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REVIEW

Potential systematic error in laboratory experiments on microbial leakage through filled root canals: review of published articles

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Abstract

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Aim To systematically evaluate whether published studies on microbial leakage through filled root canals in human teeth embedded in a two-chamber system were properly controlled. Specifically, the control for the assumption that leakage should occur through the root canal rather than other routes was investigated.

Methodology A systematic search was conducted using Medline, Biosis, Cochrane, Embase, and Web of Science databases. In addition, the reference lists of review articles pertaining to the topic were searched. No language restriction was applied. Two independent reviewers screened titles and abstracts. All articles deemed appropriate by either reviewer were included in the full-text evaluation. In case of disagreement, a referee arbitrated between the reviewers.

Results With 93.8% agreement prior to discussion and arbitration, 67 articles were included. On average, the size of the negative control group was 30% (mean) of the *n* in the experimental groups (minimum = 0.0%, maximum = 100%, SD = 27%). The majority of studies (57 of 67) used inadequate negative controls. The whole root was covered with the sealing material in these specimens, whilst the root tip was left uncovered in the experimental groups. Consequently, leakage between outer root surface and sealing material was not controlled for. The authors of the remaining 10 communications did not state clearly how negative control assessments were performed.

Conclusions Experimental investigations should be performed to assess the routes of microbial leakage in two-chamber models.

Keywords: bacteria, leakage, review, root canal.

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Introduction

There has been much dispute regarding the value of laboratory studies dealing with leakage through filled root canals (Schuurs *et al.* 1993, Wu & Wesselink 1993, Editorial Board of the Journal of Endodontics 2007, De-Deus 2008). Some of the main problems with this type of studies have been discussed. Results are not necessarily comparable between investigations apparently employing the same method (Wu & Wesselink 1993). Most studies lack the power to detect statistical differences (Schuurs *et al.* 1993). The clinical value of laboratory leakage studies has been questioned to the point where some journals do not consider this type of investigation for publication anymore (Editorial Board of the Journal of Endodontics 2007).

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In theory, different types of leakage can occur. The term coronal leakage refers to microorganisms from the oral cavity that penetrates the whole root canal system to eventually trigger a host reaction in the apical periodontium. Apical leakage refers to infiltration of the apical root segment by peptides and other molecules, which have the potential to support microbial metabolism in the filled root canal system. Because microorganisms are the cause of apical periodontitis, it is conceivable that coronal leakage through filled root canals plays a role in post-treatment infection. Hence, leakage tests with viable bacteria as markers could theoretically be used to compare the capacity of different materials to prevent coronal leakage. The common set-up to do these tests in the laboratory is to use a two-chamber system, with a tooth sealed in between the upper and the lower chamber. Turbidity or a colour reaction in the originally sterile broth contained in the lower chamber indicates leakage of viable microorgansims. The number of the leak-proof specimens is determined over time and compared between groups using either a rank test (taking the event time into consideration) or a contingency table analysis (looking at the ratios of leaking versus leak-proof specimens at termination of the experiment). This model was originally described in restorative dentistry to test for bacterial leakage around fillings (Mortensen *et al.* 1965). Later, it was adapted to endodontics (Goldman *et al.* 1980). Since then, a multitude of studies has been published in the endodontic literature comparing microbial leakage through root canals filled with different materials or techniques in a two-chamber set-up. In teeth filled with gutta-percha and a sealer, leakage usually occurred within weeks (Torabinejad *et al.* 1990). This stands against the histological observation *in situ* that few to no bacteria are found in the apical portion of root canals when they were properly filled with gutta-percha and sealer, even if these canals had been exposed to the oral cavity for extended time periods (Ricucci & Bergenholtz 2003, Ricucci *et al.* 2009). There could be two reasons for this discrepancy: It is either possible that the observations from the laboratory studies are valid, because the two-chamber method is more sensitive than histology. In theory, one viable microorganism has to reach the lower chamber to then multiply and indicate leakage. Alternatively, there could be an inherent problem with the two-chamber laboratory set-up that has not received attention. More specifically, the assumption that *leakage should occur through the root canal space rather than through other routes* could be wrong. Using proper (negative) controls, this doubt could technically be expelled. However, a superficial look at the published microbial leakage studies raises serious doubt as to whether the negative controls in these investigations were really appropriate.

It was the goal of the current communication to systematically review the literature regarding microbial leakage studies, in which a two-chamber model had been used. It was aimed to find all the laboratory studies dealing with microbial leakage through filled root canals. Studies on coronal seal and root-end fillings were not included. Identified articles were critically inspected regarding the controls that were used. Specifically, the question whether potential routes of microbial leakage were adequately controlled in the two-chamber set-ups was addressed.

Materials and methods

Search strategy

The Medline database was searched via the OvidSP search interface with a combined search strategy using keyword search and controlled vocabulary (MeSH-terms) as described in Table 1. The same strategy was adapted and applied using Biosis (OvidSP), the Cochrane library (Wiley), Embase (http://www.embase.com) and the Web of Science (Thompson). Articles from inception of these databases (Medline 1948, Biosis 1980, Cochrane 1995, Embase 1974, Web of Science 1899) up to and including March 2010, were considered. No language restriction was applied. Furthermore, the reference lists of the reviews and studies concerning the topic were searched for additional titles.

Study selection

In a first screening step, the two reviewers (D.-K. Rechenberg and G. De-Deus) independently evaluated

Table 1 Example of search strategy (here in Medline database) employed for the current literature review

Number	Search history	Results
1	((leak* or mikroleak*).mp. or penetration.mp. or exp Dental Leakage/)	93603
2	((exp 'Root Canal Filling Materials'/ or exp 'Root Canal Preparation' or exp 'Root Canal Obturation'/ or exp Dental Pulp Cavity/) or (((pulp* or root*) adj5 canal).mp.))	20674
3	((bacteria* or microorganism* or saliva).mp. or exp Bacteria/)	1229702
4	1, 2 and 3	265

titles and abstracts from the electronic search and assessed these with respect to the exclusion criteria applied. Communications were excluded when they were: (i) not on human teeth; (ii) not using microorganisms; (iii) on intracanal medication rather than permanent root fillings; (iv) on leakage through coronal restorations, temporaries or the cementum-post interface; (v) on root end fillings or MTA plugs; (vi) on perforation repair; (vii) completely off topic or used a different methodology; or (viii) review articles, conference abstracts, comment letters or case reports. Any title included by either reviewer went further to the second screening step, the full-text evaluation. Both reviewers assessed 81 full texts applying the exclusion criteria mentioned above. Two articles included for full-text evaluation were not in English (French and Chinese) and were translated with the help of a translator. Disagreement was resolved by discussion and with the help of an experienced referee (M. Zehnder).

Data extraction and presentation

Data extraction was performed with the help of a data extraction form piloted on several papers. The following data were recorded: (i) reference name; (ii) the year of publication; (iii) observation time in days; (iv) whether the purity of culture was controlled (yes/no); (v) the type of material(s) used for the seal between tooth and chambers; (vi) number of specimens per experimental group; (vii) whether there was an appropriate control for the seal between the tooth and chamber (yes/no), and (viii) whether significant differences between the experimental groups were reported (yes/no).

The data are presented in descriptive form, and descriptive statistics were applied when deemed helpful.

Results

Three hundred and eighty-seven communications were identified by the systematic search protocol (Supporting information). One additional title (Goldman *et al.* 1980) was found through the reference list search. Articles were written in English (n = 368), Chinese (n = 2), Croatian (n = 2), Czech (n = 1), Danish (n = 1), French (n = 7), German (n = 1), Greek (n = 1), Japanese (n = 1), Polish (n = 1) and Turkish (n = 2). However, all of these publications but one French communication (Marciano *et al.* 1986) contained titles and abstracts in English. Eighty-one full-text articles were included by at least one reviewer. From these, 14 were excluded after thorough reading (Fig. 1, Supporting information). With 93.8% agreement prior to discussion and arbitration, 67 articles were included in this review (Table 2). All of these publications were in English.

The first article to assess microbial leakage through root fillings was published in 1980 (Goldman *et al.* 1980). In the included studies (Table 2), the main outcome evaluated was turbidity of the broth in the lower chamber in 62 articles, whilst the authors of five publications evaluated cultivability of microorganisms from the broth in the lower chamber. Three of these research groups measured the time until microorganisms could be plated (Gish *et al.* 1994, Barrieshi *et al.* 1997, Jayalatha *et al.* 1998) and the other two the colony-forming units at different times (Clark-Holke *et al.* 2003, Jacobovitz *et al.* 2009). Observation periods ranged from 8 to 364 days. In most studies facultative bacteria that grow well under laboratory conditions were used as indicators of leakage. However, merely 19 studies used selective- or half-selective media to prevent contamination by other taxa. In 24 out of the 67 studies, purity of culture was not controlled at all. The teeth and materials used for the two-chamber models were sterilized in all but three studies (Shipper & Trope 2004, Shipper *et al.* 2004, Yazdi *et al.* 2009), in which nothing was mentioned regarding this step. In most studies (n = 42), gas sterilization was used for that purpose. Other studies used autoclaving (n = 12), gamma radiation (n = 6), immersion in sodium hypochlorite (n = 1) or a combination of different methods (n = 3) to avoid contamination. However, in 17 papers it was not mentioned whether the roots were sterilized again after they were filled or whether the materials applied were sterile.

The most common materials to seal experimental teeth between the two chambers were cyanoacrylate and sticky wax, or a combination thereof (Table 2). Nail varnish was frequently applied to the outer root surface and sometimes implemented in the seal between the two chambers (Table 2). To detect gross leakage between the chambers before the upper chamber was filled with the bacterial broth, this chamber was first filled with a dye solution in five studies (Malone & Donnelly 1997, Carratù *et al.* 2002, De-Deus *et al.* 2007b, 2008a, Jacobovitz *et al.* 2009). In these investigations, leaking specimens were either discarded or the seal was improved before continuing with the main experiment. In the remaining 62 communications, no such primary control tests were reported.

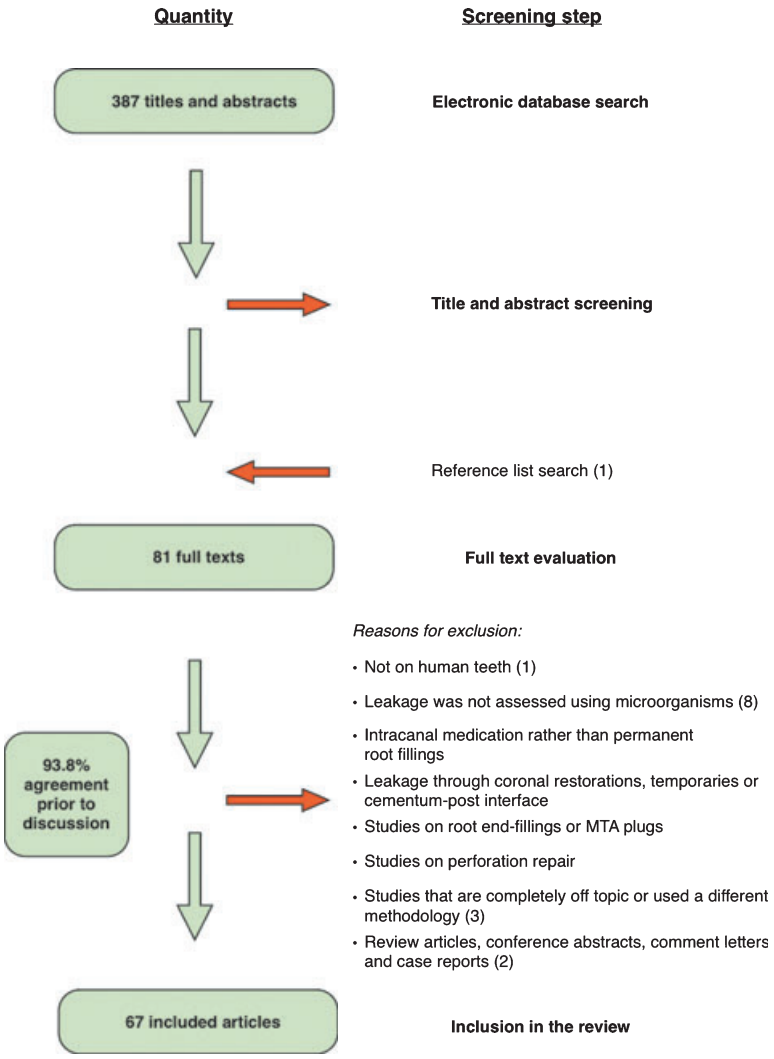

Figure 1 Flow chart depicting the primary inclusion and subsequent exclusion of articles related to the current topic.

The number of negative controls (i.e. specimens to test whether leakage could occur through routes other than the root canal) was usually fairly below the numbers of specimens in experimental groups. On average, the size of the negative control group was 30% (mean) of the *n* in the experimental groups (minimum = 0.0%, maximum = 100%, SD = 27%). Moreover, the majority of studies used inadequate negative controls. The main methodological flaw in this respect was that in almost all studies (57 of 67), the whole root including the root tip that was sealed between the chambers was covered with the sealing material(s), so that the possibility of leakage through the interface between outer root surface and sealing material was not controlled for (Fig. 2). In the remaining 10 publications, the authors did not state clearly how they proceeded with their negative controls. No study group used histological methods to trace the routes of microbial leakage.

In 31 of the 67 studies, no significant differences were found between experimental groups.

Discussion

The current systematic search of the published literature on microbial leakage through root filled teeth in a two-chamber model showed that the route of leakage between the two chambers was inadequately controlled for and thus remains unknown. In other words, negative controls used in these studies did not ensure that leakage really occurred through the root canal space rather than via another route, such as the

Table 2 Studies included in this review and evaluation criteria

Reference	Microorganisms	Observation time (days)	Purity of culture controlled (y/n)	Type of seal between tooth and chambers (material)	*n* in experimental group/s	*n* in negative control (overall)	Appropriate control for seal between tooth and chamber (y/n)	Significant differences between experimental groups (y/n)
Goldman *et al.* (1980)	*P. mirabilis, S. salivarius*	42	y	Cyanoacrylate, wire ligature	10	2	n	n
Torabinejad *et al.* (1990)	*P. vulgaris, S. epidermidis*	over 90	Y	Epoxy resin	16/17	4	n	y
Khayat *et al.* (1993)	Human whole saliva	48	n	Cyanoacrylate	15	5	n	n
Wu *et al.* (1993)	*P. aeruginosa*	50	y	Wire ligature, nail varnish	29	2	n	N/A
Gish *et al.* (1994)	*S. anginosus*	90	y	Cyanoacrylate	20	5	n	N/A
Behrend *et al.* (1996)	*P. vulgaris*	21	y	Cyanoacrylate, wire ligature	20	2	n	y
Chailertvanitkul *et al.* (1996a)	*S. sanguis*	90	y	Sticky wax, cyanoacrylate, nail varnish	20	20	n	n
Chailertvanitkul *et al.* (1996b)	*S. sanguis*	90	y	Sticky wax, cyanoacrylate, nail varnish	20	10	n	n
Chailertvanitkul *et al.* (1996c)	*F. nucleatum*	84	y	Sticky wax, cyanoacrylate, nail varnish	20	10	n	n
Barrieshi *et al.* (1997)	*F. nucleatum, P. micros, C. rectus*	90	y	Cyanoacrylate	30	5	n	N/A
Chailertvanitkul *et al.* (1997a)	Anaerobic streptococci related to *P. micros* and *P. intermedia*	90	y	Sticky wax, cyanoacrylate, nail varnish	20	10	n	y
Chailertvanitkul *et al.* (1997b)	*S. sanguis, P. intermedia*	90	y	Sticky wax, cyanoacrylate, nail varnish	20	10	n	n
Malone & Donnelly (1997)	Human whole saliva	60	n	Cyanoacrylate	10	2	n	n
Jayalatha *et al.* (1998)	*E. faecalis*	8	y	Not stated	12	3	n	n
Barthel *et al.* (1999)	*S. epidermidis*	38	y	Sticky wax, cyanoacrylate	30	3	n	n
McDougall *et al.* (1999)	*E. faecalis*	90	n	Adhesive resin, nail varnish	10	4	n	y
Siqueira *et al.* (1999)	Human whole saliva (diluted)	60	n	Cyanoacrylate	20	5	Not stated	y
Barthel *et al.* (2000)	*S. epidermidis*	364	y	Sticky wax	20	3	n	y
Padachey *et al.* (2000)	*E. faecalis*	90	Y	Adhesive resin	10	10	n	y
Siqueira *et al.* (2000)	Human whole saliva (diluted)	60	n	Cyanoacrylate	20	5	Not stated	n
Bal *et al.* (2001)	*P. vulgaris*	70	y	Cyanoacrylate	20	2	n	n
Gilbert *et al.* (2001)	*P. vulgaris*	21	n	Cyanoacrylate	20	5	n	y
Timpawat *et al.* (2001)	*E. faecalis*	60	y	Sticky wax	19	9	n	y
Carratù *et al.* (2002)	*P. mirabilis, S. epidermidis*	37	y	Cyanoacrylate, silicone paste	12	6	n	n
Jacobson *et al.* (2002)	*K. pneumoniae*	84	y	Epoxy cement	20	5	n	y
Miletić *et al.* (2002)	*S. mutans, S. mitis, P. melaninogenica, L. acidophilus, C. albicans*	90	y	Cyanoacrylate, acrylic resin	20	10	n	n
Britto *et al.* (2003)	Anaerobic streptococci related to *P. micros* and *P. intermedia*	60	y	Sticky wax, cyanoacrylate	12	12	n	y

Table 2 (*Continued*)

Reference	Microorganisms	Observation time (days)	Purity of culture controlled (y/n)	Type of seal between tooth and chambers (material)	*n* in experimental group/s	*n* in negative control (overall)	Appropriate control for seal between tooth and chamber (y/n)	Significant differences between experimental groups (y/n)
Clark-Holke *et al.* (2003)	*F. nucleatum, P. micros, C. rectus*	60	y	Sticky wax, cyanoacrylate	10	10	n	y
Saunders *et al.* (2004)	*S. sanguis*	90	n	Not stated	10	3	n	n
Shipper *et al.* (2004)	*S. mutans, E. faecalis*	30	y	Sticky wax	15	12	n	y
Shipper & Trope (2004)	*S. mutans*	30	y	Sticky wax	15	10	n	y
Al-Hezaimi *et al.* (2005)	Human whole saliva	42	n	Epoxy resin	11	3	n	y
Chogle *et al.* (2005)	*E. faecalis*	70	n	Cyanoacrylate, wire ligature	30	Less than 10	Not stated	n
De-Deus *et al.* (2006a)	Human whole saliva (diluted)	100	n	Cyanoacrylate	20	5	Not stated	n
De-Deus *et al.* (2006b)	Human whole saliva (diluted)	63	n	Cyanoacrylate	9	5	n	y
Karagenç *et al.* (2006)	*S. epidermidis*	More than 80	n	Silicone resin glue	15	3	n	y
Mohammadi *et al.* (2006)	*E. faecalis*	90	n	Not stated	10	3	n	n
Pitout *et al.* (2006)	*E. faecalis*	90	y	PVC solvent weld cement, nail varnish	25	5	Not stated	y
Wang *et al.* (2006)	*S. mutans*	30	y	Sticky wax	15	0	n	n
Yücel & Ciftçi (2006)	*E. faecalis*	60	y	Sticky wax	20	10	n	n
Yücel *et al.* (2006)	*E. faecalis*	60	y	Sticky wax	20	10	n	n
Baumgartner *et al.* (2007)	*E. faecalis*	50	y	Sticky wax	15	3	Not stated	n
De-Deus *et al.* (2007a)	Human whole saliva (diluted)	63	n	Cyanoacrylate	20	10	n	y
De-Deus *et al.* (2007b)	Human whole saliva (diluted)	63	n	Cyanoacrylate	20	10	n	y
Ghoddusi *et al.* (2007)	*S. mutans*	90	y	Cyanoacrylate	40	6	n	y
Monticelli *et al.* (2007)	*S. mutans*	100	y	Sticky wax	12	3	n	y
Muñoz *et al.* (2007)	*E. faecalis*	30	y	Cyanoacrylate, nail varnish	10	3	n	n
Pitout *et al.* (2007)	*E. faecalis*	90	y	PVC solvent weld cement, nail varnish	25	5	n	Not stated
Yang *et al.* (2007)	*P. nigrescens*	90	n	Sticky wax, cyanoacrylate	15	5	n	y
Brosco *et al.* (2008)	*E. faecalis*	120	y	Epoxy adhesive	20	5	n	y
De-Deus *et al.* (2008b)	Human whole saliva (diluted)	100	n	Cyanoacrylate	20	10	Not stated	n
De-Deus *et al.* (2008a)	Human whole saliva (diluted)	105	n	Cyanoacrylate	10	10	Not stated	n
Fransen *et al.* (2008)	*E. faecalis*	65	y	Hot glue, nail varnish	20	8	n	n
Pasqualini *et al.* (2008)	*E. faecalis*	47	y	Cyanoacrylate	22	4	n	y
Saleh *et al.* (2008)	*E. faecalis*	135	y	Sticky wax	15	10	n	y
Alves Mozini *et al.* (2009)	*E. faecalis*	60	y	Epoxy resin	11	4	n	y
Drukteinis *et al.* (2009)	Human whole saliva (diluted)	75	n	Sticky wax, cyanoacrylate	25	5	n	n
Eldeniz & Ørstavik (2009)	*S. mutans*	40	y	Sticky wax	15	12	n	y
Grecca *et al.* (2009)	*E. faecalis*	90	n	Epoxy resin	10	3	Not stated	n

Table 2 (*Continued*)

Reference	Microorganisms	Observation time (days)	Purity of culture controlled (y/n)	Type of seal between tooth and chambers (material)	*n* in experimental group/s	*n* in negative control (overall)	Appropriate control for seal between tooth and chamber (y/n)	Significant differences between experimental groups (y/n)
Hollanda *et al.* (2009)	*S. aureus, E. faecalis, P. aeruginosa, B. subtilis, C. albicans*	60	y	Cyanoacrylate, epoxy resin, nail varnish	10	5	n	y
Jacobovitz *et al.* (2009)	*E. faecalis*	30	y	Polyester resin, nail varnish	15	5	Not stated	n
Lyons *et al.* (2009)	*S. mutans*	28	n	Cyanoacrylate, orthodontic resin powder and liquid	20	20	n	n
Pitout & Oberholzer (2009)	*E. faecalis*	90	y	PVC solvent weld cement, nail varnish	25	5	n	y
Salz *et al.* (2009)	*S. mutans*	30	y	Sticky wax	16	12	n	y
Taşdemir *et al.* (2009)	*E. faecalis*	56	y	Cyanoacrylate	10	10	n	n
Williamson *et al.* (2009)	*F. nucleatum, P. micros, E. faecalis*	40	n	Cyanoacrylate	15	5	n	n
Yazdi *et al.* (2009)	Human whole saliva	120	n	Sticky wax, composite resin, sticky wax	22	5	n	N/A

outside of the tooth. This is an important issue taking into consideration that pre-clinical studies should be able to generate basic yet reliable knowledge on a given topic. In properly performed laboratory studies, confounding factors are controlled and thus the tested variable can be singled out and assessed. However, it is always wrong to make clinical conclusions based on *in vitro* studies. For instance, as has been pointed out (Wu & Wesselink 1993, Barthel *et al.* 1999), it is an inherent problem of microbial leakage studies that leakage cannot easily be quantified. If there is nutrient broth in the lower chamber, then microorganisms reaching there will immediately start to multiply. Furthermore, the number of microorganisms necessary to cause a lesion in the apical periodontium is not known. On the contrary, it could be so that the path of leakage and gap size would affect the time, at which turbidity occurs in the lower chamber. In other words: leakage through a large gap should occur quicker than through a narrow counterpart. Nonmotile microorganisms breach gaps through multiplication. Consequently, the straighter the route of leakage, the quicker it will show in the lower chamber. However, voids could be filled with air (dry void) or fluid, or both (with entrapped air). Furthermore, it cannot be excluded that bacterial growth could be inhibited by the test materials. One material may have different effects on different microbial species. Overall, as has been suggested (Wu 2008), 'the relevance of early or postponed leakage within a short experimental period (for instance 60 days) is unknown'.

If laboratory studies on microbial leakage are performed properly, then ideas for clinical research can be generated. If not, the results are misleading. Despite the apparent popularity of microbial leakage studies using a two-chamber system, little effort has been made to expel the possibility of systematic errors and to validate the method. A correct negative control in a two-chamber leakage study should test the seal between the chambers. If the whole root of a negative control tooth is sealed off, then the only statement that can be made if the broth in the lower chamber remains sterile is that the sealing material *per se* does not allow penetration of microorganisms (Fig. 2). However, what would be more important is to check the interface between outer root surface and the sealing material used to separate the chambers. To this end, intact teeth with enamel covering the dentine or coronally shortened/accessed counterparts, which are tightly sealed coronally, should be used in the negative control group (Fig. 2). Both conceivable types of coronal seal have

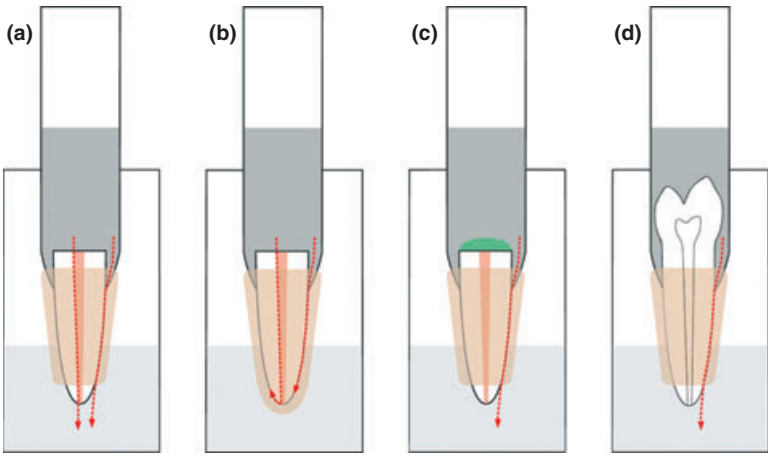

Figure 2 Schematic drawing of an experimental test set-up (a) and the counterpart that has commonly been used as negative control (b). Possible microbial leakage pathways are indicated by red arrows. If the sealing material between the chambers is placed so that the whole root is covered, as has been performed in most studies, leakage between outer root surface and sealing material is not controlled for. The control is thus incorrect. Better set-ups to control for leakage pathways other than the filled root canal are depicted on the right (c, d). Either an instrumented and root filled tooth with a microbia-tight material (green) covering the root filling should be used (c), or a virgin counterpart with an intact crown (d). Importantly, the apex of control teeth should be left uncovered like in the test specimens.

their disadvantages: intact crowns may contain microcracks in the enamel that went unnoticed and allow for leakage. Coronally shortened teeth with a seal above the root filling bear the disadvantage that materials predictably preventing microbial leakage when placed on dentine are elusive, and would need to be identified first in the context of this set-up. Importantly, negative control specimens should be placed in the sealing cuff between the chambers so that their apex is left unsealed, as is performed in the test groups with the root filled teeth (Fig. 2). This would afford confidence that, in case these negative controls do not leak, leakage in test specimens occurs through the root or the root canal. Furthermore, proper negative controls are important to exclude contamination of the set-up. The seal between the two chambers was not controlled properly in 57 of the 67 published investigations. As for the remaining 10 studies, no statement regarding control of the seal was given, so it remains unknown whether proper control measures were taken or not. The rationale for sealing the whole apical root tip in the negative control group was probably that, instead of using intact teeth as negative controls, most investigators (54 of 67 studies) used teeth with instrumented root canals. In only 11 studies intact teeth were used as negative controls; and in four of these the root tip was covered nevertheless. In the remaining seven studies with intact teeth as negative controls, the authors did not state clearly how they proceeded. One study used glass cylinders (Wu *et al.* 1993) as negative controls, one used no negative controls at all (Wang *et al.* 2006).

Based on the current findings, the assumption that leakage should occur through the filled root canal rather than the outer root surface is up for debate. As has been shown in studies on coronal bacterial leakage, sticky wax (a combination of bees wax, paraffin and resins) is not a good sealant (Barthel *et al.* 2001). Epoxy resins and silicone-based materials are commonly used as endodontic sealers (AH-plus and RoekoSeal are prominent examples of these groups of materials, respectively, which were used in many studies). However, it appears logical that using a similar material to seal a tooth between the two chambers as is used inside the root canal to connect the core filling material to the root canal wall makes no sense, if the goal is to test leakage through the root canal exclusively. The interface between the outer root surface and the sealant is much greater than that inside the canal (if potentially not equally complex, because of the dentinal tubules in the root canal wall).

In none of the included studies, the routes of microbial leakage were traced histologically. The authors of one most recent publication that was published after the initiation of the current review did

just that (Brosco *et al.* 2010). However, they did not lay any emphasis on the seal or the potential of leakage outside the root either. These investigators also covered the whole outer root surface with the sealant (epoxy resin) in the negative control group.

A potential confounding factor that has not been considered in this review is the possibility that root filling materials could impede microbial leakage not because of their sealing ability, but rather because of the antimicrobial properties that some of these materials exert (Slutzky-Goldberg *et al.* 2008). However, currently used materials have such low antimicrobial properties once they have set that obscuring of results by this factor is unlikely. Antimicrobial agents are diluted in the two-chamber model. Growth in the broth of the lower chamber will occur even if the amount that can be filled into a root canal of a strong antiseptic such as calcium hydroxide is directly placed into that chamber (Zehnder *et al.* 2007).

The conclusion of the current systematic review is that experimental investigations should be performed to specifically address the routes of microbial leakage in two-chamber models. The method under evaluation appears not to be suitable to compare different permanent root filling materials at this point. Instead, future studies should aim at the question how (if ever) roots filled with conventional filling materials can be penetrated by microorganisms. This requires proper controls and histology.

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Supporting Information

Additional supporting information may be found in the online version of this article.

Table S1. Hits from the literature search obtained with the different databases.

Table S2. Excluded studies.

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