



Contamination Controls for Analysis of Root Canal Samples by Molecular Methods: An Overlooked and Unsolved Problem

David Figdor, MDS, FRACDS, DipEndo, PhD, FASM,* and Malin Brundin, DDS, PhD†

Abstract

Introduction: It has been almost 20 years since molecular methods were first described for the analysis of root canal microbial flora. Contamination control samples are essential to establish DNA decontamination before taking root canal samples, and this review assessed those studies. **Methods:** Using PubMed, a search was conducted for studies using molecular microbial analysis for the investigation of endodontic samples. Studies were grouped according to the cleaning protocol, acquisition methods, and processing of control samples taken to check for contamination. **Results:** Of 136 studies applying molecular analysis to root canal samples, 21 studies performed surface cleaning and checking nucleotide decontamination with contamination control samples processed by polymerase chain reaction. Only 1 study described disinfection, sampling from the access cavity, and processing by polymerase chain reaction and reported the result; that study reported that all samples contained contaminating bacterial DNA. **Conclusions:** Cleaning, disinfection, and checking for contamination are basic scientific prerequisites for this type of investigation; yet, this review identifies it as an overlooked issue. On the basis of this review, we call for improved scientific practice in this field. (*J Endod* 2016;42:1003–1008)

Key Words

Contamination controls, molecular analysis, polymerase chain reaction, root canal sampling

From the *Department of Microbiology, Monash University, Melbourne, Australia; and †Department of Odontology/Endodontics, Faculty of Medicine, Umeå University, Umeå, Sweden.

Address requests for reprints to Dr Malin Brundin, Department of Odontology/Endodontics, Faculty of Medicine, Umeå University, Umeå 90187, Sweden. E-mail address: malin.brundin@umu.se

0099-2399/\$ - see front matter

Copyright © 2016 American Association of Endodontists.

<http://dx.doi.org/10.1016/j.joen.2016.04.015>

The first published article applying molecular analysis for the investigation of root canal microbiota was published in 1997 when the polymerase chain reaction (PCR) technique was used to target specific DNA sequences in samples taken from infected root canals (1). Advances in molecular techniques for microbial identification provide fast, accurate, and straightforward procedures for taxonomic characterization of species in clinical samples.

From that first publication in 1997 to October 2015, there have been more than 136 articles that use a molecular approach for laboratory processing of the microbial isolates. In this narrative review, we examine an important specific issue, namely contamination controls for the analysis of root canal samples using molecular methods.

Significance

Accurate sampling of the root canal microbiota is contingent upon a sterile field and checking for contamination prior to entering the pulp space. Molecular methods screen for microbial DNA, which is ubiquitous on tooth structure. The high binding affinity between DNA and hydroxyapatite presents a special challenge for decontamination. Review of studies using molecular analysis reveals that decontamination and controls that check for extraneous DNA are overlooked issues. Cleaning and contamination controls are essential for best practice.

A Brief History

Before the advent of sequence-based identification, microbiological analysis of root canal microbiota relied on conventional culture. The principles and scientific methods for microbial sampling from infected root canals were developed in the 1960s and published in a classic thesis (2). Möller's work was significant for several reasons, not the least being the scientific rigor he applied to controlling the field before taking a sample from the subject area. Numerous articles using culture techniques for processing root canal samples had been published before that time. What changed was the logical yet simple refinements made by Möller to rigorously control the sampling and subsequent laboratory processing for the accurate recovery of bacteria.

An essential preparatory step was to work in a sterile field that excluded extraneous microbes and then check the field with a contamination control sample to confirm the absence of contaminants before entry into the pulp space. This seemingly simple but hitherto overlooked step was based on the premise that if microbes were present on the tooth surface, they may be picked up when taking the sample, thus contaminating the results of the root canal sample. Möller asked 2 essential questions: How does one clean and disinfect the tooth surface? and, when taking the sample, How can one check that the operating field is free from surface-contaminating microbes? Möller answered those questions by exhaustively developing and testing various cleaning and disinfection protocols that could be applied in a reproducible way. He then confirmed the validity of his methods by taking contamination controls to check the effectiveness of cleaning and disinfection steps. Before his careful pioneering work, many studies were poorly controlled for contamination, and there were many gaping technical problems that negated accurate sampling of the infected root canal space.

What You Can't See Won't Matter or Will It?

It is important to recognize the essential differences between culture and molecular-based studies. Conventional culture reveals the presence of living, growing microbes that can be seen as colonies on agar plates or broth culture. Cultivated colonies can then be processed by phenotypic and biochemical tests to identify the species. Molecular studies record the presence of DNA, usually specific nucleotide sequences targeted by PCR. In recent years, advances in DNA sequencing mean that recovered DNA can be partially or wholly sequenced for species identification. Because these are such disparate methods, it is worth noting that sterility means something different for each method. A sterile field for culture means that there are no cultivable viable microbes, whereas a sterile field for molecular work means an absence of recoverable microbial nucleotides from the respective sampled region.

The relevant questions posed and effectively answered by Möller in his 1966 work are as pertinent today as they were in the 1960s. Is the tooth surface free from contaminating microbial DNA before taking the root canal sample? The corollary question is can it be confirmed that cleaning and decontamination (of microbial nucleotides) have been effective? Have contamination controls been taken to ensure that extraneous DNA is not picked up when taking a sample from the root canal space?

A Challenging Issue: DNA Binding to Hydroxyapatite

The chemical composition of teeth presents a special issue in relation to nucleotide sampling because of the strong binding affinity of hydroxyapatite for DNA. This discovery (3) provided great benefit for the separation of DNA in early studies (4). It provides a unique scientific advantage, such as the ability to recover ancient DNA from animal and human remains (5). Yet, it also presents special challenges in endodontic microbiology.

Recent studies have confirmed both the binding affinity of hydroxyapatite and dentin for DNA (6, 7). One of the implications from this work is that microbial biofilms, which contain extracellular DNA (8), potentially leave long-lasting traces of DNA highly bound to the tooth surface. Thus, any microbial biofilm that has had intimate contact with any part of the tooth has the potential to leave detectable traces of microbial DNA for extended periods. Reliable removal of the bound DNA presents an as-yet unsolved challenge.

In this review, we assessed studies that have used molecular approaches for the analysis of root canal microbiota and evaluated their cleaning, disinfection, and nucleotide decontamination protocol; whether a contamination control sample was taken; and how it was processed in the laboratory. Finally, we critically evaluated the results of the contamination controls.

Materials and Methods

Using PubMed, a search was conducted for the following terms: "PCR + root canal," "PCR + endodontic," "molecular + endodontic," and "molecular + root canal." Of those articles describing *in vivo* investigation of root canal microbes, 136 were selected as fulfilling the search criteria and suitable for analysis. The articles were grouped according to whether the surface was cleaned, if it was checked with a contamination control sample, and how samples were processed in the laboratory and then divided into categories. Studies that undertook surface cleaning, acquired a contamination control sample, and processed it by PCR were selected for detailed analysis.

Results and Discussion

There were 136 articles that fulfilled the search criteria. These studies were separated initially according to whether a contamination

control sample was taken and how the control sample was processed in the laboratory (Fig. 1). Eight of 136 articles used no or had no information about a surface decontamination procedure (Fig. 1). The application of appropriate controls is a fundamental requirement in any scientific inquiry, so without controls it calls into question how those articles reached scientific publication.

The majority of articles (89/136) reported cleaning of the tooth surface but no description of a contamination control sample (Fig. 1). An obvious limitation of cleaning without taking a control sample is that there are no data to support the contention that the root canal samples actually disclose microbes exclusively located within the root canal space.

In 16 of 136 articles, the tooth surface was cleaned, and the sterility was checked by culture (Fig. 1). Providing that the results were negative (ie, no samples were positive by culture), this means that there were no living, cultivable bacteria, which is a positive step to confirm a sterile field. However, a control sample processed by culture is insufficient for molecular work because the molecular approach targets nucleotide sequences. For the study to be effective and provide authentic sampling results, the operating field must be tested for the presence of microbial DNA. There should be no amplifiable DNA, which helps verify that the sequences isolated are validly derived from the root canal space.

Only 23 of 136 articles were identified that performed surface cleaning and checking nucleotide decontamination by taking a contamination control sample processed by PCR (Fig. 1). These 23 articles were evaluated according to their reporting of contamination controls (Fig. 2). Of these, 2 articles (9, 10) used the same material from earlier studies (11, 12) (Fig. 2), so they were not analyzed further. In 3 of 23 articles (11, 13, 14), no results were reported from contamination controls, which leaves the findings unknown. It would have been a sound scientific procedure for these articles to report the results of

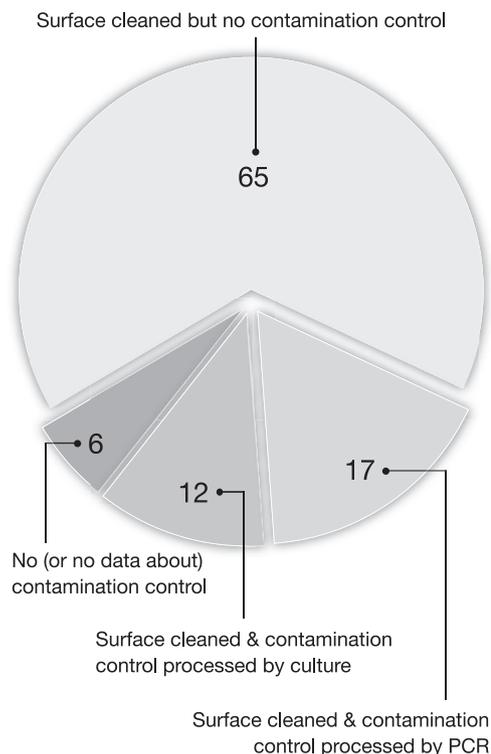


Figure 1. A pie chart showing analysis of 136 articles separated by whether a contamination control sample was taken and whether the control sample was laboratory processed by culture or PCR. The numbers are the percent of articles out of total 136 articles.

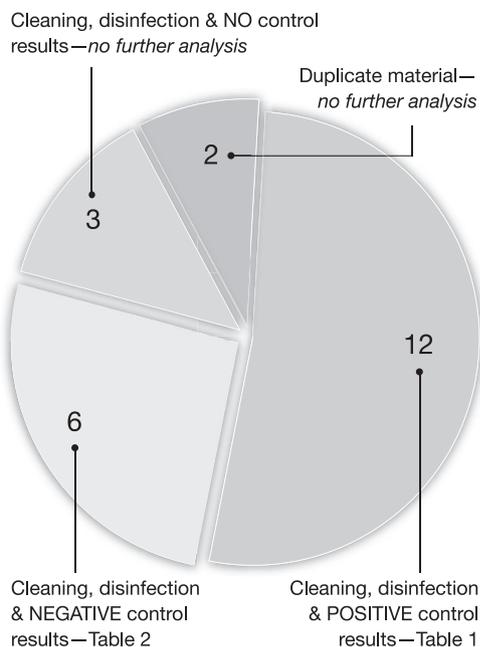


Figure 2. A pie chart showing analysis of 23 articles that performed surface cleaning and checking nucleotide decontamination by taking a contamination control sample processed by PCR. Figures shown are the number of articles in each group (from a total of 23 articles) and how 18 articles are assigned to Tables 1 and 2.

such controls. Thus, there was no further analysis of these 3 studies (Fig. 2). From these 23 articles, there were 18 that performed surface cleaning, checked with a contamination control sample processed by PCR, and reported the results. These 18 papers were further divided into 2 groups (Fig. 2): 12 articles with some contamination control results that were positive for residual DNA (Table 1) and 6 articles with all-negative control results (Table 2).

Of the 12 articles with positive results for DNA contamination, 10 described exclusion of those teeth from the case material (Table 1). One article (16) described all contamination control samples as positive for bacterial DNA, which might negate the reported findings from the root canal samples; however, the species selected for investigation in that study, *Enterococcus faecalis*, was not detected in species-

specific contamination controls, so the authors proceeded with analysis of the root canal samples (16). In another article (15), control samples were taken in only 7 of 58 teeth, and 1 case with a positive contamination control result was not excluded from the study (Table 1).

In 6 of 18 articles that included the contamination control results, all contamination control samples were reported as negative (Table 2). This finding stands in stark contrast to the majority of other work that consistently indicates great difficulty in obtaining nucleotide-free tooth surfaces. The specific reasons are unknown but may include PCR primer sensitivity, the number of amplification cycles (too low may fail to amplify DNA), the method of DNA extraction (eg, boiling cells, used in 1 study, may not release DNA from some species), and whether the primers were species specific or universal (for recovery of all bacterial DNA). How and where the control sample was taken may also significantly influence the result. Some of these issues are further addressed later.

Are All Cleaning Protocols Effective?

Another important question is what were the cleaning and disinfection protocols applied to the tooth surface before taking a sample? For culture-based studies, Möller showed that cleaning with hydrogen peroxide (H_2O_2) and disinfection with tincture of iodine were highly effective for producing a sterile tooth surface. The iodine was inactivated with sodium thiosulfate (to prevent carryover of iodine into culture media) before taking a contamination control sample. Does the same protocol apply for decontamination before taking a control sample for molecular analysis?

The only study known to the authors conducted on cleaning protocols for molecular sampling (32) showed that after cleaning with H_2O_2 , the use of iodine for disinfection resulted in almost half the teeth still being contaminated with recoverable DNA. An improvement occurred when sodium hypochlorite (NaOCl) was used for decontamination, resulting in DNA detection in 13% of samples compared with 45% after using iodine (32). Interestingly, 1 article described the use of iodine for disinfection (Table 2) yet reported no positive findings out of 100 cases (26), which stands in contrast to the findings described in the cleaning protocols study (32).

The fallibility of current decontamination methods for molecular work is also shown in a study (16) that used H_2O_2 and 6% NaOCl to clean and disinfect the tooth surfaces of 23 teeth before sampling the root canal for *E. faecalis*. After cleaning, contamination control samples taken from the access cavity and processed with universal primers

TABLE 1. Analysis of 12 Articles that Describe Cleaning, Disinfection, and a Positive Surface Contamination Control Result by Polymerase Chain Reaction

Study	Method of disinfection*	Site of control sample	Primer type used for analysis	Control results (positive/total)
Kaufman et al, 2005 (15)	30% H_2O_2 , 5% I ₂ , 6% NaOCl	Surface	Enterococcal specific	1/7 [†]
Sedgley et al, 2006 (16)	30% H_2O_2 , 6% NaOCl	Cavity	Universal and Enterococcal specific	23/23 [‡]
Rôças et al, 2011 (17)	3% H_2O_2 , 2.5% NaOCl	Surface	Universal	3/50
Rôças et al, 2011 (18)	3% H_2O_2 , 2.5% NaOCl	Surface	Universal	3/27
Paiva et al, 2012 (12)	6% H_2O_2 , 2% I ₂ , 6% H_2O_2 , 2.5% NaOCl	Cavosurface angle	Universal	3/30
Rôças et al, 2012 (19)	3% H_2O_2 , 2.5% NaOCl	Operative field	Universal	8/50
Neves et al, 2013 (20)	6% H_2O_2 , 2.5% NaOCl	Cavosurface angle	Universal	5/50
Paiva et al, 2013 (21)	6% H_2O_2 , 2% I ₂ , 6% H_2O_2 , 2.5% NaOCl	Cavosurface angle	Universal	4/18
Rôças et al, 2013 (22)	3% H_2O_2 , 2.5% NaOCl	Surface	Universal	1/50
Rôças et al, 2013 (23)	6% H_2O_2 , 2% I ₂ , 6% H_2O_2 , 2.5% NaOCl	Cavosurface angle	Universal	5/45
Rôças et al, 2014 (24)	6% H_2O_2 , 2.5% NaOCl	Cavosurface angle	Universal	3/28
Pinheiro et al, 2015 (25)	30% H_2O_2 , 2.5% NaOCl	Surface	Enterococcal specific	2/20

H_2O_2 , hydrogen peroxide; I, iodine; NaOCl, sodium hypochlorite.

*Two cleans described in all articles but not stated in 1 study (16).

[†]Controls done in 7 cases only (from a total 58); 1 case with a positive contamination control result was not excluded from the study.

[‡]23/23 for Universal; 0/23 for Enterococcal primers. All cases were included in the study material.

TABLE 2. Analysis of 6 Articles that Describe Cleaning, Disinfection, and Negative Surface Contamination Control Results by Polymerase Chain Reaction

Study	Method of disinfection	No. of cleans	Site of control sample	Primer type used for analysis	Processed controls (n/total)
Tomazhino et al, 2007 (26)	30% H ₂ O ₂ , 5% I ₂	1	Surface	BPR specific	100/100
Montagner et al, 2012 (27)	30% H ₂ O ₂ , 2.5% NaOCl	1	Surface	Universal	20/20
Gomes et al, 2013 (28)	30% H ₂ O ₂ , 2.5% NaOCl	1	Operative field	Universal	15/15
Stojanović et al, 2014 (29)	30% H ₂ O ₂ , 2.5% NaOCl	2	Operative field	Universal	51/51
Teles et al, 2014 (30)	3% H ₂ O ₂ , 3% NaOCl	2	Operative field	NS	69/69
Moraes et al, 2015 (31)	30% H ₂ O ₂ , 5.25% NaOCl	1	Operative field and surface	Universal	27/27

BPR, black-pigmented rods; H₂O₂, hydrogen peroxide; I₂, iodine; NaOCl, sodium hypochlorite; NS, not stated.

(for all bacterial DNA) revealed recoverable DNA in 100% of samples (16), which led the authors to raise the question as to whether total physical removal of DNA remaining after disinfecting endodontic access cavities might ever be achievable.

Of 136 articles using molecular methods for analysis of root canal samples, there was a nonuniform approach used for surface cleaning and disinfection. Most studies used a combination of H₂O₂ and NaOCl for cleaning and disinfection of the operative field and tooth (Fig. 3). However, 16% of articles (Fig. 3) used cleaning protocols that did not describe the use of NaOCl, which is generally regarded as essential for the removal of DNA.

When and Where Should a Contamination Control Sample Be Taken?

In the oral cavity, microbial DNA is everywhere. When a sample is taken for the purpose of molecular microbial analysis of the root canal space, the aim should be to first exclude all extraneous DNA and then to verify its exclusion by taking a contamination control sample. That sample must be processed using molecular methods to ascertain the absence of any residual microbial DNA.

When should the sample be taken? If it is taken after cleaning the tooth surface but before access cavity preparation, this would test for residual DNA before cutting the access cavity. Yet, it is likely that prepa-

ration of the access cavity will lead to further microbial DNA deposition or uncover existing DNA within the entry site. Therefore, after access cavity preparation, a second cleaning and disinfection should be performed before taking a sample (33). This is the reason for the recommendation of a 2-step disinfection protocol (32). Briefly, the tooth and surrounding field are cleansed with 30% H₂O₂ and decontaminated with a 2.5% (or higher concentration) NaOCl solution. After access cavity preparation using sterile burs without water spray, the operative field, including the pulp chamber, is then swabbed again with 2.5% NaOCl. NaOCl is then inactivated by sterile 5% sodium thiosulfate (2, 32).

It is interesting to note that a 2-step clean was performed in 11 of 12 studies that described some positive results for contamination control samples (Table 1); yet, 4 of 6 studies that reported negative results for their control (Table 2) used a single-clean protocol.

Where should the sample be taken? It is expected that the operative field (rubber dam and retainer) should be sterile after decontamination and disinfection. In 4 of 6 studies that had negative contamination control results (Table 2), samples were taken from the operative field so it is not surprising that these sites were free of contamination. It is also reasonable to expect that the tooth surface should have no recoverable microbial DNA before taking a root canal sample.

Of the 18 articles in Tables 1 and 2, 13 described samples taken at the tooth surface (6 of which described cavosurface angles, presumably at the surface side of the access cavity); 10 of 15 described some positive contamination control samples (Table 1), and 3 reported negative results (Table 2). However, the external tooth surface is not the site for taking the root canal sample.

Because the aim is to sample the root canal contents, the site of greatest relevance is the access cavity; it is reasonable to expect that the cavity walls are free of contaminating DNA before entry is made into the pulp chamber. Only 2 of the 136 studies identified in this review (11, 16) described 2-step cleaning, taking a contamination control sample from the access cavity before entry into the pulp space and processing the sample with universal PCR primers targeted broadly for bacterial DNA. One of these (11) did not report the results, so the findings from these controls are unknown. The other (16) reported that 100% of access cavities tested were positive for contaminating bacterial DNA.

If a sample is taken after breaching the infected pulp chamber, there is either the risk that microbial DNA from the root canal will be picked up in the control sample or that disinfection of the access cavity before taking the control will ruin the subsequent root canal sample.

From the foregoing analysis, it is apparent that it is difficult to eliminate all traces of bacterial DNA even if the bacteria in the access cavity are dead. It is also evident that contamination control sampling has been mostly taken at sites that are less than relevant for taking a contamination control sample. The site of greatest importance is the access cavity—after it is prepared and before breaching the pulp chamber. Only 1 of 136 studies has done so and reported the findings (16). On the basis of what is currently known, the optimal time and site are to take the control sample of the dentin walls of the access cavity after a 2-step cleaning and disinfection and before entry into the root canal space.

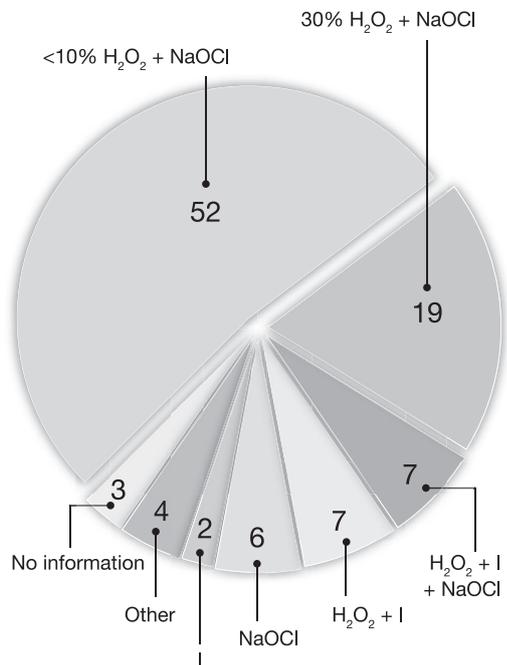


Figure 3. A pie chart showing analysis of 136 articles based on the cleaning and disinfection protocol. The numbers are the percent of articles out of total 136 articles.

Sensitivity of PCR

The PCR technique is widely regarded as highly sensitive for the recovery of DNA. There are many factors that influence the ability of PCR to detect nucleotide sequences, and these include primer design, amplicon size, inhibition factors, and cycle number (34–36). Higher cycle numbers increase sensitivity, and it is generally regarded that 35 to 40 cycles provide optimum amplification with limited artifacts. All but 1 of the 18 studies described in Tables 1 and 2 used ≥ 35 cycles. One study used 30-cycle amplification and reported no recoverable DNA from 69 contamination control samples (30).

How sensitive is PCR when applied to samples taken for contamination controls? One way to calibrate sensitivity is to measure the detection limit (ie, the minimum number of copies/cells that can be detected by that method). Of the studies in Tables 1 and 2, 16 of 18 did not describe a detection limit. Two studies did so; both articles (17, 18) report a thousand cell detection limit.

Live or Dead?

The subject of whether the contaminating DNA is derived from live or dead cells is outside the scope of this review. However, it is worth noting briefly that although a molecular marker for DNA that delineates living from dead cells would be ideal, no such reliable technology is currently available. Various molecular approaches for the preferential detection of viable cells have been proposed, such as ethidium monoazide and propidium monoazide (37, 38), but there are no unanimously agreed-upon molecular markers for living cells. Because messenger RNA (mRNA) is expressed in living cells and has a short half-life, the presence of measurable mRNA is one such putative molecular indicator of living microbes. However, there are few studies that have applied it to endodontic microbiology (39, 40), probably because of the many challenges that make it difficult to accurately detect and measure mRNA in clinical samples.

Plain Speaking on Conducting Suitable Molecular Decontamination Controls

The paucity of studies using satisfactory molecular decontamination controls raises the question as to whether it is possible to satisfactorily clean microbe-covered enamel, restorative materials, dentin, and the access cavity to achieve a nucleotide-free field. Is there an unstated reason why so many studies have not done or reported on the results of DNA decontamination controls? It seems so, and it ought to be brought out. As mentioned previously, only 1 of 136 studies identified in this review described current best practice cleaning, disinfection, and site-specific (ie, access cavity) contamination control samples and processed them with universal primers that detect all bacterial DNA (16). That study reported that 100% of contamination control samples were positive for contaminating microbial DNA (16). In our own preliminary investigations, we found that cleaning the surface with 30% H₂O₂, 2.5% NaOCl, and 5% iodine followed by inactivation of NaOCl with thiosulfate is insufficient to eliminate bacterial DNA from *in vivo* access cavities. Contamination control samples from the access cavity were positive for bacterial DNA in 35%–97% depending on primer choice (unpublished data).

Based on these reports and our own observations, it is time to bring the issue of contamination controls to the surface. Failure to satisfactorily clean and disinfect the tooth surface and access cavity and then checking it with a contamination control before taking a root canal sample for molecular analysis is a problem that must not be overlooked. It is an issue that readers should be aware of when reading endodontic microbiology studies in scientific journals, and it is reasonable to expect that authors and journal referees ensure that all appropriate steps have been performed to a validated scientific standard. When this has not

been done, it ought to be raised in the discussion section so that it is appropriately acknowledged and discussed in the article.

Where to from Here?

Fifty years after Möller's contribution, it is time for a bold re-examination of the controls applied in molecular analysis of samples from the root canal space. Tooth structure, in particular the root canal space, has unique challenges for microbial sampling and molecular processing, so changes are warranted in the way we deal with this issue. Based on the foregoing discussion, it is important to review and reassess the problem. Those conducting a clinical study of this kind know the enormous amount of work to plan it, recruit patients, collect the clinical material, and undertake the laboratory work. It makes good sense to ensure that the data obtained are accurate, reliable, and true.

Now is the time to re-establish basic scientific principles. Valid sampling must begin with proper cleaning and nucleotide disinfection of the tooth surface. Proper protocols are needed for this purpose, and the sterility of the field must be checked by appropriate molecular contamination controls. The results should be clearly articulated in articles prepared for publication. Thus, for cleaning, disinfection, and contamination controls, we propose the following:

1. Clinicians and researchers working in this field of inquiry reintroduce the practice.
2. Referees and editors should insist on it.
3. Readers look for and place greater value on articles that adhere to and describe the practice.

Conclusions

This review assesses the practice of disinfection in studies using molecular analysis of the root canal microbiota and the procedures applied for verifying nucleotide decontamination. Microbial DNA is ubiquitous on tooth surfaces, and the high binding affinity between DNA and hydroxyapatite poses a special challenge for decontamination. The lessons learned 50 years ago for culture methods (excluding and eliminating contamination and then checking for it) appear to have been largely overlooked in studies using molecular methods for the analysis of root canal microbiota. Scrutiny of these studies revealed that only 1 of 136 reported current best practice cleaning, disinfection, and taking a site-specific (access cavity) contamination control sample processed by PCR (16). A salient finding from that study was that all control samples taken from the access cavity were positive for contaminating DNA (16), which highlights just how difficult the task is to achieve a fully nucleotide-decontaminated site. It is a basic scientific principle that the relevant site for sampling undergo thorough nucleotide disinfection and that there are contamination controls to ensure that it is effective. After almost 20 years and over 136 publications of molecular techniques for sampling the infected root canal, it is time for advanced methods to get back to basics.

Acknowledgments

The authors thank Drs Göran Sundqvist and Ramachandran Nair for critical review of the manuscript.

The authors deny any conflicts of interest related to this study.

References

1. Conrads G, Gharbia SE, Gulabivala K, et al. The use of a 16s rDNA directed PCR for the detection of endodontopathogenic bacteria. *J Endod* 1997;23:433–8.

2. Möller Å. *Microbiological examination of root canals and periapical tissues of human teeth. Methodological studies* [Odontological dissertation]. Umeå, Sweden: Umeå University; 1966.
3. Tiselius A, Hjerten S, Levin O. Protein chromatography on calcium phosphate columns. *Arch Biochem Biophys* 1956;65:132–55.
4. Bernardi G. Chromatography of nucleic acids on hydroxyapatite. *Nature* 1965;206:779–83.
5. Sosa C, Vispe E, Nunez C, et al. Association between ancient bone preservation and DNA yield: a multidisciplinary approach. *Am J Phys Anthropol* 2013;151:102–9.
6. Brundin M, Figdor D, Sundqvist G, Sjögren U. DNA binding to hydroxyapatite: a potential mechanism for preservation of microbial DNA. *J Endod* 2013;39:211–6.
7. Brundin M, Figdor D, Johansson A, Sjögren U. Preservation of bacterial DNA by human dentin. *J Endod* 2014;40:241–5.
8. Whitchurch CB, Tolker-Nielsen T, Ragas PC, Mattick JS. Extracellular DNA required for bacterial biofilm formation. *Science* 2002;295:1487.
9. Vianna ME, Horz HP, Gomes BP, Conrads G. *In vivo* evaluation of microbial reduction after chemo-mechanical preparation of human root canals containing necrotic pulp tissue. *Int Endod J* 2006;39:484–92.
10. Paiva SS, Siqueira JF Jr, Rôças IN, et al. Molecular microbiological evaluation of passive ultrasonic activation as a supplementary disinfecting step: a clinical study. *J Endod* 2013;39:190–4.
11. Vianna ME, Horz HP, Gomes BP, Conrads G. Microarrays complement culture methods for identification of bacteria in endodontic infections. *Oral Microbiol Immunol* 2005;20:253–8.
12. Paiva SS, Siqueira JF Jr, Rôças IN, et al. Supplementing the antimicrobial effects of chemomechanical debridement with either passive ultrasonic irrigation or a final rinse with chlorhexidine: a clinical study. *J Endod* 2012;38:1202–6.
13. Gomes BP, Montagner F, Jacinto RC, et al. *Gemella morbillorum* in primary and secondary/persistent endodontic infections. *Oral Surg Oral Med Oral Pathol Oral Radiol Endod* 2008;105:519–25.
14. Rodrigues RC, Antunes HS, Neves MA, et al. Infection control in retreatment cases: *in vivo* antibacterial effects of 2 instrumentation systems. *J Endod* 2015;41:1600–5.
15. Kaufman B, Spångberg L, Barry J, Fouad AF. *Enterococcus* spp. in endodontically treated teeth with and without periradicular lesions. *J Endod* 2005;31:851–6.
16. Sedgley C, Buck G, Appelbe O. Prevalence of *Enterococcus faecalis* at multiple oral sites in endodontic patients using culture and PCR. *J Endod* 2006;32:104–9.
17. Rôças IN, Siqueira JF Jr. Comparison of the *in vivo* antimicrobial effectiveness of sodium hypochlorite and chlorhexidine used as root canal irrigants: a molecular microbiology study. *J Endod* 2011;37:143–50.
18. Rôças IN, Siqueira JF Jr. *In vivo* antimicrobial effects of endodontic treatment procedures as assessed by molecular microbiologic techniques. *J Endod* 2011;37:304–10.
19. Rôças IN, Siqueira JF Jr. Characterization of microbiota of root canal-treated teeth with posttreatment disease. *J Clin Microbiol* 2012;50:1721–4.
20. Neves MA, Rôças IN, Siqueira JF Jr. Clinical antibacterial effectiveness of the self-adjusting file system. *Int Endod J* 2014;47:356–65.
21. Paiva SS, Siqueira JF Jr, Rôças IN, et al. Clinical antimicrobial efficacy of NiTi rotary instrumentation with NaOCl irrigation, final rinse with chlorhexidine and interappointment medication: a molecular study. *Int Endod J* 2013;46:225–33.
22. Rôças IN, Siqueira JF Jr. Detection of antibiotic resistance genes in samples from acute and chronic endodontic infections and after treatment. *Arch Oral Biol* 2013;58:1123–8.
23. Rôças IN, Lima KC, Siqueira JF Jr. Reduction in bacterial counts in infected root canals after rotary or hand nickel-titanium instrumentation — a clinical study. *Int Endod J* 2013;46:681–7.
24. Rôças IN, Neves MA, Provenzano JC, Siqueira JF Jr. Susceptibility of as-yet-uncultivated and difficult-to-culture bacteria to chemomechanical procedures. *J Endod* 2014;40:33–7.
25. Pinheiro ET, Candeiro GT, Teixeira SR, et al. RNA-based assay demonstrated *Enterococcus faecalis* metabolic activity after chemomechanical procedures. *J Endod* 2015;41:1441–4.
26. Tomazinho LF, Avila-Campos MJ. Detection of *Porphyromonas gingivalis*, *Porphyromonas endodontalis*, *Prevotella intermedia*, and *Prevotella nigrescens* in chronic endodontic infection. *Oral Surg Oral Med Oral Pathol Oral Radiol Endod* 2007;103:285–8.
27. Montagner F, Jacinto RC, Signoretti FG, et al. Clustering behavior in microbial communities from acute endodontic infections. *J Endod* 2012;38:158–62.
28. Gomes GB, Sarkis-Onofre R, Bonow ML, et al. An investigation of the presence of specific anaerobic species in necrotic primary teeth. *Braz Oral Res* 2013;27:149–55.
29. Stojanović N, Krnić J, Popović B, et al. Prevalence of *Enterococcus faecalis* and *Porphyromonas gingivalis* in infected root canals and their susceptibility to endodontic treatment procedures: a molecular study. *Srp Arh Celok Lek* 2014;142:535–41.
30. Teles AM, Manso MC, Loureiro S, et al. Effectiveness of two intracanal dressings in adult Portuguese patients: a qPCR and anaerobic culture assessment. *Int Endod J* 2014;47:32–40.
31. Moraes LC, Fatturi-Parolo CC, Ferreira MB, et al. Saliva, supragingival biofilm and root canals can harbor gene associated with resistance to lactic agents. *Braz Oral Res* 2015;29:1–6.
32. Ng YL, Spratt D, Sriskantharajah S, Gulabivala K. Evaluation of protocols for field decontamination before bacterial sampling of root canals for contemporary microbiology techniques. *J Endod* 2003;29:317–20.
33. Sundqvist G. Endodontic microbiology. In: Spångberg L, ed. *Experimental Endodontics*. Boca Raton: CRC Press; 1990:131–53.
34. Stolovitzky G, Cecchi G. Efficiency of DNA replication in the polymerase chain reaction. *Proc Natl Acad Sci U S A* 1996;93:12947–52.
35. Nandakumar R, Mirchandani R, Fouad A. Primer sensitivity: can it influence the results in *Enterococcus faecalis* prevalence studies? *Oral Surg Oral Med Oral Pathol Oral Radiol Endod* 2007;103:429–32.
36. Opel KL, Chung D, McCord BR. A study of PCR inhibition mechanisms using real time PCR. *J Forensic Sci* 2010;55:25–33.
37. Nogva HK, Dromtorp SM, Nissen H, Rudi K. Ethidium monoazide for DNA-based differentiation of viable and dead bacteria by 5'-nuclease PCR. *Biotechniques* 2003;34:804–13.
38. Nocker A, Cheung CY, Camper AK. Comparison of propidium monoazide with ethidium monoazide for differentiation of live vs. dead bacteria by selective removal of DNA from dead cells. *J Microbiol Methods* 2006;67:310–20.
39. Williams JM, Trope M, Caplan DJ, Shugars DC. Detection and quantitation of *E. faecalis* by real-time PCR (qPCR), reverse transcription-PCR (RT-PCR), and cultivation during endodontic treatment. *J Endod* 2006;32:715–21.
40. Rôças IN, Siqueira JF Jr. Identification of bacteria enduring endodontic treatment procedures by a combined reverse transcriptase-polymerase chain reaction and reverse-capture checkerboard approach. *J Endod* 2010;36:45–52.