Journal of Endodontics
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Printed in U.S.A.
Vol. 29, No. 5, May 2003

Evaluation of Protocols for Field Decontamination Before Bacterial Sampling of Root Canals for Contemporary Microbiology Techniques

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The effectiveness of sodium hypochlorite (NaOCl) (2.5%) or iodine (10%) for decontamination of the operation field (tooth, rubber dam, and gasket [Oraseal]) was compared by using bacterial cultivation. In addition, the final samples were also assessed for bacteria by using polymerase chain reaction. Teeth (*n* = 63) receiving root canal treatment were polished with pumice, isolated with rubber dam, and their margins sealed with Oraseal. The operation field was disinfected with hydrogen peroxide (30%), followed by iodine (*n* = 31) or NaOCl (*n* = 32), before and after access cavity preparation. The operation field was sampled before and after each decontamination, giving four samples per field. After the final decontamination, there was no significant difference (p = 0.602, 0.113, 0.204) in recovery of cultivable bacteria from various sites in either group. However, bacterial DNA could be detected significantly (p = 0.010) more frequently from the tooth surfaces after iodine (45%) compared with NaOCl (13%) decontamination, although on the rubber dam or Oraseal surfaces there was no difference. Root canal sampling for polymerase chain reaction might be better preceded by NaOCl decontamination than by iodine, based on the findings.

Studies of root canal infection may be compromised at various preliminary stages, such as decontamination of the field, access cavity preparation, during sampling, transportation of the sample to the laboratory, and finally during laboratory processing for cultivation (1). The protocols used at each of these stages should ideally be optimal and standardized both within and between studies, enabling valid comparison of results between workers. Möller's (1) comprehensive work on the methodology for microbiological examination of root canals and periapical tissues is accepted as the gold standard. Decontamination of the sampling field is mandatory to avoid false-positive results during microbiological analysis. Möller (1) recommended cleaning the field with 30% hydrogen peroxide followed by swabbing with 5% or 10% iodine tincture before root canal sampling. Curiously, these agents are not favored for intracanal debridement, whereas sodium hypochlorite (NaOCl) is universally favored (2) and is also preferred as a working-surface disinfectant (3). The antibacterial effect of NaOCl is due to its powerful oxidizing action (4). It also has the ability to disintegrate organic tissue (5) and consequently may be able to disrupt the integrity of the tooth surface plaque biofilm. Its ready availability in dental clinics makes it an obvious candidate for decontamination of the sampling field as well. The efficacy of NaOCl in decontamination of the tooth surface has not been directly compared with the protocol recommended by Möller (1).

Rubber dam isolation is a prerequisite to root canal treatment. It provides protection against foreign material entering the oro-pharynx, chemical irritation of oral soft tissues, and salivary contamination of the pulp chamber. On occasions, however, contamination of the operation site by saliva, gingival fluid, or blood may occur because of leakage (6). Such leakage is more common with the single-tooth isolation often used for root canal treatment. It has been suggested that seepage of fluid may be prevented by using a paste gasket material, such as a mixture of zinc oxide powder and denture adhesive between tooth and dam (7). A commercially available cellulose/zinc oxide material (Oraseal; Ultradent Products, Inc., South Jordon, UT, U.S.A.), which forms a firm gel on contact with saliva, is able to patch small leaks conveniently. The role of such a material in bacterial sampling of root canals has not been considered.

Until the late 1980s, detection of bacteria was reliant upon their growth in the laboratory. The invention of polymerase chain reaction (PCR) by Mullis in 1987 (8) brought about the possibility of detection of bacteria by amplification of their DNA, in particular the 16S RNA gene (9, 10). Studies using this method of detection must therefore use precautions to eliminate bacterial DNA in the operation field, during decontamination.

The objectives of this study were to compare the effectiveness of NaOCl (2.5% v/v) and iodine tincture (10% w/v) for decontamination of the surfaces of teeth, rubber dam, and gasket material (Oraseal) using both cultivation and molecular techniques for detection of bacteria.

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TABLE 1. No. of samples with bacterial contamination for both iodine (n = 31) and sodium hypochlorite (n = 32) decontamination protocols as assessed by culture and PCR. Figures in parenthesis indicate percentages.

Sample	Method of detection	Tooth surface (T)			Rubber dam (R)			Oraseal (O)		
		Iodine	NaOCl	Iodine vs NaOCl p value	Iodine	NaOCl	Iodine vs NaOCl p value	Iodine	NaOCl	Iodine vs NaOCl p value
1st set	Culture	21 (68)	17 (53)	0.353	8 (26)	12 (38)	0.468	20 (65)	17 (53)	0.508
2nd set	Culture	9 (29)	11 (34)	0.853	3 (10)	6 (19)	0.504	5 (16)	6 (19)	1.000
3rd set	Culture	9 (29)	21 (66)	**0.004**	9 (29)	7 (22)	0.828	14 (45)	18 (56)	0.450
4th set	Culture	6 (19)	9 (28)	0.602	4 (13)	0	0.113	3 (10)	8 (25)	0.204
4th set	PCR	14 (45)	4 (13)	**0.010**	5 (16)	4 (13)	0.959	14 (45)	7 (22)	0.090

NB p values in bold are significant at 5% level.

MATERIALS AND METHODS

Sixty-three adult patients scheduled for root canal treatment in the Department of Conservative Dentistry, Eastman Dental Institute were randomly divided into two groups (iodine and NaOCl). The tooth under treatment was polished with pumice and isolated with rubber dam. Oraseal was applied around the dam margin to prevent leakage of saliva.

The surfaces of the tooth, rubber dam, clamp, and Oraseal were decontaminated by scrubbing with 30% (v/v) hydrogen peroxide (H_2O_2) (Sigma-Aldrich Company Limited, Poole, UK) until no further effervescence was evident. Either iodine tincture (10% w/v) (Betadine; Seton Healthcare Group plc, Oldham, UK) (n = 31) or NaOCl (2.5% v/v) (Sainsbury's household bleach; J. Sainsbury plc, London, UK) (n = 32) were then applied for 1 min. The solutions were inactivated by sterile sodium thiosulphate solution (5% w/v) (Sigma Chemical Co., St. Louis, MO, U.S.A.). The decontamination procedures were repeated after access cavity preparation using a sterile tungsten carbide bur in an air-turbine handpiece (W&H Topair 195 RM; Dentalwerk Bürmoos Ges.m.b.H., Bürmoos, Austria).

The field at three sites (tooth/restoration [T], rubber dam [R], and Oraseal [O]) was sampled for bacterial contamination at four stages. At each point, bacterial samples were taken by "swabbing" the respective sites with three sterile paper points (ROEKO D-89122; ROEKO, Langenau, Germany) for each site. The first set of samples (T1, R1, O1) was taken before decontamination, the second (T2, R2, O2) after the first decontamination procedure, the third (T3, R3, O3) after accessing the pulp chamber, and the fourth (T4, R4, O4) after the second decontamination procedure following access cavity preparation. The soaked portions of the three paper points were cut off with sterile scissors into a sterile vial containing 400 μl of reduced transport fluid (RTF) (11) under the perfusion of anaerobic gas consisting of 10% H_2, 10% CO_2, and 80% N_2 (BOC Gases, Guildford, UK).

Samples were transported to the microbiology laboratory on ice and processed within 10 min. Paper points were vortexed to resuspend the bacterial cells and 100-μl aliquots were dispersed in duplicate onto Anaerobe Agar (Bioconnections, Leeds, UK) and blood agar (Bioconnections) plates containing 5% v/v defibrinated horse blood (Oxoid Limited, Basingstoke, UK). The remainder of the final samples (T4, R4, O4) were frozen (−70°C) for subsequent molecular analysis. The plates were incubated either anaerobically or aerobically at 37°C for 7 and 3 days, respectively. The presence of bacterial colonies on either agar plate indicated contamination of the sampled site.

A PCR technique was also used to detect bacteria or bacterial DNA in the final paper point samples for all three sites (T4, R4, O4). DNA was extracted from the samples by routine laboratory methods and amplification of the 16S ribosomal RNA gene by PCR was performed (10) using the global primers 27F and 1492R (12). PCR products were separated by agarose gel electrophoresis, stained with ethidium bromide, and observed under ultraviolet light. Presence of an amplicon of c1500 bases indicated contamination of the sampled site. Positive controls of *Escherichia coli* DNA and negative controls containing water and PCR reagents only were always included.

TABLE 2. Results of statistical comparison between samples taken at different stages using cultivation data

	Iodine group	NaOCl group
Tooth samples		
T1 versus T2	**0.005**	0.208
T2 versus T3	1.000	**0.024**
T3 versus T4	0.553	**0.006**
Rubber dam samples		
R1 versus R2	0.184	0.164
R2 versus R3	0.108	1.000
R3 versus R4	0.212	**0.016**
Oraseal™ samples		
O1 versus O2	**<0.001**	**0.009**
O2 versus O3	**0.028**	**0.005**
O3 versus O4	**0.004**	**0.022**

NB p values in bold are significant at 5% level.

Chi-square tests were used to compare: (i) NaOCl or iodine decontamination, and (ii) the culture and PCR methods for detecting contamination.

RESULTS

Baseline data (T1, R1, O1) revealed no significant ($p = 0.353$, 0.468, 0.508, respectively) difference between the iodine and NaOCl groups in the frequency of detection of cultivable microorganisms before decontamination procedures (Table 1).

A significant reduction in the number of samples showing contamination was only evident (Table 2) on the tooth surface (T1 versus T2, $p = 0.005$) and Oraseal (O1 versus O2, $p < 0.001$) samples from the iodine group and Oraseal (O1 versus O2, $p = 0.009$) samples from the NaOCl group.

There was a significant increase in the number of samples showing contamination of Oraseal (O2 versus O3) after access cavity preparation for both iodine ($p = 0.028$) and NaOCl ($p = 0.005$) groups (Table 2). Only 10% and 25% of the final Oraseal samples (Table 1) showed contamination after the second decon-

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TABLE 3. Comparison between culture and PCR technique for detecting contamination in the final samples

Protocol used	Method of detection	Total number of final contaminated samples (T4 + R4 + O4)
Iodine $n = 31 \times 3$ (93)	Culture	13 (14%)
	PCR	33 (35%)
	Comparison between culture and PCR	p = **0.003**
NaOCl $n = 32 \times 3$ (96)	Culture	17 (20%)
	PCR	15 (16%)
	Comparison between culture and PCR	p = 0.846

NB p value in bold is significant at 5% level.

tamination step (O3 versus O4) using iodine (p = 0.004) or NaOCl (p = 0.022), respectively (Table 2).

There was also a significant increase in the number of samples showing contamination of the tooth surfaces (T2 versus T3, p = 0.024) after access cavity preparation in the NaOCl group (Table 2). However, only 28% (T3 versus T4, p = 0.006) (Tables 1 and 2) showed contamination after the second decontamination step. Such a degree of recontamination and therefore subsequent decontamination was not evident for the iodine group.

The rubber dam was not significantly recontaminated during access cavity preparation (R2 versus R3) in either group (Table 2). There was, however, a further significant (p = 0.016) reduction (R3 versus R4) in number of contaminated rubber dam samples after the second decontamination step in the NaOCl group.

The fourth and final samples (T4, R4, O4) showed no significant difference between the final iodine and NaOCl decontamination steps, in the number of samples positive for viable bacterial contaminants after (Table 1).

In contrast, when PCR was used as the bacterial detection method for the fourth and final samples, those from the tooth surface revealed a significantly (p = 0.010) higher proportion of contamination using the iodine protocol (45%) compared with the NaOCl protocol (13%) (Table 1). Such a difference between these decontamination protocols was not found for the rubber dam and Oraseal samples using PCR.

The PCR technique detected bacterial (DNA) contamination significantly (p = 0.003) more frequently than the culture technique (35% versus 14%) after using the iodine decontamination protocol, considering all samples together (n = 93) (Table 3). There was, however, no significant difference (p = 0.846) in the frequency of detection of contaminated samples by PCR (16%) compared with the culture (20%) technique after using the NaOCl decontamination protocol (Table 3).

DISCUSSION

The use of culture techniques is widely established for the detection of bacteria. Indeed the original tooth decontamination studies used these criteria for protocol development (1). More recently however, PCR techniques have been used increasingly for studies of the root canal flora (13, 14). They offer a more sensitive threshold of detection (15, 16) and do not require the presence of cultivable bacteria (17). Even the presence of bacterial DNA released by breakdown of dead cells may give a positive test.

The initial samples (T1, R1, O1) gave baseline data for the degree of contamination of the three sites before the decontamination protocols and were similar between the test groups (Table 1). Viable bacteria could only be detected in 53% and 68% of the T1 samples in the NaOCl and iodine groups, respectively. That 100% contamination was not evident is attributable to plaque removal by polishing of the tooth surface with pumice. Contamination of the applied rubber dam and Oraseal, on the other hand, is likely to occur during and after oral application.

After the first decontamination step, all sites had reduced numbers of contaminated samples but the reduction was not statistically significant for the rubber dam samples using both protocols and the tooth surface using the NaOCl protocol. The former observation is explained by the fact that rubber dam was usually not heavily contaminated at the outset, whereas the latter by the fact that fewer teeth in the NaOCl group were initially contaminated and that there were slightly more contaminated samples after the first decontamination step. Only 66% and 71% of the samples from the teeth/restorations were "sterile" after using the first NaOCl or iodine decontamination, respectively. In contrast, Möller (1) showed that 83.3% to 98.2% decontamination was achieved after use of his iodine protocol on teeth with or without restorations. More consistent with this study, he found teeth restored with temporary cements were more difficult to decontaminate (sterility achieved in 75–81% of cases). Such temporary restorations were present in a large proportion (89%) of the teeth in the present study and a second decontamination step was able to achieve levels of decontamination comparable to Möller's (1) observations, that is 72% and 81% for the NaOCl and iodine groups, respectively.

Of the samples taken from Oraseal, 81% and 84% were successfully decontaminated in the iodine and NaOCl groups, respectively, with the reduction being significant (Table 2). This compares favorably with Möller (1) who reported 83% decontaminated samples from the junctions between the tooth and the rubber dam without the aid of any gasket material. Oraseal is a proprietary product sold for the purpose of sealing the junction of rubber dam and the tooth to prevent leakage of saliva. This gasket material, which absorbs moisture from saliva or irrigant to form a gel, prevents leakage of fluids by bulk flow in either direction. The porous nature of the material, however, may potentially allow it to form a reservoir for microorganisms leaking through the rubber dam margin from the oral cavity. Equally, the results suggested that decontaminating agents were able to penetrate the body of the material to exert their bactericidal effects.

Access cavity preparation included the removal of any defective/leaking restoration together with carious dentine. The latter may potentially serve to recontaminate the sampling field. The recontamination was found to be significant on the surfaces of teeth (T2 versus T3) for the NaOCl group and the Oraseal (O2 versus O3) for both groups (Table 2). This observation reinforces the necessity for the second decontamination step.

Statistical analyses revealed no significant differences between the use of iodine or NaOCl as decontaminating agents when the bacterial culturing method was used for detection (Tables 1 and 3).

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Such a comparison has not been reported in the literature, although Möller (1) compared iodine tincture with chloramine and preferred the former for decontamination.

Although iodine was found to be no different from NaOCl as a decontaminant when assessed by culture, a significant difference was evident in one area by PCR. In this study, PCR detected contamination of samples from iodine-treated tooth surfaces more frequently than NaOCl-treated surfaces (Table 3). This may be attributed to the mode of action of the antimicrobial agents. NaOCl has been shown not only to kill bacteria but also to generate single strand breaks in DNA (18). Conversely, the bactericidal nature of iodine tincture has been shown not to be due to DNA damage (19). In addition, although ethanol is bactericidal, it has no deleterious effects on DNA, indeed it is used in the isolation and purification of nucleic acids. Because successful PCR depends on continuous lengths of DNA (at least 1500 base pairs in this case), highly fragmented DNA would provide poor template for PCR.

On the basis of the results of this study, it can be concluded that both iodine and sodium hypochlorite protocols do not ensure complete decontamination of all sites. Therefore, when sampling for microbiological studies, it is essential to perform sterility control tests of the sampling field to account for false positives. It is also important to include the second decontamination stage after access cavity preparation, because the removal of temporary filling material and carious dentine may recontaminate the field. Because NaOCl is more effective in some areas at reducing PCR-detected contamination, it might be recommended as the decontaminant of choice for such studies of root canal infection. Alternatively, it may be added as an additional step to remove or denature DNA.

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