flooding but at the same time, make sample collection feasible.28
- The absorbent points were then removed and transferred directly into a tube containing RTF.42
- This tube was taken to the Dr. Prabhakar Kore Basic Science Research Centre where it was processed within 2 hours of sample collection for culture and PCR assay.
- Reduced transport fluid in eppendorf tubes supplemented was used as transport medium. It was collected from BSRC as soon as a patient was recruited for the study and consent was taken; prior to sample collection.
Materials and Methods

- After thoroughly shaking the endodontic biomaterial sample in a mixer for 60 secs, a part (required amount) of each sample were used for culture and the rest of the sample was frozen immediately at -20 °C until assayed by PCR. \(^{43,44}\)

Step III

For Microbial Isolation and Identification:

- Samples were processed microaerobically by the candle (CO\(_2\)) jar system and anaerobically inside an anaerobic chamber (85% N\(_2\), 10%H\(_2\), 5% CO\(_2\)) by evacuation replacement procedure.

- In the evacuation replacement; anaerobiosis was achieved by the formation of the hydrogen and carbon dioxide gas mixture using a mixture of 500mg of sodium carbonate, 500mg of citric acid and 500mg of sodium borohydride.

- The liberated hydrogen combines with oxygen in the presence of palladium catalyst to form water. At the same time, traces of carbon di oxide released from the first reaction stimulate growth of anaerobic bacteria.

- Each sample was inoculated on a 5% sheep-blood agar plate with Vitamin K and Haemin in a CO\(_2\) jar observed after 48 hours, a 5% sheep-blood agar plate with Vitamin K and Haemin along with a 5% sheep-blood agar plate with Kanamycin incubated anaerobically and Enterococcus Confirmatory Agar incubated at 37 °C in CO\(_2\) jar, for 72 hours each till growth was observed.

- Colonies were counted in CFUs and pure colonies were picked and gram staining was done in order to confirm the targeted microorganism.

- Strains were identified based on Gram staining and classified by colony morphology, oxygen tolerance and biochemical test. \(^{45}\)

- These isolated pure colonies were picked up using a sterile nichrome loop and subcultured to get pure colonies for \textit{ex-vivo} tests.
Materials and Methods

- All isolated microbial strains of *Streptococcus spp*, *Enterococcus faecalis*, *Porphyromonas gingivalis* were subjected to antimicrobial susceptibility testing using *ex-vivo* model.
- Treatment was completed in the children from whose teeth biomaterial has been collected in the Department of Pedodontics and Preventive Dentistry.

Microbial Analysis:

- The endodontic biomaterial sample was vortexed for uniform mixing of the sample with RTF.
- The media and incubation done for the culture were:
  a. Blood agar for *Streptococcus Spp* incubated in CO$_2$ jar for 48 hours.
  b. Blood agar and Kanamycin blood agar for *Porphyromonas gingivalis* incubated in anaerobic jar for 72 hours.
  c. Enterococcus Confirmatory Agar for *Enterococcus faecalis* incubated in CO$_2$ jar for 72 hours.

**Confirmation of *Streptococcus spp*, *P. gingivalis* and *Enterococcus Faecalis by gram staining***:

- Gram staining procedure and the SOP was developed. Gram staining was done for confirmation of *Streptococcus spp*, *P. gingivalis* and *Enterococcus faecalis* initially followed by biochemical tests.

Step IV

**Antimicrobial activity assessed using ex-vivo model:**

- **Procurement of extracted teeth and their preparation**
  1. A sufficient number of extracted, multi-rooted human deciduous teeth, including primary maxillary and mandibular molars with almost 2/3$^{rd}$ of the roots present were selected for the study.
2. The teeth were cleaned with periodontal curettes to remove periodontal tissues and bone and stored in saline.

3. The teeth were then sterilized in autoclave at 121°C and 15 psi for 20 minutes.

4. Access cavity preparation was done using sterile burs with air-rotor under continuous water cooling.

5. After access cavity preparation the extracted deciduous molars were again stored in saline and were subjected to sterilization by autoclave prior to their use in the ex-vivo model.

➢ Ex-vivo Model for antimicrobial activity of various combinations of intracanal medicaments and vehicles on selected pathogens cultured from deciduous molars with necrotic pulp

1. The identified colonies of microorganisms namely Streptococcus spp and Enterococcus faecalis were then picked up by means of a loop, grown in broth and were adjusted to 0.5 McFarland standard. Porphyromonas gingivalis inoculum was adjusted to 1.0 McFarland standard.

2. Streptococcus spp, and Porphyromonas gingivalis was lawn cultured on Blood agar whereas Enterococcus faecalis was on Brain heart infusion agar.

3. After the agar plates were made, checked for sterility and inoculated in vertical laminar airflow unit, the sterilized teeth were placed in the agar with a pair of sterile forceps.

Preparation of the 2Mix:

For the Ciprofloxacin, Metronidazole and Amoxicillin clavulanate tablet, the enteric coating is removed with a scalpel. The tablet is pulverized using mortar and pestle and, the powder is segregated. Antibiotics are prepared freshly before every use. Antibiotic paste remains are discarded and single antibiotic powders are stored
Materials and Methods

sealed in air tight containers separately. The 2 antibiotics are mixed together with respective vehicles in a ratio of 1:1:1. A creamy consistency is prepared with good handling characteristics. Then this mix is used wherever essential.46,38,39

**Gel preparation:**

To make the handling characteristics similar to the DAP and modified DAP antibiotic pastes, instead of using 2% CHX which was available commercially as irrigating solution, 2% CHX gel was formulated.47

**Preparation of DAP:** Ciprofloxacin, and Metronidazole was taken in a ratio of 1:1.

**Preparation of Modified DAP:** Amoxicillin clavulanate, and Metronidazole was taken in a ratio of 1:1.

**Preparation of Chlorhexidine gluconate:** 2% Chlorhexidine gluconate will be taken in one increment using a standard plastic filling or cement carrier. It will be taken in the ratio of 1:1 by volume with respect to the vehicle.

These medicaments were placed on the floor of the pulp chamber of the deciduous molars and a temporary restoration (CAVIT) was placed to seal the access opening in the teeth.

Zones of inhibition were noted after 24 hrs for *Streptococcus spp* whereas 48 hrs for *Enterococcus faecalis* and *Porphyromonas gingivalis*. The following zones of inhibition of the various combinations were recorded for:

- Group A: Ciprofloxacin and Metronidazole(Double antibiotic paste) with glycerine in the ratio of 1:1 by volume,
- Group B: Ciprofloxacin and Metronidazole(Double antibiotic paste) with polyethylene glycol in the ratio of 1:1 by volume.
- Group C: Ciprofloxacin and Metronidazole(Double Antibiotic Paste-DAP) with propylene glycol in the ratio of 1:1 by volume,
Materials and Methods

- Group D: Amoxicillin clavulanate plus Metronidazole (Modified Double Antibiotic Paste) with glycerine in the ratio of 1:1 by volume.
- Group E: Amoxicillin with clavulanate plus Metronidazole (Modified Double Antibiotic Paste) with polyethylene glycol in the ratio of 1:1 by volume.
- Group F: Amoxicillin with clavulanate plus Metronidazole (Modified Double Antibiotic Paste) with propylene glycol in the ratio of 1:1 by volume.
- Group G: 2% Chlorhexidine gluconate gel with glycerine in the ratio of 1:1 by volume.
- Group H: 2% Chlorhexidine gluconate gel with polyethylene glycol in the ratio of 1:1 by volume and
- Group I: 2% Chlorhexidine gluconate gel with propylene glycol in the ratio of 1:1 by volume

These zones of inhibitions were measured with the help of a measuring scale and entered in an Excel sheet. The data was entered, coded and recoded by the principal investigator under the guidance of an experienced statistician and epidemiologist prior to entry in the computer.

B. **Secondary Objective (Polymerase Chain Reaction):**

**Step V:**

Lab Protocol for Polymerase chain reaction

1. Isolation of Genomic DNA
2. Quantification of DNA by Bio photometer
3. Polymerase chain reaction
4. Agarose Gel Electrophoresis or Gel Documentation
1. Step-wise Isolation of Genomic DNA from biomaterial obtained from infected root canals of teeth was done using standardized protocol and the extracted DNA was stored at -20°C.\textsuperscript{43,44}

2. Quantification of DNA by Bio photometer was done at the wavelengths 260 and 280 nm using UV spectrophotometer. The ratio A\textsubscript{260}/A\textsubscript{280} of 1.6-1.8 is indicative of good purity.\textsuperscript{43,44}

Step VI:

3. Polymerase chain reaction (PCR)

   ➢ Conventional PCR assay for \textit{E. faecalis}\textsuperscript{48}:

   1) The primer sequences for \textit{E. faecalis} were designed based on literature from Sedgley et al, 2005. The primers specificity was confirmed by National Centre for Biotechnology Information (NCBI) by BLAST analysis.

   2) Following is a list of the PCR primers used in the current study for \textit{Enterococcus faecalis}:

<table>
<thead>
<tr>
<th>Microorganism</th>
<th>Primer (5’ to 3’)</th>
</tr>
</thead>
<tbody>
<tr>
<td>\textit{Enterococcus faecalis}</td>
<td>CCGAGTGCTTGCACTCAAATTGG</td>
</tr>
<tr>
<td></td>
<td>CTCTTATGCCATGCGGCATAAAC</td>
</tr>
</tbody>
</table>

   3) To determine temperature for optimal primer annealing for PCR specificity experiment, temperature gradient PCR assays were performed, using positive control DNA from \textit{E. faecalis} ATTC 35550 and molecular grade water as negative control in the Veriti 96-Well Fast Thermal cycler (Applied Biosystems NY).
4) 100 ng total DNA template was prepared for 25 µl PCR amplification. 2X Master Mix (Amplicon) containing 2.5 U of red Taq DNA polymerase was used as per manufacturer’s instructions. 7.5 pmole of each primer was added to the reaction mixture.

5) The PCR amplification protocol was as follows:-

Five minutes DNA denaturation step at 95 ºC was followed by 35 consecutives cycles at 94 ºC for 30 sec; 60 ºC for 45 secs and 72 ºC for 15 secs. Following is the tabular representation:

<table>
<thead>
<tr>
<th>Steps</th>
<th>Temperature</th>
<th>Time</th>
<th>Number of cycles</th>
</tr>
</thead>
<tbody>
<tr>
<td>Initial Denaturation</td>
<td>95 ºC</td>
<td>5 minutes</td>
<td>1 cycle</td>
</tr>
<tr>
<td>Denaturation</td>
<td>94 ºC</td>
<td>30 sec</td>
<td>35 cycles</td>
</tr>
<tr>
<td>Annealing</td>
<td>60 ºC</td>
<td>45 sec</td>
<td></td>
</tr>
<tr>
<td>Extension</td>
<td>72 ºC</td>
<td>15 sec</td>
<td></td>
</tr>
<tr>
<td>Final Extension</td>
<td>72 ºC</td>
<td>10 minutes</td>
<td>1 cycle</td>
</tr>
<tr>
<td>Soak</td>
<td>4 ºC</td>
<td>Indefinite</td>
<td></td>
</tr>
</tbody>
</table>

- PCR assay for Multiplex of *P gingivalis* and *Treponema denticola*:

1) The multiplex PCR was performed by using specific primers for the 16S rRNA gene of each bacterium.

2) Following is a list of the PCR primers used in the current study for *P. gingivalis* and *Treponema denticola*:
<table>
<thead>
<tr>
<th>Microorganism</th>
<th>Primer (5’ to 3’)</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>P. gingivalis</em></td>
<td>GG CAG CTT GCC ATA CTG CG</td>
</tr>
<tr>
<td></td>
<td>ACT GTT AGC AAC TAC CGA TGT</td>
</tr>
<tr>
<td><em>Treponema denticola</em></td>
<td>TAA TAC CGA ATG TGC TCA TTT ACA T</td>
</tr>
<tr>
<td></td>
<td>TCA AAG AAG CAT TCC CTC TTC TTC TTA</td>
</tr>
</tbody>
</table>

3) PCR amplification reactions were carried out in a reaction mixture in a final volume of 25 μl consisting of 3 μl of DNA sample, and 22 μl of reaction mixture containing 7.5 pmol of each primer, 200 μM of a mixture of deoxynucleoside triphosphates, 1.5 mM MgCl₂, 10X PCR buffer (10 mM Tris-HCl, pH 8.0), 50 mM KCl, 2.5 U RED Taq DNA Polymerase.

4) The PCR amplification protocol was as follows:-

- 95 °C for 5 min followed by 40 cycles of 95 °C for 30 secs, 60 °C for 1 min, 72 °C for 1 min, and a final step of 72 °C for 10 min. Following is the tabular representation:

<table>
<thead>
<tr>
<th>Steps</th>
<th>Temperature</th>
<th>Time</th>
<th>Number of cycles</th>
</tr>
</thead>
<tbody>
<tr>
<td>Initial Denaturation</td>
<td>95 °C</td>
<td>5 minutes</td>
<td>1 cycle</td>
</tr>
<tr>
<td>Denaturation</td>
<td>95 °C</td>
<td>30 secs</td>
<td></td>
</tr>
<tr>
<td>Annealing</td>
<td>60 °C</td>
<td>1 min</td>
<td>40 cycles</td>
</tr>
<tr>
<td>Extension</td>
<td>72 °C</td>
<td>1 min</td>
<td></td>
</tr>
<tr>
<td>Final Extension</td>
<td>72 °C</td>
<td>10 minutes</td>
<td>1 cycle</td>
</tr>
<tr>
<td>Soak</td>
<td>4 °C</td>
<td>Indefinite</td>
<td></td>
</tr>
</tbody>
</table>
4. **Agarose Gel Electrophoresis to visualize isolated DNA**

The PCR amplicons were analyzed by electrophoresis on a 2 % agarose gel and stained with 0.5 µg/ml ethidium bromide.\textsuperscript{45,44}

**GELDOCUMENTATION\textsuperscript{45,44}:**

**Procedure**

1) PCR products were visualised under ultraviolet light using Syngene gel documentation. A 100-bp DNA ladder served as the molecular weight marker.

2) The identity of each band was concluded by visual comparison with a molecular weight ladder. Using a 302nm UV transilluminator and orange emission filter, images of ethidium bromide stained DNA gels are captured in a fraction of second.

3) Gel documentation of PCR generated DNA bands of *Enterococcus faecalis* positive samples following standardization by electrophoresis are confirmed.

4) Gel documentation of PCR generated DNA bands of *P. gingivalis* and *Treponema denticola* samples following standardization by electrophoresis are confirmed.

**Outcome Measures:**

1. Comparison of all 9 medicament groups for each organism isolated, namely *Streptococcus spp, Porphyromonas gingivalis* and *Enterococcus faecalis*.

2. Comparison of the medicaments with the same vehicle against the above mentioned organisms.

3. Comparison of the vehicles with the same medicament against the above mentioned organisms.
4. To identify and compare selected micro-organisms from root canals of infected deciduous molars through microbial culture and PCR.

**Data analysis plan:**

The data was entered in the excel sheet for statistical analysis. Statistical analysis was done using SPSS software version 22. Mean, Median and Standard Deviation of the data was assessed wherever necessary.

**Phase 1:**

**In vitro study**

- Mean Value & SD
- Kruskal Wallis test
- Pairwise comparison using post hoc Mann Whitney U test (p<0.05).

**Phase 2:**

**Main study**

- Tests of Normality by Shapiro-Wilk tests.
- Kruskal Wallis test – Intergroup comparison
- Post-hoc comparison by Mann-Whitney U test.
- Statistical Difference in detection of *P. gingivalis* and *E. faecalis* by culture and PCR by McNemar test.

Level of significance was set at p<0.05 for all the above tests applied.

We also compared the results using parametric tests and results were the same except for the difference between vehicles which was statistically significant only for Modified DAP combined with glycerine and propylene glycol. Though the data is Quantitative on applying Tests of Normality, and even after log transformation, the distribution was not normal, hence Non-parametric tests were applied and analysed.
RESULTS

Phase 1:

A. *In vitro* Determination of Bactericidal activity of vehicles against ATCC strains by broth dilution

The results for the Minimum Bactericidal activity of the vehicles PG, Glycerine, PEG 400, PEG 1000 and PG+PEG 400. All vehicles exhibited bactericidal activity though at different concentrations on the ATCC strains of *Staphylococcus aureus*, *S. mutans*, *E. faecalis* and *E. coli*. Propylene glycol exhibited bactericidal activity at 50% against *S. mutans*, 100% against *Staphylococcus aureus*, 25% against *E. faecalis* and 50% against *E. coli*. Glycerine, PEG 400 and PG + PEG 400 exhibited bactericidal activity at only 100% against all the organisms making them the least bactericidal vehicle amongst above mentioned vehicles. PEG 1000 was bactericidal against *S. mutans* and *E. coli* at 25%, while against *Staphylococcus aureus* and *E. faecalis* at 100%.

Of all the ATCC strains of microorganisms, *S. mutans* and *E. coli* were the most susceptible to the vehicles, *E. faecalis* exhibited intermediate susceptibility and *S. aureus* was the most resistant to all the vehicles. Propylene glycol showed bactericidal activity against maximum number of organisms i.e. three of which *E. faecalis*, commonly associated with root canal treatment failure was terminated at a very low concentration of 25 %. PEG 1000 exhibited bactericidal activity against *S. mutans* and *E. coli* at the lowest concentration and maximum dilution i.e. 25%. Glycerine and Combination of Propylene glycol and PEG 400 were the vehicles with least bactericidal activity against selected pathogens. Combination of Propylene glycol and PEG 400 did not show any synergistic antimicrobial activity and infact its
efficacy decreased against *S. mutans*, *E. faecalis* and *E. coli* in comparison to Propylene glycol alone.

**B. In vitro Antibacterial activity of endodontic medicaments and vehicle combinations against ATCC strains by agar well diffusion method using pure drugs**

Initially MIC was evaluated for single endodontic medicaments only and all organisms were resistant to Metronidazole. MIC of Chlorhexidine against *S. mutans*, *S. aureus* and *E. coli* was 0.078% whereas against *E. faecalis* and *P. gingivalis* was 0.156% and 0.019% respectively. MIC of Ciprofloxacin against *S. mutans* was 7.81µg/ml, *S. aureus* was 31.25µg/ml, and *P. gingivalis* was 0.019µg/ml. MIC of Ciprofloxacin against *E. faecalis* and *E. coli* was 1.95µg/ml. MIC of Amoxicillin clavulanate against *S. mutans* was 7.8125µg/ml, *S. aureus* was 3.90µg/ml, and *P. gingivalis* was 0.019µg/ml. MIC of Amoxicillin clavulanate against *E. faecalis* and *E. coli* was 15.625µg/ml. All organisms exhibited resistance to Metronidazole when used alone as depicted by the extremely high MIC values.

Later MIC of combination of endodontic medicaments i.e. Ciprofloxacin with Metronidazole and Amoxicillin clavulanate with Metronidazole were carried out. Following combination of Ciprofloxacin with Metronidazole, MIC values of *S. mutans*, *S. aureus* and *P. gingivalis* further reduced while Amoxicillin clavulanate with Metronidazole combination reduced MIC values against *S. mutans* only. MIC of Ciprofloxacin with Metronidazole against *S. mutans*, *E. faecalis* and *E. coli* was 1.95µg/ml; where as against *S. aureus* and *P. gingivalis* was 7.81µg/ml and 0.039µg/ml respectively. MIC of Amoxicillin clavulanate with Metronidazole against *S. mutans* and *P. gingivalis* was 3.90µg/ml and 0.019µg/ml respectively. MIC of
Amoxicillin clavulanate with Metronidazole against *S. aureus*, *E. faecalis* and *E. coli* was 15.625µg/ml.

**Antimicrobial activity of the endodontic medicaments and vehicle combinations against selected pathogens using agar well diffusion method**

The zones of inhibition of all of the selected organisms were recorded to measure their antibacterial activity using agar well diffusion method. Amongst the mean zones of inhibition against *S. mutans* using the CHX combination with the four vehicles, the highest was for CHX+PG (32.00±1.00) but the difference in comparison to CHX and other vehicles combination was not significant. Similarly, the highest zone of inhibition for A+M and the four vehicle combination against *S. mutans* was of A+M+PG (26.33±0.57) again. C+M with all the four vehicles against *S. mutans* exhibited resistance which is shown by the no zones of inhibition recorded in the triplicates performed.

Amongst the mean zones of inhibition against *S. aureus* using the CHX endodontic medicament combination with the four vehicles, the highest was for CHX+PEG (39.00±1.00) but the difference between CHX and other vehicles was not significant. The highest zone of inhibition amongst C+M and the four vehicle combination against *S. aureus* was of C+M+PEG (25.00±1.00) again. Similarly, the highest zone of inhibition for A+M and the four vehicle combination against *S. aureus* was of A+M+PG and A+M+PEG (29.00±1.00). The difference amongst all four vehicle combinations was not statistically significant.

Amongst the mean zones of inhibition against *E. faecalis* using the CHX endodontic medicament combination with the four vehicles, the highest was for CHX+PG (31.67±1.52) but the difference between CHX and other vehicles was not significant. The highest zone of inhibition amongst C+M and the four vehicle
Results

Combination against *E. faecalis* was C+M+PEG (34.00±1.00). Similarly, the highest zone of inhibition for A+M and the four vehicle combination against *E. faecalis* was of A+M+Glycerine (28.00±1.00). The difference amongst all four vehicle combinations was not statistically significant.

Amongst the mean zones of inhibition against *P. gingivalis* using the CHX endodontic medicament combination with the four vehicles, the highest was for CHX+Glycerine (27.67±1.52) but the difference between CHX and other vehicles was not significant. The highest zone of inhibition amongst C+M and the four vehicle combination against *P. gingivalis* was C+M+PG+PEG (39.00±1.00). Similarly, the highest zone of inhibition for A+M and the four vehicle combination against *P. gingivalis* was of A+M+PG (46.33±1.52). There was no statistically significant difference in the same antimicrobial drug and vehicles i.e. PEG, PG, PEG + PG and Glycerine except *P. gingivalis*. There existed significant difference in C+M+PG and C+M+Glycerine only on *P. gingivalis*.

All the organisms tested were more susceptible to one of the endodontic medicament and vehicle combinations but the difference was not statistically significant except for *P. gingivalis*. *S. mutans* and *S. aureus* were more susceptible to Chlorhexidine than C+M and A+M as endodontic medicaments which is shown by the larger zones of inhibition. *E. faecalis* was most susceptible to C+M followed by Chlorhexidine and lastly A+M. Also, A+M was most effective against *P. gingivalis*, followed by C+M and lastly, Chlorhexidine. The zones of inhibition of *P. gingivalis* were larger in comparison to other facultative anaerobes, which shows that the obligate anaerobes are more susceptible.
C. *In vitro* Antibacterial activity of innovative endodontic medicaments and different vehicle combinations against ATCC strains by agar well diffusion method using commercial drugs

The zones of inhibition against *S. mutans*, *S. aureus*, *E. faecalis* and *E. coli* were recorded using commercial drugs for preparing the combinations of the intracanal medicaments and vehicles to measure their antibacterial activity by agar well diffusion method.

Amongst the mean zones of inhibition against *S. mutans* using the 2% CHX combination with the four vehicles, the highest was for CHX+PEG+PG (20.67±0.577) followed by CHX+PEG (20.33±0.577) and lastly CHX+Glycerine and CHX+PG were (20.00±0.000). The highest zone of inhibition for C+M and the four vehicle combination against *S. mutans* was of C+M+Glycerine (22.33±0.577). A+M with all the four vehicles against *S. mutans* exhibited maximum zone of inhibition with A+M+PG (33.00±0.000).

Amongst the mean zones of inhibition against *S. aureus* using the CHX endodontic medicament combination with the four vehicles, the highest was for CHX+PEG (21.33±0.577). The highest zone of inhibition amongst C+M and the four vehicle combination against *S. aureus* was of C+M+Glycerine (23.33±0.577). Similarly, the highest zone of inhibition for A+M and the four vehicle combination against *S. aureus* was of A+M+Glycerine (33.67±0.577).

Amongst the mean zones of inhibition against *E. faecalis* using the CHX endodontic medicament combination with the four vehicles, the highest was for CHX+PEG+PG (21.67±0.577). The highest zone of inhibition amongst C+M and the four vehicle combination against *E. faecalis* was C+M+Glycerine (26.00±1.00) and
Results

C+M+PG+PEG (26.00±1.000). Similarly, the highest zone of inhibition for A+M and the four vehicle combination against *E. faecalis* was of A+M+PG (31.33±0.577).

Lastly, *E. coli* was tested as a control as per CLSI guidelines for antimicrobial susceptibility testing while *P. gingivalis* being susceptible to the drug and vehicle combinations was not selected. Amongst the mean zones of inhibition against *E. coli* using the CHX endodontic medicament combination with the four vehicles, the highest was for CHX+PG+PEG (21.33±0.577). The highest zone of inhibition amongst C+M and the four vehicle combination against *E. coli* was of C+M+PG and C+M+PEG (25.00±0.000). Similarly, the highest zone of inhibition for A+M and the four vehicle combination against *E. coli* was of A+M+PG (32.33±1.528).

Overall observations can be summarized as the most effective medicament-vehicle combination effective against *S. mutans* is A+M+PG, for *S. aureus* is A+M+Glycerine, for *E. faecalis* is A+M+PG and *E. coli* is A+M+PG closely followed by C+M+PG or PEG. Chlorhexidine is the least effective while A+M is the most effective intracanal medicament. Of all the vehicles, PG is the most effective vehicle when used in combination with intracanal medicaments.
Phase 2:

The distribution of males was higher (57%) in comparison to the female children (43%) who participated in the study on basis of inclusion and exclusion criteria. The deciduous mandibular molar teeth showed a higher distribution of necrotic pulp (75%) as compared to deciduous maxillary molars (25%)

A. Primary Objective-Antimicrobial activity of various combinations of intracanal medicaments and vehicles

Organisms detected and their association with signs and symptoms present in subjects

There was no significant relation between any of the organisms detected and the signs and symptoms present including radiolucency detected in furcation or periapical area radiographically. Though the association was not statistically significant, the following are the highest percentages of detection of organism combinations in relation to each sign and symptom recorded.

History of pain was detected in 92.3% of root canals detecting Streptococcus spp and E. faecalis, tenderness to percussion was detected in 32.7% of root canals detecting Streptococcus spp and P. gingivalis. Swelling was detected in 66.7% of root canals detecting all the selected microorganisms, followed by 55.8% in root canals detecting Streptococcus spp and P. gingivalis and 23.1% with root canals detecting Streptococcus spp and E. faecalis. Teeth with draining sinus had the highest percentage of 16.7% in root canals detecting all the selected microorganisms. Vital root canals have lesser microbial load(27.7%) as compared to non-vital root canals(72.3%). Lastly, as only teeth diagnosed as necrotic due to the presence of periradicular radiolucency were included in the study; association of organisms detected with it and its statistical significance could not be estimated; as there exists
no comparison for the teeth with periradicular radiolucency.

**Comparison of antimicrobial activity in terms of zones of inhibition (mms) of all nine groups of medicaments and vehicle combinations against *Streptococcus spp*, *Porphyromonas gingivalis* and *Enterococcus faecalis***

During evaluation of parameters related to primary objective, comparison of antimicrobial activity in terms of zones of inhibition (mms) of all nine groups of medicaments and vehicle combinations against *Streptococcus spp*, *Porphyromonas gingivalis* and *Enterococcus faecalis* was done. There was statistically significant difference ($p<0.001$) between the nine groups against *Streptococcus spp* and the maximum zone of inhibition was of A+M+PG was $32.26 \pm 7.77$. There was statistically significant difference ($p<0.001$) between the nine groups against *Porphyromonas gingivalis* and the maximum zone of inhibition was of A+M+PG was $39.37 \pm 9.27$. There was statistically significant difference ($p<0.001$) between the nine groups against *Enterococcus faecalis* the maximum zone of inhibition was of A+M+PEG was $34.53 \pm 7.34$.

**Comparison of antimicrobial activity in terms of zones of inhibition (mms) of medicaments in each vehicle against *Streptococcus spp*, *Porphyromonas gingivalis* and *Enterococcus faecalis***

To know exactly which intracanal medicament and vehicle were effective, comparisons were done between all three medicaments keeping vehicles constant and all three vehicles keeping medicaments constant. This would give the idea of the effectiveness of each medicament and vehicle individually and which of the two to combine for maximum bactericidal effect and thus, effective disinfection of the root canals of deciduous necrotic molars. The difference between antimicrobial activity of all the three medicaments with each of the three vehicles as vehicle against selected
microorganisms was statistically significant.

Following are the intracanal medicament and vehicle combinations using same vehicle but having statistically significant differences in antimicrobial activity or their zones of inhibition. For *Streptococcus spp*, A+M+G (31.32 ± 6.85) had greater antimicrobial activity as compared to C+M+G (28.75 ± 7.11) (p=0.009), A+M+PEG (31.95 ± 7.13) had greater antimicrobial activity as compared to C+M+PEG (26.03 ± 8.36) (p=0.001), A+M+PG (32.26 ± 7.77) had greater antimicrobial activity as compared to C+M+PG (28.60 ± 7.82) (p=0.009).

For *P. gingivalis*, A+M+G (35.75 ± 9.28) had greater antimicrobial activity as compared to C+M+G (29.67 ± 8.41) (p=0.02), A+M+PEG (35.25 ± 7.61) had greater antimicrobial activity as compared to C+M+PEG (29.75 ± 7.39) (p=0.002), A+M+PG (39.37 ± 9.27) had greater antimicrobial activity as compared to C+M+PG (30.42 ± 7.01) (p<0.001).

For *E. faecalis*, A+M+G (33.58 ± 7.24) had greater antimicrobial activity as compared to C+M+G (30.21 ± 7.25) (p=0.15), A+M+PEG (34.53 ± 7.34) had greater antimicrobial activity as compared to C+M+PEG (28.47±6.96) and the difference was significant (p=0.007), A+M+PG (33.79 ± 8.54) had greater antimicrobial activity as compared to C+M+PG (31.11 ± 10.66) (p=0.39).

**Comparison of antimicrobial activity in terms of zones of inhibition(mms) of vehicles in each medicament against* Streptococcus spp*, *Porphyromonas gingivalis* and *Enterococcus faecalis***

The difference between antimicrobial activity of all the three vehicles with each of the two medicaments i.e. C+M and A+M against *Streptococcus spp* and *Porphyromonas gingivalis* was statistically significant.

For *Streptococcus spp*, C+M+G (28.75 ± 7.11) had greater antimicrobial
activity as compared to C+M+PEG (26.03 ± 8.36) (p=0.02). C+M+G (28.75 ± 7.11) and C+M+PG (28.60 ± 7.82) had similar antimicrobial activity (p=0.87). C+M+PG (28.60 ± 7.82) had greater antimicrobial activity than C+M+PEG (26.03 ± 8.36) (p=0.04).

For *P. gingivalis*, A+M+G (35.75 ± 9.28) had greater antimicrobial activity as compared to A+M+PEG (35.25 ± 7.61) and the difference was not significant (p=0.94). A+M+G (35.75 ± 9.28) had lesser antimicrobial activity to A+M+PG (39.37 ± 9.27) and the difference was not significant (p=0.06). A+M+PG (39.37 ± 9.27) had greater antimicrobial activity than A+M+PEG (35.25 ± 7.61) and the difference was statistically significant (p=0.009).

The mean zones of inhibition in mm of the three medicaments with PG as vehicle against *E. faecalis* were, CHX+G was 26.58 ± 6.31, CHX+PEG was 25.68 ± 7.17, CHX+PG was 25.16 ± 8.46 and the difference between them was not significant (p=0.79).

Overall to summarize the findings of this study, modified DAP (A+M) is the most effective intracanal medicament and can replace the currently utilized DAP (C+M) for clinical purposes. The possibility of CHX being explored further as at times there was no significant difference in its antimicrobial activity as compared to DAP (C+M) especially against virulent microbial species like *Enterococcus faecalis*. Among the vehicles studied in this research work, Propylene glycol(PG) shows promising results consistently during the *in vitro* and *ex-vivo* studies; with all the medicaments and is recommended to be utilized to dispense intracanal medicaments.
B. Secondary Objective- PCR assay

- *Streptococcus spp* were detected in 100% of the endodontic samples from necrotic deciduous teeth by culture alone.

- *Treponema denticola* was detected in 63% of the endodontic samples from necrotic deciduous teeth by PCR alone.

**Comparison of detection of *E. faecalis* by PCR and culture**

- *Enterococcus faecalis* was isolated in 18% of the endodontic samples from necrotic deciduous by culture and 41% by PCR and the difference in detection was statistically significant (p<0.001; McNemar test).

**Comparison of detection of *P. gingivalis* by PCR and culture**

- *Porphyromonas gingivalis* was isolated in 58% of the endodontic samples from necrotic deciduous teeth by culture and 76% by PCR and the difference in detection was statistically significant (p<0.001; McNemar test).
Phase 1:

A. *In vitro* Determination of Bactericidal activity of vehicles against ATCC strains by broth dilution

In our study, 100% concentration of all vehicles showed antimicrobial activity. Micro-broth dilution was performed as diffusion through agar of these viscous vehicles is difficult. *Enterococcus faecalis* was most susceptible to propylene glycol but resistant to other vehicles. Glycerine had least antimicrobial activity at 100% concentration only, similar to observations of no activity by Gomes et al in 2002. Both PEG and propylene glycol have low toxic potential and are common carriers for drugs. Also, they enhance handling properties of drugs and aid in ease of placement.

Combination of PG and PEG 400 exhibited no synergistic effect. PEG 400 is advantageous in not interacting with other components, and antibacterial activity.

Following are the list of authors with findings in accordance and contrary to our research related to antimicrobial activity and toxicity of vehicles namely, Glycerine, Polyethylene glycol 400(PEG 400):

<table>
<thead>
<tr>
<th>Vehicles studied/ Authors</th>
<th>Method/ Organism Tested</th>
<th>Conclusion</th>
</tr>
</thead>
<tbody>
<tr>
<td>I. Glycerine</td>
<td></td>
<td></td>
</tr>
<tr>
<td>1) Barr M and Tice L, 1971&lt;sup&gt;52&lt;/sup&gt;</td>
<td>Broth Dilution Test/ Multiple Tube Dilution Test ATCC strains of <em>Pseudomonas aeruginosa</em>, <em>Escherichia coli</em>, <em>Salmonella typhi</em>, <em>Staphylococcus aureus</em>.</td>
<td>Possesses antimicrobial activity. Bacteriostatic for <em>Salmonella typhi</em> at 37.8% after 48 hrs and bactericidal at 23.6% after 7 days</td>
</tr>
<tr>
<td>2) Gomes et al, 2002&lt;sup&gt;33&lt;/sup&gt;</td>
<td>Modified agar diffusion test</td>
<td>No antimicrobial effect.</td>
</tr>
<tr>
<td>Vehicles studied/ Authors</td>
<td>Method/ Organism Tested</td>
<td>Conclusion</td>
</tr>
<tr>
<td>---------------------------</td>
<td>-------------------------</td>
<td>------------</td>
</tr>
<tr>
<td><strong>II. PEG 400</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1) Vaamonde et al, 1982</td>
<td>Agar Dilution Method</td>
<td>Significant inhibitory effect independent of water activity ( a_w ) so, good antimicrobial activity.</td>
</tr>
<tr>
<td></td>
<td><em>Staphylococcus aureus.</em></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>2) Chirfe et al, 1983</td>
<td>Agar Dilution Method</td>
<td>Significant antibacterial activity due to lowering ( a_w ) and phase–contrast microscopy revealed clumping and morphological changes.</td>
</tr>
<tr>
<td></td>
<td><em>S. aureus, Klebshiella pneumoniae, P. aeroginosa, E. coli</em></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>3) Bozzini JP et al, 1986</td>
<td>Electron Microscopy</td>
<td>Severe plasmolysis in <em>K. pneumoniae</em> cells, cell wall collapse and fingerlike extrusions to emerge from the bacterial cell hence, good antimicrobial activity.</td>
</tr>
<tr>
<td></td>
<td><em>Klebshiella pneumoniae</em></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>4) Ambrose et al, 1991</td>
<td>Agar Dilution Method</td>
<td>PEG 400 has good antimicrobial activity.</td>
</tr>
<tr>
<td></td>
<td>Clinical isolates of <em>S. aureus, S. epidermis, E. faecalis, E. coli</em>, group C ( \beta )-hemolytic <em>Streptococcus, P. mirabilis</em> and <em>Klebshiella spp.</em></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>5) Gomes et al, 2002</td>
<td>Modified agar diffusion test</td>
<td>No antimicrobial effect.</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>6) Li BQ et al, 2011</td>
<td>Systemic toxicity and toxicokinetics of a high dose of polyethylene glycol 400 in dogs following iv injection.</td>
<td>The toxicity of PEG-400 is low, and alterations produced are reversible.</td>
</tr>
</tbody>
</table>
### Vehicles studied/ Authors

<table>
<thead>
<tr>
<th>III. PEG 1000</th>
<th>Method/ Organism Tested</th>
<th>Conclusion</th>
</tr>
</thead>
<tbody>
<tr>
<td>1) Carreira et al, 2007&lt;sup&gt;20&lt;/sup&gt;</td>
<td>Agar Dilution Method Standard Strains Of <em>S. aureus, S. mutans, E. faecalis, E. coli, P. aeruginosa, Klebsiella pneumoniae, E. cloacae, C. tropicalis</em> and <em>C. albicans</em>.</td>
<td>Of all the vehicles, PEG 1000 maximum antimicrobial activity, allows greater penetration. Antimicrobial action may be related to the hydrophilic property of PEG, removing water necessary for microbial growth.</td>
</tr>
</tbody>
</table>

### IV. PG

| 1) Olitzky et al, 1965<sup>38</sup> | ----------- | Propylene glycol is shown to have antimicrobial activity when used as a dermatological vehicle |
| 2) Kinnunen et al, 1991<sup>59</sup> | Broth dilution method *Candida albicans, Staphylococcus aureus, Staphylococcus epidermidis, Streptococcus pyogenes A, Streptococcus mitis*, and *E. coli* | Antibacterial and antifungal properties of propylene glycol exists but are lower when compared to hexylene glycol in cosmetic and dermatological vehicles |
| 3) Rowe et al, 2009<sup>30</sup> | ------ | Low toxic potential and are common carriers for pharmaceutical drugs, cosmetics and food industry |
B. *In vitro* Antibacterial activity of endodontic medicaments and vehicle combinations against ATCC strains by agar well diffusion method using pure drugs

Following are the studies which have studied the antimicrobial activity and tissue toxicity of Chlorhexidine, Ciprofloxacin and Amoxycillin clavulanate and have findings supporting and contrary to our research observations:

<table>
<thead>
<tr>
<th>Vehicles studied/ Authors</th>
<th>Method/ Organism tested</th>
<th>Conclusion</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>I. 2% Chlorhexidine</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1) Mistry et al, 2014&lt;sup&gt;10*&lt;/sup&gt;</td>
<td>Microbroth dilution method <em>S. mutans</em>, <em>S. aureus</em> and <em>E. faecalis</em></td>
<td>Chlorhexidine is an effective antimicrobial with MIC&lt;0.0625 %</td>
</tr>
<tr>
<td>2) Gomes et al, 2006&lt;sup&gt;12&lt;/sup&gt;</td>
<td>Agar diffusion method &amp; Direct contact test ATCC strains of <em>S. aureus</em>, <em>C. albicans</em>, <em>E. faecalis</em>, <em>P. gingivalis</em>, <em>P. endodontalis</em>, <em>Prevotella intermedia</em></td>
<td>2 % CHX gel alone demonstrated strongest antimicrobial action.</td>
</tr>
<tr>
<td>3) Filho et al, 2008&lt;sup&gt;14&lt;/sup&gt;</td>
<td>Agar diffusion method &amp; Direct contact test ATCC strains of <em>S. mutans</em>, <em>S. sobrinus</em>, <em>C. albicans</em>, <em>E. faecalis</em> and clinical isolates of <em>P. gingivalis</em>, <em>Prevotella intermedia</em>.</td>
<td><em>S. mutans</em> showed significantly larger zones of inhibition as compared to <em>P. gingivalis</em>, <em>Prevotella intermedia</em>. <em>C. albicans</em>, and <em>E. faecalis</em> were most resistant.</td>
</tr>
<tr>
<td><strong>II. Ciprofloxacin</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1) Oboh IE et al, 2007&lt;sup&gt;61&lt;/sup&gt;</td>
<td>Agar diffusion method standard strain of <em>S. aureus</em>, and clinical isolates of <em>S. aureus</em>, <em>C. albicans</em>, <em>E. faecalis</em>,</td>
<td>5 µg/ml showed higher antimicrobial activity as compared to the 100 µg/ml of Ciprofloxacin used in our study.</td>
</tr>
<tr>
<td>Vehicles studied/ Authors</td>
<td>Method/ Organism tested</td>
<td>Conclusion</td>
</tr>
<tr>
<td>--------------------------</td>
<td>-------------------------</td>
<td>------------</td>
</tr>
<tr>
<td><strong>Klebsiella pneumoniae, Pseudomonas aeruginosa, Bacillus subtilis, E. coli</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>2) Jain et al, 2009\textsuperscript{62}</td>
<td>Kirby-Bauer Disc diffusion method. Three Standard Strains of <em>S. mutans</em></td>
<td><em>S. mutans</em> showed resistance to Ciprofloxacin as in our study.</td>
</tr>
<tr>
<td>3) Choudhary et al, 2011\textsuperscript{63}</td>
<td>Kirby-Bauer Disc diffusion method. Clinical isolates of <em>S. mutans</em>.</td>
<td>Greater antimicrobial activity as compared to Tetracyclines.</td>
</tr>
</tbody>
</table>

### III. Amoxycillin Clavulanate

| 1) Baumgartner et al, 2003\textsuperscript{64} | E- test. Antibiotic susceptibility of 98 strains of bacteria isolated from 12 endodontic abscesses | ➢ Amoxycillin clavulanate was 100% effective against all 98 species of microorganisms. 
➢ Metronidazole had greatest resistance but if combined with Amoxicillin, sensitivity increased from 91% to 99%. |
| 2) Salinas et al, 2006\textsuperscript{65} | ---------- | ➢ Amoxicillin clavulanate had greatest sensitivity and lowest resistance amongst the commonly prescribed antibiotics followed by amoxicillin alone. |
### Vehicles studied/ Authors | Method/ Organism tested | Conclusion
--- | --- | ---
3) Ruparel et al, 2012<sup>66</sup> | MTT assay SCAPs from 2 extracted immature mandibular third molars. | High concentrations of TAP, DAP and Augmentin (Amoxycillin clavulanate) have a detrimental effect on SCAP survival.

Principles of Antibiotic Therapy advise the use of narrow spectrum and bactericidal drugs to prevent antibiotic resistance development.<sup>19</sup> Bactericidal drug combination overcome selective pressure and development of antibiotic resistance in microorganisms.<sup>67</sup> The most common reason for resistance in Enterobacteriaceae is β-lactamase production, so a β-lactamase inhibitor, clavulanic acid can be incorporated. So, Amoxycillin clavulanate, found to be 100% effective against endodontic bacteria<sup>64,66</sup> in comparison to Ciprofloxacin. In this *in vitro* study, modified DAP with Amoxycillin clavulanate was studied for the first time as an intracanal medicament.

Amoxycillin clavulanate has MIC values similar to the Indian Council Medical Research guidelines for *S. mutans* and *P. gingivalis*.<sup>68</sup> Also, combination of Amoxycillin clavulanate with Metronidazole had MIC values, lower or similar for *S. mutans, E. faecalis, P. gingivalis* and *E. coli* except *S. aureus*. Antimicrobial activity of A+M when combined with the four different vehicles were the largest in terms of zones of inhibition against *P. gingivalis*.

*P. gingivalis*, an obligate anaerobe exhibits larger zones of inhibition as they are extremely susceptible to Metronidazole which targets anaerobes.<sup>18</sup> 2% CHX exhibited antimicrobial activity against all selected pathogens contrary to the antibiotic medicaments used with an added advantage no resistance development.<sup>15,69</sup>
Discussion

PG when used as vehicle to carry intracanal medicaments improves the medicament penetration through dentinal tubules as first noted by Cruz et al.\textsuperscript{21} Carreira et al observed that PEG when used as vehicle to carry Metronidazole made resistant microbes; sensitive.\textsuperscript{21} Vehicles improve handling properties of the resulting intracanal medicament paste and enhance the release of the intracanal medicaments from the paste.\textsuperscript{58} The results of the previous in vitro study(Phase 1 A.) highlight that all the selected vehicles possessed bactericidal activity.\textsuperscript{70}

Intracanal medicaments in combination with vehicles no significant difference in their antimicrobial activity except for PG which was significantly better than glycerine when used alongwith C+M intracanal medicament combination against \textit{P. gingivalis}. PEG, PG+PEG and glycerine as vehicles with the three intracanal medicaments i.e. 2\% Chlorhexidine gluconate, C+M and A+M might be lacking synergistic activity.

The agar well diffusion method used in this study is the most widely used method of antibacterial activity assessment of newer substances like plant extracts, new drug formulations, dental materials, medicaments etc.\textsuperscript{51,56,71} This is one of the standard methods of comparing the antibacterial activity and the latest being the E-test. Limitation of E-test is that standardized strips for the combination of antimicrobial drugs with vehicles are unavailable, hence agar well diffusion method was performed in this in vitro study.

C. \textit{In vitro} Antibacterial activity of innovative endodontic medicaments and different vehicle combinations against ATCC strains by agar well diffusion method using commercial drugs

It was observed that all commercial preparations except A+M had lesser antimicrobial activity in terms of zones of inhibition as compared to the pure drugs.
used in (Phase 1 B). 2% CHX and C+M had lesser zones of inhibition respectively. This might have been due to the fact that same drug concentrations utilized in the in vitro study in pure form has been not equal to drug concentration in 0.5mg of commercial drugs due to the presence of filler, binder and other substance used in the preparation of tablets from pure drugs. C+M was also effective against *S. mutans* contrary to our findings. Amongst all the three intracanal medicaments, A+M was the most effective irrespective of the vehicle utilized. Amongst the vehicles it was interesting to note that either PG or PEG was the most effective vehicle with all the intracanal medicaments. Their combination i.e. PG+PEG did not have any synergistic effect hence, is not recommended as per this study. Glycerine had the lowest antibacterial activity hence, is not recommended as per the observations from this study. Hence, as per the findings of this study we suggest the combination of A+M with preferably with PG as vehicle and 2% CHX with PEG as vehicle. The combination of C+M had no predilection for the vehicle to be used to carry. The zones of inhibition were similar for C+M and 2% CHX with no difference due to use of the four different vehicles to carry them. Amongst all the medicaments A+M was most effective and with vehicle PG.

There are no studies comparing antibacterial activity of DAP consisting of combinations like A+M and C+M using commercially available preparations as in this study. Antibacterial activity of TAP has been assessed using agar well diffusion method\(^2\) and the zones of inhibition recorded are lower but comparable to the zones of inhibition of DAP(C+M) and modified DAP(A+M) recorded in our study. Agar well diffusion method was used inorder to compare the results to the study using ex-vivo model (Phase 2A.), to compare if diffusion of endodontic medicaments is hampered through dentinal tubules when used in vivo.
Phase 2:

In this study though the distribution of males was higher (57%) in comparison to the female children (43%) but it has no influence on the organisms detected as organisms present depends on the environment present within the root canals. The deciduous mandibular molar teeth showed a higher distribution of necrotic pulp 28 (75%) as compared to deciduous maxillary molars 9 (25%) which is in accordance with the study by Fabris et al. This might be due to the fact that mandibular molars erupt earlier in the cavity than maxillary molars and are in function for longer period of time and due to food retention and hence, get carious more often. A. Primary Objective-Antimicrobial activity of various combinations of intracanal medicaments and vehicles

Organisms detected and their association with signs and symptoms present in subjects

As organisms were always detected along with Streptococcus spp, the association with signs and symptoms is not true association with each organism but with the interaction between the organisms detected in each sample. Also, Treponema denticola was detected by means of PCR but was not considered as it was not detected by culture. Previous studies have shown an association between T. denticola and E. faecalis with periapical radiolucency while P. gingivalis with tenderness to percussion. When a total of 224 cultivable isolates were recovered belonging to 56 different bacterial species, association were observed between anaerobes especially gram negative microbes and pain, tenderness on percussion and swelling. P. gingivalis was associated with pain, wet canals and tenderness to percussion; P. gingivalis and E. faecalis were associated with swelling and E. faecalis and Streptococcus spp was associated with previous endodontic treatment.
Though the association was statistically not significant, it is interesting to note that few organism combinations had higher percentage in relation to specific signs and symptoms.

For instance, history of pain was detected in 92.3% of root canals detecting *Streptococcus spp* and *E. faecalis*, tenderness to percussion was detected in 32.7% of root canals detecting *Streptococcus spp* and *P. gingivalis* which is in accordance with the study by Gomes et al. Swelling was detected in 66.7% of root canals detecting all the selected microorganisms, followed by 55.8% in root canals detecting *Streptococcus spp* and *P. gingivalis* which is in accordance with the study by Gomes et al but contrary to the findings by the same author in low association i.e. 23.1% with root canals detecting *Streptococcus spp* and *E. faecalis*. Teeth with draining sinus had the highest percentage of 16.7% in root canals detecting all the selected microorganisms which might be related to the fact that now the environment in the root canals becomes less anaerobic.

The findings of this study that vital root canals have lesser microbial load (27.7%) as compared to non-vital root canals (72.3%) further supported the findings by various authors. Lastly, as only teeth diagnosed as necrotic due to the presence of periradicular radiolucency were included in the study; association of organisms detected with it could not be estimated.

**Comparison of antimicrobial activity in terms of zones of inhibition (mms) of all nine groups of medicaments and vehicle combinations against *Streptococcus spp*, *P. gingivalis*, and *E. faecalis***

A+M+PG had the greatest zone of inhibition i.e. 32.26 ± 7.77 mms depicting maximum antimicrobial activity against *Streptococcus spp*, A+M+PG had greatest zone of inhibiton of 39.37 ± 9.27 mms against *P. gingivalis* and A+M+PEG had
greatest zone of inhibition of 34.53 ± 7.34 mms against *E. faecalis*. Amoxycillin Clavulanate being the most effective intracanal medicament is in accordance with the findings of Baumgartner et al, whereas PG and PEG being the ideal vehicles was pointed out by Cruz et al and Carreira et al respectively. 21,20,54,59

To know exactly which intracanal medicament and vehicle were effective, comparisons were done between all three medicaments keeping vehicles constant and all three vehicles keeping medicaments constant. This would give the idea of the effectiveness of each medicament and vehicle individually and which of the two to combine for maximum bactericidal effect and thus, achieve effective disinfection of the root canals of deciduous necrotic molars.

**Comparison of antimicrobial activity in terms of zones of inhibition(mms) of medicaments in each vehicle against Streptococcus spp, P. gingivalis, and E. faecalis**

When comparison was done amongst the intracanal medicaments with the same vehicle, i.e. C+M, A+M, CHX in the same vehicle namely, glycerine followed by PEG and lastly, PG; it was observed that A+M had significantly more antimicrobial activity as compared to both C+M and CHX. These results are in accordance with study on antimicrobial susceptibility of endodontic organisms, which observed that Amoxicillin clavulanate was effective against most of the microorganisms especially anaerobes developing resistance. 64,17 This has also been observed clinically in a case report using Amoxycillin in triple antibiotic paste instead of Minocycline to overcome the problem of discoloration inherent with tetracycline usage. 14 Also, combination with Metronidazole could be more effective compared with administration of single drug.64,68 Also, study by Jardim et al showed
that Amoxicillin clavulanate was effective against β-lactams resistant isolates too except *Proteus spp.*

Similarly, when the same three intracanal medicaments were used with either PEG or PG, A+M was the most effective medicament irrespective of the vehicle used to dispense. Though there are no studies on using A+M as an intracanal medicament, it can be concluded from antibiotic susceptibility testing against endodontic pathogens that most effective is Amoxycillin clavulanate with no resistance. Chunduri et al concluded that Amoxycillin still possesses activity against major pathogens in orofacial odontogenic infections but for severe infections Amoxycillin clavulanate is useful. The vehicles did not influence the antimicrobial activity against these organisms like *Streptococcus spp.*, and *P. gingivalis*. These observations were common to the the selected organisms i.e. *Streptococcus spp.*, and *P. gingivalis*; as these organisms are also more susceptible as compared to *E. faecalis*. Also, *P. gingivalis* is more susceptible being an obligate anaerobe which is observed by the overall larger zones of inhibition and literature supports this observation.

When the same three intracanal medicaments were used with either glycerine, PEG or PG against *E. faecalis* A+M and C+M were similar in antimicrobial activity, the differences being statistically significant only when used with PEG. This shows that A+M can replace C+M i.e. the DAP and vehicles do play a role in enhancing the antibacterial efficacy especially against resistant organisms like *E. faecalis*. Also, these findings are in accordance to studies by Samuelsson DG et al (2000), ICMR Bulletin (2009), Al-Badah AS et al (2015) that *E. faecalis* is resistant to Cephalosporin, semi-sythetic penicillin, Ampicillin, Metronidazole and Tetracyclines etc thus supporting the combination of drugs used as in this study and antimicrobials like 2% Chlorhexidine. Numerous studies regarding antibiotic susceptibility of
bacteria associated with endodontic, dentoalveolar and odontogenic infections have clinical evidence of Amoxicillin clavulanate being most effective antibiotic.  

Comparison of antimicrobial activity in terms of zones of inhibition (mms) of vehicles in each medicament against *Streptococcus spp*, *P. gingivalis*, and *E. faecalis*

Glycerine and Propylene glycol (PG) were significantly better vehicles than PEG to carry (C+M) medicament against *Streptococcus spp*. Propylene glycol has been a ideal vehicle and has consistently showed good antimicrobial properties and also, when combined with intracanal medicaments throughout the various phases of the study (*in vitro* and *ex-vivo*). Though in the initial *in vitro* studies glycerine did not show promising results as a vehicle alone but when combined with intracanal medicaments especially antibiotic based medicaments, it possessed antimicrobial activity.

PG was significantly better vehicle as a carrier for (A+M) medicament than PEG and Glycerine when used against *P. gingivalis*. Hence PG can be recommended as the ideal vehicle to carry these medicaments against both *Streptococcus spp* and *P. gingivalis*. This is in accordance with the findings of Cruz et al (2002) and Ganesh MR et al (2014) though these studies did not compare it with other vehicles included in this study.

Hence, PG could be used as an ideal vehicle for all the above intracanal medicaments on the basis of the numerous *in vitro* and *ex-vivo* experiments carried out in this study.
B. Secondary Objective- PCR assay and its comparison to Culture:

- Detection of selected micro-organisms by Culture:

  *Streptococcus spp* were detected in all the samples i.e. 100% of the endodontic samples from necrotic deciduous teeth by culture. These findings are in accordance with studies by Hegde et al, Pazelli et al, da Silva et al, Marsh SJ et al and Faria G; which found *Streptococcus spp* in 100%, 96.7%, 85%, 82% and 85% of root canals of primary teeth with necrotic pulp and periapical lesions respectively. Also, few of the older studies have also found a slightly lower prevalence of *Streptococcus spp* like Cohen et al (70%), and Tomic-Karovic K et al (76%). This might be due to the difference in technique used for culture to identify the microorganisms.

  *Enterococcus faecalis* was isolated in 19 out of 111 samples i.e. 17% of the endodontic samples from necrotic deciduous teeth by culture. This observation is in accordance with the study by Cogulu et al who detected 18% by means of culture only. This similarity is inspite of using different culture techniques for detection of *Enterococcus faecalis*. It is interesting to note that this is in primary infections of deciduous teeth whereas initially it was believed that *Enterococcus faecalis* was present only in teeth with failed endodontic treatment. Hegde et al found a relatively higher percentage of 35% and it might be due to the use of Enterococcus confirmatory agar in our study; which has a high specificity and low sensitivity hence should not be preferred for initial culture of samples.

  *Porphyromonas gingivalis* was isolated in 58 out of 111 samples i.e. 52% of the endodontic samples from necrotic deciduous teeth by culture. This is a higher percentage as compared to studies by Gomes et al who used VMGA transport media which found a relatively low percentage of 6.7%. This might be due to the use of a different transport media and the one used in our study i.e. RTF is proven to be an
efficient transport media. Other studies by da Silva et al and Pazelli et al found the percentage of black pigmented bacilli (BPB consisting of mainly the *Porphyromonas spp* and *Prevotella spp*) to be 30% and 35.5%. The higher rate of detection might be even due to advancement in anaerobic culture techniques in the last decades.

- **Detection of selected micro-organisms by PCR:**

  *Enterococcus faecalis* was isolated in 45 out of 111 samples i.e. 41% of the endodontic samples from necrotic deciduous teeth by PCR. These findings are higher when compared to the detection rate of 14% by Cogulu et al and similar to findings of *Enterococcus spp* of 50% by Fabris et al. Both the authors have used PCR for detection. This also highlights the importance of thorough disinfection of root canals of deciduous necrotic teeth in order to achieve successful pulp therapy as *Enterococcus faecalis* is closely related to failure of pulp therapy.

  *Porphyromonas gingivalis* was isolated in 87 out of 111 samples i.e. 78% of the endodontic samples from necrotic deciduous teeth by PCR. This is similar to results by Fabris et al (49%) and Gomes et al (100%) who detected *Porphyromonas gingivalis* using PCR. Though our percentage of detection is higher, it is also important to note that our detection by culture was higher too and all samples positive for *P. gingivalis* by culture were also positive by PCR. Cogulu et al detected 16% of *P. gingivalis* by PCR and these findings are contrary to our findings.

  *Treponema denticola* was detected in 79 out of 111 samples i.e. 71% of the endodontic samples from necrotic deciduous teeth by PCR. These findings are contrary to the findings of Cogulu et al which detected a low i.e. 16% of *Treponema denticola* by PCR where as Gomes et al detected 40% of *Treponema denticola* in the root canal samples from necrotic deciduous teeth by means of PCR.
Comparison of Detection of selected micro-organisms by Culture and PCR:

*Enterococcus faecalis* and *Porphyromonas gingivalis* were detected by both culture and PCR. *Enterococcus faecalis* was isolated in 17% of the endodontic samples from necrotic deciduous by culture and 41% by PCR.

*Porphyromonas gingivalis* was isolated in 52% of the endodontic samples from necrotic deciduous teeth by culture and 78% by PCR. The difference between the detection rates by culture and PCR for both *Enterococcus faecalis* and *Porphyromonas gingivalis* was statistically significant (p< 0.001, Mc Nemar test). This is contrary to the findings of Cogulu et al as they did not find the difference between detection by culture and PCR to be statistically significant and this is the only study comparing culture and PCR for detection of specific microorganisms in deciduous teeth. Many studies in permanent dentition already agree upon the fact that PCR is a more rapid, sensitive and specific technique for detection of specific microorganisms.
CONCLUSION

The present study showed that the Modified Double Antibiotic Paste (A+M) was more effective than the currently recommended Double Antibiotic Paste (C+M). Hence, A+M consisting of Amoxycillin clavulanate with Metronidazole can be used as an intracanal medicament for effective disinfection of root canals prior to root canal filling, revascularization procedures, non-surgical treatment of chronic periapical lesions to name a few. Also, Chlorhexidine (CHX) was effective in disinfection of root canals but had lower antimicrobial activity in comparison to the antibiotic-based intracanal medicaments. But we still believe that Chlorhexidine has a potential antimicrobial activity which should be utilized as it being a antimicrobial biocide, has the inherent advantage of not developing resistance. It also exhibits the property of substantivity which when combined with vehicles can lead to the slow, longer release and appropriate diffusion of the intracanal medicaments. As per the findings of this study, A+M is the most effective intracanal medicament with possibility of CHX being explored further.

Among the vehicles studied in this research work, Propylene glycol (PG) shows promising results consistently; with all the medicaments and is recommended to be utilized to dispense intracanal medicaments. Other vehicles also exhibited bactericidal activity and can be used to carry intracanal medicaments. These vehicles sometimes have a synergistic action while at other times are compatible with only a few medicaments. Hence, PG should be used as the ideal vehicle to carry intracanal medicament for clinical purposes like disinfection, revascularization etc. Vehicles to dispense intracanal medicaments not only, improve handling characteristics but aid in placement, diffusion and sustained release of intracanal medicaments. Thus, vehicles
should be considered an equally important constituent of intracanal medicament pastes.

It was noticed that *Enterococcus faecalis*, *Porphyromonas gingivalis* and *Treponema denticola* were detected in higher percentages by PCR i.e. 41%, 78% and 71% respectively; as compared to previous studies. Thus, it emphasizes the role of intracanal medicaments to kill these organisms prevalent in root canals for the success of pulp therapy in deciduous necrotic teeth.

Also, for detection of microbiota in deciduous root canals, PCR is a better detection tool in comparison to culture, the difference being statistically significant (p<0.001). It also aids in more rapid, sensitive and specific detection of microorganisms.

The present study had certain limitations. Formulations of Chlorhexidine could have been studied and the one with better bactericidal activity could have been selected. Also, higher percentage of CHX gel could have been prepared for greater antibacterial activity as its antibacterial activity is concentration dependant. Also, when zones of inhibition of 2% CHX gel were compared to that of 2% CHX irrigating solution, zones of inhibition of the gels were smaller and this may be attributed to the viscous nature of the gels which hamper the diffusion of CHX.

Further studies using better formulations of Chlorhexidine as an intracanal medicament as compared to antibiotics could be tried out with better polymers to improve the efficacy of Chlorhexidine gel. As in a recent study, gels loaded with antibiotics namely modified triple antibiotic paste consisting of (ciprofloxacin, metronidazole and clindamycin) and DAP (ciprofloxacin and metronidazole) were prepared at a concentration of 1mg/mL. Similarly modified DAP consisting of Amoxycillin clavulanate gels should be studied in comparison to Chlorhexidine gel.
Once *ex-vivo* studies are done, clinical trials can be undertaken. Also, this study suggests the antimicrobial activity against commonly isolated organisms from endodontic infections and would help in choosing the prescription of systemic antibiotics judiciously.
REFERENCES


spectrum and its clinical impact on severe deep space head and neck infections.


