Inhibition of pure cultures of oral bacteria by root canal filling materials

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Abstract

This study compared the antimicrobial effectiveness of nine dental materials and a negative control agent against 21 strains or species of bacteria using an agar diffusion assay. The materials were:

1. *Camphorated parachlorophenol mixed with calcium hydroxide (CPC+$Ca(OH)_2$)*
2. *CPC mixed with zinc oxide (CPC+ZnO)*
3. *Formocresol mixed with zinc oxide and eugenol (FC+ZOE)*
4. *Chlorhexidine mixed with ZOE (CHX+ZOE)*
5. *Kri™ paste*
6. *ZOE*
7. *Zinc oxide mixed with sterile water (ZnO+H_2O)*
8. *Calcium hydroxide mixed with sterile water ($Ca(OH)_2$+H_2O)*
9. *Vitapex™*
10. *Vaseline™ (control).*

The test bacteria represented species commonly isolated from nonvital primary and permanent tooth root canals. The antimicrobial effectiveness of the materials was divided into five groups based on the diameters of the zones of inhibition against all test bacteria and distribution of the data. All materials except Vaseline™ showed antimicrobial activity against some of the 21 organisms. Generally, all materials inhibited gram-negative anaerobic bacteria more effectively than aerotolerant gram-negative or gram-positive bacteria. Materials containing CPC or FC (except Kri™ paste) produced strong or medium strong inhibition against most bacteria. CHX+ZOE, Kri™ paste, ZnO+H_2O, and ZOE inhibited all or most bacteria, but to lesser extent than CPC+$Ca(OH)_2$, CPC+ZnO, or FC+ZOE. $Ca(OH)_2$ +H_2O, Vitapex™, and Vaseline™ generally were noninhibitory. The findings should allow a comparative evaluation of antimicrobial effectiveness to be made of materials commonly used in pulpectomy procedures with primary teeth. (Pediatr Dent 18:444–49, 1996)

Success of endodontic therapy of primary teeth depends, in part, on the elimination or reduction of bacteria present in the root canal.[1] This may be accomplished by mechanical debridement and use of antibacterial irrigating agents and root canal filling materials.

Antimicrobial activity of root canal filling materials has been studied extensively by agar diffusion techniques using pure cultures of oral bacteria.[2–17] Most of these investigations focused on facultative streptococci and staphylococci, but a few utilized anaerobic species, i.e., *Bacteroides gingivalis*,[18] *B. endodontalis*[13] (both are currently classified in the genus *Porphyromonas*), *B. fragilis*, and *Veillonella* species.[2,9,18] Previous in vitro studies have not reflected adequately the microbial composition of root canals which recent literature indicates is predominately anaerobic.[19–25] A number of these anaerobes, e.g., species of *Peptostreptococcus*, which frequently have been found to predominate in necrotic pulpal tissue, have not been included in agar diffusion testing of dental materials.

Most cultural studies of infected root canals have employed permanent teeth. However, two comprehensive cultural investigations of primary teeth [23,24] reported isolation of species similar to inhabitants of permanent teeth. In the latter investigations a majority of the isolates were obligate anaerobes with *Bacteroides* (currently *Porphyromonas* or *Prevotella*) species and anaerobic streptococci representing a large proportion of the microbial populations.

Our investigation is an in vitro evaluation of antimicrobial activity of nine root canal filling materials that have been used clinically against 21 bacterial strains or species known to inhabit infected root canals, and include anaerobic species that frequently predominate the microbiota. The results are intended to show the relative antimicrobial effectiveness of each material and indicate those that are noninhibitory against particular species.

Methods and materials

Zones of inhibition were measured around wells containing root canal filling materials or Vaseline™ in agar plates. Each plate was seeded previously with one of 21 test microorganisms.

Dental materials

Nine dental materials and one control material (Vaseline™) were tested. These were:

1. Camphorated parachlorophenol (parachlorophenol 35%, camphor 65%, U.S.P. Sultan Chemists Inc, Englewood, NJ) mixed with calcium hydroxide (AMEND Drug and Chemical Co Inc, Irrigation, NJ) (CPC+$Ca(OH)_2$)
2. Calcium hydroxide mixed with sterile water ($Ca(OH)_2$+H_2O)
3. CPC mixed with zinc oxide (U.S.P. Sultan) (CPC+ZnO)
4. Zinc oxide mixed with eugenol (U.S.P. Sultan) (ZOE)
5. Formocresol (Buckley's formocresol, formaldehyde 19%, cresol 35%, U.S.P. Sultan) mixed with ZOE (FC+ZOE)
6. Zinc oxide mixed with sterile water (ZnO+H_2O)
7. Chlorhexidine dihydrochloride (Sigma Chemical Co, St Louis, MO) mixed with ZOE (CHX+ZOE)
8. Kri™ paste (iodoform, camphor, p-chlorophenol, menthol, Pharmachemie AG, Zurich, Switzerland)
9. Vitapex™ (iodoform, $Ca(OH)_2$, NEO Dental Chemical Products Co, LTD, Tokyo, Japan)
10. Vaseline™ (Chesebrough Ponds USA, Greenwich, CT) (control).

Abbreviations and formulas for these agents are summarized in Table 1.

Pure cultures

Pure cultures of bacteria that have been reported to inhabit nonvital root canals of primary teeth were employed in inhibition experiments (Table 2). Clinically isolated or American type Culture Collection (ATCC, Rockville, MD) reference strains of 10 obligate anaerobic bacteria, *Prevotella melaninogenica, Bacteroides fragilis, Prevotella intermedia* (two strains), *Veillonella parvula, Peptostreptococcus micros* (four strains), *Peptostreptococcus anaerobius, Peptostreptococcus magnus, Streptococcus constellatus, S. morbillorum* and *Prevotella buccae* and seven facultative anaerobic or aerobic bacteria, *S. mutans, Staphylococcus aureus, Lactobacillus casei, Escherichia coli, Pseudomonas aeruginosa, Candida albicans* and an oral *Neisseria* isolate were cultured in Schaedler broth (Difco, Detroit, MI) or Brain Heart Infusion broth (BBL, Cockeysville, MD), both enriched with hemin and menadione, at 37°C, either aerobically or anaerobically for 18–24 hr, according to the growth requirement of the organism. The purity of each test strain was checked by Gram's stain and colony morphology during each trial.

Diffusion assay

The broth culture suspension was adjusted to a No. 1 McFarland standard (approximately $3x10^8$ cells/ml). Aliquots of the suspension containing the bacteria (100 µl) were spread on 90-mm diameter Petri dishes containing a nonselective medium, Brucella Agar™ (BBL, Cockeysville, MD) enriched with 3–5% sheep blood, hemin (5 mg/ml), and menadione (1 mg/ml). Small wells (4 mm diameter and 3 mm deep) were made in the agar using a sterile amalgam carrier. Freshly mixed root canal filling materials were placed into the wells using a syringe or amalgam carrier. Two filling materials were assayed on each plate. The 10 agents were assayed using a single broth culture and media prepared from a single batch. The experiments were repeated with new cultures of the test organisms and media prepared from new batches. Anaerobic and some facultative strains were incubated at 37°C for 7 days using an Anaerobic GasPak™ jar (B-D Microbiology Systems, Cockeysville, MD) and gas-generating envelopes (GasPak Plus, B-D Microbiology Systems) to achieve anaerobiosis. An anaerobic indicator strip (BBL) was placed in the jars to monitor oxygen contamination of the environment. *Neisseria* spp., *E. coli, P. aeruginosa* and *C. albicans* were incubated in air at 37°C for 7 days.

After incubation, zones of inhibition (no growth of bacteria) were examined around wells containing filling materials. These appeared as clear, circular halos surrounding the wells. Diameters of the zones were measured with a Boley gauge by one investigator. The mean of four measurements minus the 4-mm diameter

TABLE 1. ABBREVIATIONS AND FORMULAS FOR TEST FILLING MATERIALS

1. CPC + $Ca(OH)_2$	Camphorated parachlorophenol: $Ca(OH)_2$= 16 drops: 1 scoop = 0.16 cc: 0.17 g
2. CPC + ZnO	Camphorated parachlorophenol: ZnO = 8 drops: 1 scoop = 0.08 cc: 0.2g
3. FC + ZOE	Formocresol: ZnO: Eugenol = 2 drops: 1 scoop: 6 drops = 0.02 cc: 0.2 g: 0.06 cc
4. CHX + ZOE	Chlorhexide dihydrochloride: ZnO: Eugenol = 0.002 g: 0.198 g: 0.07 cc
5. Kri™ Paste	Commercial product: Iodoform: Camphor: P-chlorophenol: Menthol
6. ZOE	Zinc oxide: Eugenol = 1 scoop: 7 drops = 0.2 g: 0.07 cc
7. ZnO + H_2O	Zinc oxide: Sterile water = 1 scoop: 7 drops = 0.2 g: 0.07 cc
8. $Ca(OH)_2$ + H_2O	Calcium hydroxide: Sterile water = 1 scoop: 10 drops = 0.17 g: 0.1 cc
9. Vitapex™	Commercial product: Iodoform: $Ca(OH)_2$
10. Vaseline™	Commercial product: Petroleum jelly

TABLE 2. MICROORGANISMS TESTED

Species	Source	
Anaerobic Gram Positive		
Peptostreptococcus micros (Pmic)	clinical/child	(CC1)*
	clinical/adult	(CA1)*
	clinical/adult	(CA2)*
	ATCC† 33270	(ATC)
Peptostreptococcus anaerobius (Pana)	ATCC 27337	(ATC)
Peptostreptococcus magnus (Pmag)	ATCC 15794	(ATC)
Streptococcus constellatus (Scon)	clinical/adult	(CA3)*
Streptococcus morbillorum (Smor)	clinical/adult	(CA4)*
Anaerobic Gram Negative		
Veillonella parvula (Vpar)	clinical/adult	(CA5)*
Prevotella (Bacteroides) *intermedia* (Pint)	ATCC 25611	(ATC)
	clinical/child	(CC2)*
Prevotella melaninogenica (Pmel)	ATCC 25845	(ATC)
Prevotella buccae (Pbuc)	clinical/child	(CC3)*
Bacteroides fragilis (Bfra)	ATCC 25285	(ATC)
Facultative or Aerobic Gram Positive		
Streptococcus mutans (Smut)	clinical/adult	(CA6)‡
Lactobacillus casei (Lcas)	ATCC 11578	(ATC)
Staphylococcus aureus (Saur)	ATCC 6538	(ATC)
Candida albicans (Calb)	ATCC 26366	(ATC)
Facultative or Aerobic Gram Negative		
Escherichia coli (Ecol)	ATCC 25922	(ATC)
Neisseria species (Nspe)	clinical/adult	(CA7)§
Pseudomonas aeruginosa (Paer)	ATCC 27853	(ATC)

* ATCC: American Type Culture Collection (Rockville, MD).

† CC1, CC2, CC3, CA1, CA2, CA3, CA4, CA5: endodontic abscess.

‡ CA6: Supragingival plaque of a healthy adult.

§ CA7: Saliva of a healthy adult.

of the well represented the inhibition value of the tested product. Because several of the dental materials produced distinctive odors, no attempt was made to obscure the identity of the test agents. It should be noted that the inhibition zone size diameters did not necessarily represent absolute inhibitory values of a particular agent/species combination, but rather general indications of the agents' potency or lack thereof in relation to other materials. In addition, slight variations in zone sizes may have resulted from errors made in judgment of well depth and angulations in the agar.

Statistical analysis

Measurements of inhibitory zone were ranked into the following five inhibition categories according to the proportional distribution of the data set:

1. No
2. Weak
3. Medium
4. Medium strong
5. Strong antimicrobial activity.

The Friedman repeated measures of analysis of variance on ranks was conducted to compare the statistical difference of antimicrobial effects between materials tested with each of four bacterial groupings (anaerobic gram positive, anaerobic gram negative, facultative anaerobic gram positive, and facultative or aerobic gram negative), as well as combined data. The Duncan multiple comparison test then was used to sort the 10 materials into statistically distinct groups.

Results

The inhibitory potential of each material was categorized arbitrarily as strong, medium strong, medium, weak, or noninhibitory depending on the average size of the zones. Zone size categories and proportions of data represented in each are presented in Table 3. Data are presented according to each microbial strain/filling materials reaction in Table 4. Vaseline™ was the only material to show no inhibition against all test strains. Vitapex™ showed strong or medium strong activity against all *Prevotella* species, but no inhibition against the remaining organisms. $Ca(OH)_2+H_2O$ inhibited two of four *P. micros* (CA1 and CA2), and *P. intermedia* and *P. buccae*, but did not inhibit the remaining organisms. $ZnO+H_2O$ generally inhibited all bacterial strains except *P. anaerobius*, *P. magnus*, *S. aureus*, *Candida albicans*, *P. aeruginosa*, and *B. fragilis*. ZOE inhibited all test strains except *P. micros* (ATC), *P. magnus*, and *S. mutans*. Kri™ paste inhibited all test strains except *P. magnus*, *B. fragilis*, and *L. casei*. CHX+ZOE, FC+ZOE, CPC+ZnO, and $CPC+Ca(OH)_2$ inhibited all test strains.

Statistically significant grouping of inhibitory effects of the materials against categories of microbial species (i.e., anaerobic gram positive, anaerobic gram negative, facultative anaerobic gram positive, and facultative anaerobic gram negative) revealed the following:

TABLE 3. RANKING SCHEME FOR MICROBIAL INHIBITION

Rank	Range of Zone Diameters (mm)	% of Data Set Represented*	Frequency (N = 210)
No	0	31	65
Weak	1.4–6.2	20	40
Medium	6.3–10.3	20	42
Med. strong	10.4–26.8	20	42
Strong	> 26.8	10	21

* Data consisted of mean zone measurements of 21 test organisms and 10 materials.

TABLE 4. INHIBITION RESULTS OF 10 MATERIALS AGAINST 21 TEST MICROORGANISMS

Test Species†	Filling Materials*									
	CPC+Ca‡	CPC+ZnO	FC+ZOE	CHX+ZOE	Kri™	ZOE	ZnO+H_2O	Ca‡+H_2O	Vitapex™	Vaseline™
Pmic(CC1)	S§	MS	S	M	MS	MS	MS	No	No	No
Pmic(CA1)	S	S	W	M	W	W	M	No	No	No
Pmic(CA2)	M	M	MS	W	MS	M	W	M	No	No
Pmic(ATC)	W	W	W	M	W	No	W	W	No	No
Vpar	W	M	MS	M	W	W	W	No	No	No
Pint(ATC)	S	S	MS	MS	S	M	MS	M	S	No
Pint(CC2)	S	S	MS	MS	S	W	W	MS	S	No
Pmel	MS	MS	MS	M	S	M	M	No	MS	No
Smut	MS	MS	M	M	W	No	W	No	No	No
Lcas	M	MS	M	M	No	W	W	No	No	No
Calb	MS	MS	MS	M	M	M	No	No	No	No
Saur	M	M	MS	M	W	W	No	No	No	No
Bfra	MS	MS	MS	W	No	W	No	No	No	No
Pana	M	M	W	W	M	W	No	No	No	No
Pmag	M	M	MS	W	No	No	No	No	No	No
Scon	M	M	MS	W	W	W	W	No	No	No
Smor	MS	MS	MS	M	W	W	M	No	W	No
Ecol	MS	M	M	W	M	W	W	No	No	No
Nspe	MS	MS	MS	MS	MS	MS	M	No	No	No
Paer	MS	MS	W	M	M	M	No	No	No	No
Pbuc	S	S	S	S	S	MS	S	S	S	No

* See Table 1 for key to abbreviations. † See Table 2 for key to abbreviations. ‡ Ca: $Ca(OH)_2$ + H_2O. § Inhibition: S = strong; MS = medium strong; M = medium; W = weak; No = no inhibition.

1. Anaerobic gram positive—CPC+$Ca(OH)_2$, CPC+ZnO, and FC+ZOE were most inhibitory; CHX+ZOE, Kri™ paste, and ZnO+H_2O were less inhibitory; and $Ca(OH)_2$+H_2O, Vitapex™, and Vaseline™ were noninhibitory.
2. Anaerobic gram negative—all materials were inhibitory except $Ca(OH)_2$ and Vaseline™; $Ca(OH)_2$ was less inhibitory and Vaseline™ was noninhibitory.
3. Facultative anaerobic gram positive—CPC+$Ca(OH)_2$, CPC+ZnO, FC+ZOE and CHX+ZOE were inhibitory; Kri™ paste, ZOE, and ZnO+H_2O were less inhibitory; and $Ca(OH)_2$+H_2O, Vitapex™, and Vaseline™ were noninhibitory.
4. Facultative anaerobic gram negative—CPC+$Ca(OH)_2$, CPC+ZnO, FC+ZOE, CHX+ZOE, Kri™ paste, and ZOE were inhibitory; ZnO+H_2O was less inhibitory; and $Ca(OH)_2$+H_2O, Vitapex™, and Vaseline™ were noninhibitory.

Discussion

Most of the previous studies that tested antibacterial activity of filling materials against pure cultures used gram-positive cocci, gram-negative rods, or other bacteria that may not have represented the predominant bacterial species found in infected root canals, i.e., peptostreptococci, *Prevotella* (*Bacteroides*) spp., and obligate and facultative anaerobic streptococci.[19–23] While our objective was to test bacteria that were more representative of endodontic microbiota, comparing our data with previous studies[2–18] is difficult because of the different test strains, media and culture conditions involved. In addition, as our study used some materials that were prepared in our laboratory (e.g., CHX+ZOE), the formulations may have been slightly different from similar products used in other investigations.

All of the filling materials showed stronger antibacterial effectiveness against obligate anaerobic gram-negative microorganisms than gram-positive, facultative anaerobic, or aerobic bacteria. Generally, both obligate and facultative anaerobic gram-negative bacteria were more sensitive to filling materials than were gram-positive bacteria.

Black-pigmented anaerobes (*Prevotella, Porphyromonas,* and *Bacteroides* spp.) are believed to be pathogens in periapical infections,[25] and have demonstrated resistance to penicillin. Brook[26] and Crook et al.[27] reported the rates of penicillin resistance up to 50% in the oral *Bacteroides.* Wasfy et al.[28] found beta-lactamase production in approximately 40% of strains of *B. melaninogenica, B. intermedia,* and *B. gingivalis* in children. As the iodoform base materials (Kri™ paste and Vitapex™) showed strong or medium-strong inhibitory activity against *P. melaninogenica, P. intermedia,* and *P. buccae* in our investigation, these may be clinically indicated for treating infected teeth where penicillin resistance has been demonstrated or is suspected.

Anaerobic streptococci, which also are predominant species in infected root canals,[23,24] showed more resistance to the materials than did the *Prevotella* species.

Only CPC- and FC-containing materials demonstrated antibacterial effectiveness against anaerobic streptococci, but inhibition varied according to the species or strain employed.

The materials containing CPC or FC inhibited all of the test bacterial species in this investigation, and showed strong inhibition against one-third of all the test species. This agreed with the results of Stuart's study (CMCP against *S. mutans*)[18] and Orstavik's study (CPC against *S. aureus* and *P. aeruginosa*).[3] Both our study and Cox, Jr. et al.'s study[14] found an inhibitory effect against *E. coli* and *S. aureus* by FC+ZOE. Pupo et al.[7] and Seow[10] also demonstrated that FC+ZOE could inhibit *S. aureus*. We supported Thomas et al.'s[29] finding that FC+ZOE could inhibit *P. aeruginosa*, and Stuart et al.'s[18] finding that FC could inhibit *S. mutans*.

Kri™ paste showed stronger antibacterial effectiveness than did ZOE against pure cultures, especially against *Prevotella* species, but ZOE exhibited medium to weak inhibition, generally, against all test species. This differs from the Wright et al.[30] study, which found the antibacterial effect of ZOE to be more inhibitory to *S. faecalis* than Kri™ paste. Although *S. faecalis* was not employed as a test species in this study, Kri™ paste was more inhibitory than ZOE against the six gram-positive streptococci tested.

Some studies showed Kri™ paste to be inhibitory to *S. aureus*,[3, 10, 12] but we found it to be only minimally inhibitory against this organism. Both our study and Orstavik's study,[3] however, showed Kri™ paste to inhibit *P. aeruginosa*.

Cox Jr. et al.[14] found that ZOE could not inhibit *E. coli*, which conflicts with our finding and that of Tobias et al.,[6] but could inhibit *S. aureus*, which agrees with our and Orstavik's findings.[3] Also, both Orstavik's study and this study found that ZOE could inhibit *P. aeruginosa*.[3]

ZOE containing chlorhexidine dihydrochloride showed antibacterial activity against all of the test bacterial species, but the activity was of medium or weak strength.

We found that Vitapex™ did not inhibit the growth of *S. mutans*, *S. aureus*, or *L. casei*—results that conflict with Ninomiya et al.'s study,[31] and showed no antibacterial activity against most pure cultures. The weak activity may be partially explained by the fact that calcium hydroxide, an ingredient of Vitapex™, has been demonstrated to interfere with the antiseptic capacity of dyadic combinations of endodontic medicaments.[10]

$Ca(OH)_2+H_2O$ demonstrated no or weak antibacterial effects in the agar diffusion assays. This is surprising due to the high pH of calcium hydroxide that should inhibit bacteria. It is possible that the pH was neutralized by blood or buffers in culture media in the in vitro experiments, a phenomenon that may also occur in vivo where blood and buffering systems are present. Stuart et al.[18] found $Ca(OH)_2+H_2O$ to inhibit *S. mutans*, *Actinomyces viscosus*, *B. gingivalis*, and *B. fragilis*. Al-Khatib et al.[13] found that $Ca(OH)_2$ could inhibit *S. mutans*, *S. aureus*, and *B. endodontalis*, but $Ca(OH)_2+H_2O$ could not. We also found no inhibition zones against *S. mutans* or *S. aureus* by $Ca(OH)_2+H_2O$, but this agent produced medium or medium-strong inhibition of *P. intermedia* strains and strong inhibition of *P. buccae*.

In conclusion, the strongest inhibitory activity against all test organisms was observed with CPC+$Ca(OH)_2$, CPC+ZnO, and FC+ZOE. The next most inhibitory were CHX+ZOE and Kri paste followed by ZOE and $ZnO+H_2O$. $Ca(OH)_2+H_2O$, Vitapex™ and Vaseline™ showed minimal or no inhibition. These findings coincided with our earlier study using mixed microbial specimens obtained directly from nonvital primary teeth.[32]

Average inhibition zone diameters for each filling material exposed to 13 specimens in the previous study[32] were similar to the average zone diameters for the same filling materials against all 21 test organisms in this study, with the exception that Kri™ paste, Vitapex™, and $Ca(OH)_2+H_2O$ produced larger mean zone diameters in the present investigation. The strong or medium-strong inhibition of anaerobic gram-negative bacteria by these three materials may explain the latter finding. While specimens from nonvital primary teeth certainly harbor anaerobic gram-negative bacteria, the zones of inhibition could be limited by more resistant bacteria in the specimens.

The clinical relevance of findings from this study and our previous investigations,[32] however, can only be determined in clinical trials. Our data may be useful as a guide for relative antimicrobial effectiveness or noneffectiveness of the materials employed.

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