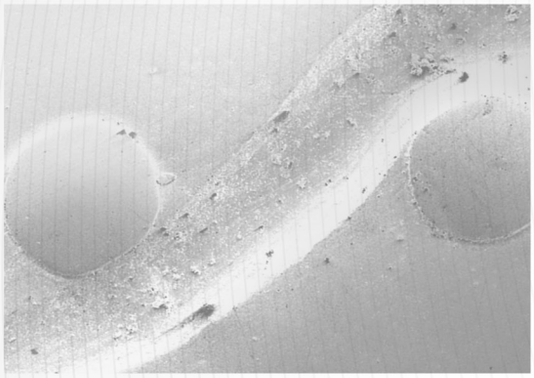
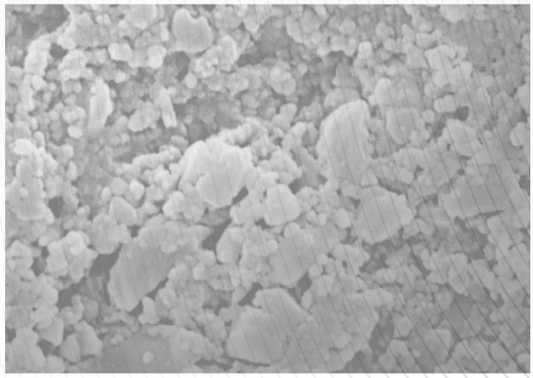
Reviews

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A review of microbiological root canal sampling: updating an emerging picture

Uma revisão de amostragem microbiológica de canal radicular: atualizando um quadro emergente

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Abstract

Objectives: The significance of microorganisms in root canals with regard to the aetiology of periapical infection and the need for crucial bacteria control during treatment are undeniable. In this study, we report and discuss a review of the literature on Microbiological Root Canal Sampling (MRS). The procedure is analyzed in detail, discussing its powers, limitations and the influence of sample collection procedures on the incidence of true and false positive results. **Data sources**: MEDLINE/PUBMED, B-On and library files of Oporto University were accessed. **Selection**: Papers were selected using the keywords: "root canal sampling"; "apical periodontitis"; "endodontic pathogens"; "root canal infection"; "Culture"; "molecular biology". The references were selected under inclusion criteria such as English language, accessibility, relevance to the theme and scientific rigor. **Conclusions**: This review illustrated the absolute need to adhere to strict methodology procedures if valid samples are to be obtained. A combination of Culture and molecular identification approaches have confirmed the polymicrobial nature of endodontic infections with a predominance of anaerobic bacteria. Nucleic acid-based techniques provide significant additional information particularly regarding the not-yet-cultivable species of the microbial community, but greatly increase the budget of the procedure. Thus, assessment of the endodontic microflora, in the context of a polymicrobial biofilm ecosystem, and its relevance to endodontic treatments must rely in the complementariness of Culture and Metagenomics approaches as they are neither mutually exclusive nor competitive, but strongly complementary.

Keywords: Apical periodontitis. Endodontic pathogens. Root canal infection. Root canal sampling.

Resumo

Objectivos*: A importância de microrganismos em canais radiculares no que diz respeito à etiologia da infeção periapical e a necessidade de controlar bactérias durante o tratamento são incontestáveis. Neste estudo, relata-se e discute-se uma revisão da literatura sobre a amostragem microbiológica de canal radicular. O procedimento é analisado em detalhes, discutindo suas atribuições, limitações e influência de procedimentos de coleta de amostra sobre a incidência de verdadeiros e falsos resultados positivos.* **Fontes de dados***: Foram usados MEDLINE/PubMed, B-On e arquivos da biblioteca da Universidade do Porto.* **Seleção***: Os trabalhos foram selecionados utilizando as palavras-chave: "root canal sampling"; "apical periodontitis"; "endodontic pathogens"; "root canal infection"; "Culture"; "molecular biology". As referências foram selecionadas de acordo com critérios de inclusão como o idioma inglês, acessibilidade, relevância para o tema e rigor científico.* **Conclusões***: Esta revisão ilustrou a absoluta necessidade de aderir aos procedimentos metodológicos rigorosos se se pretende obter amostras válidas para análise. Uma combinação de cultura e abordagens de identificação molecular confirmaram a natureza polimicrobiana das infeções endodônticas com predominância de bactérias anaeróbias. Técnicas baseadas em ácidos nucleicos fornecem informação adicional significativa, particularmente em relação às espécies não cultiváveis da comunidade microbiana, mas aumentam muito o orçamento do procedimento. Assim, a avaliação da microflora endodôntica, no contexto de um ecossistema polimicrobiano em biofilme, e sua relevância para tratamentos endodônticos devem confiar na complementaridade entre a abordagem de cultura e de metagenômica, pois não são mutuamente exclusivas nem competitivas, mas fortemente complementares.*

Palavras-chave*: Periodontite apical. Patógenos endodônticos. Infecção do canal radicular. Amostragem do canal radicular.*

Introduction

We have come a long way since the seventeenth century studies by Antony van Leeuwenhoek into establishing the role of bacteria, predominantly anaerobic, and their by-products in the pathogenesis of apical periodontitis (1). Several investigators such as Kakehashi et al. (2), Sundqvist et al. (3) and Möller et al. (4, 5) have demonstrated a strong association between periapical disease and intracanal microbiota.

Unlike other parts of the oral cavity, there is no supposedly indigenous endodontic microflora (6). All bacteria inside the infected root canal are opportunistic pathogens that can be either the commensal oral bacteria associated with a healthy oral cavity or the pathogenic bacteria associated with a diseased oral cavity, such as dental caries and periodontal disease.

The same combination of bacterial species, loads and virulence may give rise to different responses in different individuals. Santos et al. (7) seemed also reasonable to conclude that the severity of the disease may be related to the bacterial community composition topped off with host resistance, as the latter is another important factor with impact on disease pathogenesis.

Socransky and Haffajee (8) affirmed that the host may influence the microbiota, but in turn the microbiota influences the host at a local and perhaps at a systemic level. Additionally, it has been shown that in the same subject, marked differences can occur in the microbial composition both from one type of intracanal location to another (e.g. coronal vs. apical) and from similar types of locations (e.g. two distinct periapical lesions). An example of this is the observation by Özok et al. (9) that the apical part of the root canal system drives the selection of a more diverse and more anaerobic community than the coronal part. Also, several recent studies have demonstrated a less diverse microflora in endodontic infection than in saliva and supragengival plaque, data confirmed by Li et al. (6), who observed that endodontic microflora is a restricted community, supposedly a subset derived from the total oral microbiota.

Dalhén (10) affirmed that the primary endodontic infection is, therefore, a polymicrobial, predominantly anaerobic infection with little microbial specificity. This heterogeneous aetiology results in

the absence of a dominant species, which has strong diagnostic and treatment implications.

The infection of the root canal is far from being a random event. The microbial flora develops in response to the surrounding selective environment (11) with selection pressure attributable to dynamic cooperative and antagonistic relationships between different bacteria as well as the host and bacteria (12).

Nair et al. (13) observed microbial organizations in the root canal system, which very often give rise to a complex network embedded in a matrix, the so-called biofilm communities adherent to the root canal walls, isthmuses and ramifications, according to Ricucci and Siqueira (14). Costerton et al. (15) told that bacteria organized this way are more resistant to host defence mechanisms and disinfectants than planktonic bacteria and therefore are reported to be the most common cause of persistent inflammation, underpinning again the concept argued by Siqueira and Rôças (16) that the eradication of bacteria from the root canal system is critical to the endodontic treatment of teeth with apical periodontitis.

Despite conflicting technologies, the principles for root canal treatment laid at the beginning of the last century, according to Hall (1928) cited by Ng et al. (17), and remain consistent with contemporary quality guidelines approved by Endodontic Societies in Europe and North America (17). The final objective of root canal treatment is prevention (in the case of pulp inflammation) or resolution (in case of pulp infection) of periapical disease, by eradication of bacteria and their sources of nutrient supply from the root canal system (18, 19). That is to say elimination of the source of infection in an effort to obtain conditions to promote the cure and health of the root and the tissues around it. Accordingly, endodontic infections are treated by intracanal procedures such as chemomechanical preparation (instrumentation with files with copious antibacterial irrigants) in an excellent isolation field supplemented or not by an interappointment intracanal medication, followed by filling and ended with the definitive restoration.

Although sterilization seems practicable, the fact that we are dealing with a complex anatomy renders the available clinical resources frequently unable to free all canal space from all microorganisms, as was first described by Byström and Sundqvist (20). A more realistic goal of reducing the bacterial populations to a level below the clinically relevant threshold (the level necessary to induce or sustain disease) has been accepted by many clinicians and researchers in an almost dogmatic manner (14). The persisting bacteria can survive in treated canals and are able to induce or sustain periradicular tissue inflammation and longstanding endodontic infections (16, 21): microorganisms, like Enterococcus faecalis and Candida albicans, are true "persisters" since they seem to have a natural ability to survive more harsh environments and stressed conditions (22). Later, Siqueira and Rôcas (23) affirmed that some members may occupy critical niches within a complex microbial community and, therefore, are potentially important in maintaining the stability and virulence of the microbial community.

For many years, the major technique available to researchers to identify bacteria was the culturing of microorganisms and identification of sampled species by their phenotypic traits. Undoubtedly, knowledge of endodontic infections, based on data involving Microbiologic Root canal Sampling (MRS), has increased significantly during the last 30 years (16), but several questions still await elucidation.

Sampling is important to determine the composition of the endodontic microflora, because in accordance with Gomes et al. (24) this may be related to the various clinical presentations and symptoms or stages of development of an endodontic infection as well as its responses to different treatments.

In spite of the adoption of molecular approaches with the promising emergence of new data about endodontic microbiology, we are still currently faced with an old controversy: to perceive the usefulness of the MRS over the endodontic performance.

Mollander's concept: reasons to perform MRS

MRS was an early recommendation for clinical routine use, but did not become widespread among general dental practitioners as noticed by Molander et al. (25). By contrast, it has been extensively used in scientific assessments of antimicrobial intracanal treatment strategies and for the characterization of endodontic microbiology (18).

Mollander et al. (25) reminded that Onderdonk first suggested the concept that MRS should form an essential part of endodontic treatment strategy in

1901 and that Coolidge in 1919 proposed it to be part of the clinical routine. However, it was not until 30 years later that the technology received widespread recognition, mainly through the public applications of Appleton (1932) and Grossman (1938).

In Sweden, for instance, after the influential work of Engström (1964) and Möller (1966), the technology became compulsory for undergraduate endodontic education in all four Swedish Dental Schools, accordingly to Molander et al. (25).

The American Board of Endodontics attempted to assess the attitude of their members toward microbiologic assay of root canals prior to obturation. Questionnaire answers indicated that the Culture was currently being used along with the endodontist's clinical assessment of the tooth and not necessarily alone as the final mandatory for obturation of the root canal(s). Culturing was also recommended as a check on one's aseptic technique and, in cases that do not respond to routine endodontic treatment, for identification of the microorganism and antibiotic-sensitivity testing (26).

However, despite these efforts to disseminate the "new idea", the methodology is complex and its diagnostic accuracy has been questioned early on by Reit et al. (18), Buchbinder (27) and Sims (28).

Nowadays, there is another reason to persist with MRS in the clinical protocol. In fact, we must keep alert to the increased resistance of oral microflora to antibiotics probably associated with an antibiotic overprescription in daily dental practise, often without sufficient rationale for choosing a particular drug. Thus, any effort to study the microbial composition and the susceptibilities of endodontic pathogens will most likely facilitate the choice of appropriate clinical protocols or, in occasional cases, of an antibiotic as adjunctive to the clinical treatment of the infection.

How to correctly perform sampling

Inclusion and exclusion criteria

Sampling includes only one tooth with complete root formation per person and only one root canal per tooth. If the tooth is multi-rooted, either the largest one or the one with periradicular radiolucency is sampled to confine the microbial assessment to a single ecological environment.

According to authors such as Molander et al. (29), Shatorn et al. (30) and Rôças and Siqueira (31), generally, patients must be excluded in the analysis of the endodontic microflora in cases of:

- Systemic debilitating diseases such as diabetes mellitus, liver disease, chronic infections, rheumatoid arthritis or any other systemic disease that compromises the immune system;
- Use of systemic steroids or chemotherapeutic agents;
- Requirement of prophylactic antibiotic before dental treatment;
- Active chronic or aggressive marginal periodontitis;
- Pregnancy at the time of initial treatment.

Besides the single-rooted, before the sample collection and according to the analysis of the scope, the following features must be checked for each patient: response to sensitivity tests, tenderness to percussion, presence of a sinus tract or swelling, depth of periodontal pocket, history of previous antibiotic medication and root canal treatment.

For those investigations on primary endodontic infections, the criteria comprises inclusion of asymptomatic necrotic pulps (neither spontaneous pain nor response to pulpal tests or sinus tract, although tenderness to percussion may be present) with or without radiographic evidence of periapical lesions. Patients who received antibiotic therapy in the last 3 months or presented periodontitis (presence of periodontal pockets deeper than 4 mm) must be excluded, due to putative influences on the results. Also excluded are badly broken teeth (with extensive caries at the time of endodontic treatment) that could not be suitably isolated from the gingiva and saliva by the application of rubber dam: a straight probe is mandatorily suggested by Rôças and Siqueira (31) and by Gomes et al. (32) to investigate for pulp space exposure and, if necessary, restorations ought to be replaced before root canal treatment is initiated.

In investigations about treatment failure, root-fillings should end within 5 mm of the radiographic apex (29). Furthermore, Endodontic European Society (33) recommend that the recall period must be at least 1 year for the symptomatic cases and 4 years for asymptomatic ones, because no case with residual radiolucency can be assessed as failure before a 4-year observation period, unless the lesion increases in size or signs and symptoms of infection

arise. This selection must include teeth with apical periodiontitis diagnoses based on strict clinical and radiographic criteria: in agreement with Sjögren et al. (34), a diagnosed apical radiolucency is measured horizontally and vertically with a ruler to the nearest millimetre and its size is determined as the mean value of the two measurements.

When to sample?

Root canal treatment alters root canal microflora profiles in both quantity (bacterial amount) and quality (bacterial composition). According to Ito et al. (35), these differences could be due to the drastic environmental changes in root canals brought about by both mechanical cleaning with files and topical application of drugs.

As a result, to assess those differences, studies of the bacteria occurring in the root canal system involve several basic conditions:

- Pre-instrumentation samples (collected immediately after the execution of the access cavity);
- Post-instrumentation samples (collected immediately after the completion of chemomechanical procedures);
- Post-medication samples (collected immediately after the removal of interappointment dressings);
- Post-obturation samples (collected from root canal-treated with associated periapical periodontitis lesion at a given time, months to years after the initial treatment).

Basically, MRS is a passive way to obtain a sample of the pulpal space at varying moments before, during or after pulpar treatment. Aseptic techniques must be used throughout the endodontic sample acquisition with special care being taken in the control of leakage between the rubber dam and the tooth.

The sampling and bacterial assessment methodology is invariant, regardless of the sampling time (before, during or after root canal treatment).

Independently of the sampling technique, samples should be processed in the laboratory no later than four hours after the collection, as suggested by Alsunaien et al. (36).

How to Sample?

In order to obtain MRS one can use charcoaled paper points to absorb the fluid of the root canals or small files and swabs or aspiration in acute cases when soft tissues are affected.

Collection of pre- and post-instrumentation and post-medication samples *in vivo*

Studies investigating bacteria remaining in the root canals after chemomechanical procedures or intracanal medication potentially allow identification of species with the potential to influence treatment outcome (16).

Once the access cavity is created using sterile burs, the canals to be sampled cannot be dry. One can achieve humidification by irrigation with 5 ml of sterile saline solution. This is crucial if an adequate collection of microorganisms with paper cones is to be achieved. If the canal is wet, all fluid inside the pulpal space should be absorbed, using as many paper points as necessary.

The most widely used technique in root canal sampling involves the insertion of 3 or 4 sterile paper points ISO 25 or 30. The paper points should be inserted 1 mm shorter than the estimated radiographic length. Each cone must stay inside the root canal for 60 seconds with pumping movements to generate a suspension with the bacteria of the main pulpal area. Collection of the soaked paper points should be performed without any contact with potential external contaminators. The paper points should immediately be placed in a sterile microtube with 2 ml of Reduced Transport Fluid (RTF) as it offers protection against oxidation, fact demonstrated by Byström and Sundqvist (20) and by Spiegel et al. (37). This procedure is repeated for each cone.

Other studies focusing on root canal infections developed by Siqueira et al. (38), Pazelli et al. (39), de Souza et al. (40), da Silva et al. (41), Ruviere et al. (42), Sassone et al. (43) and Cogulu et al. (44) often employ “K” or “H” type files followed by two to four paper points to collect the samples.

The described method may not target the microbiota in the apical third of the root canal. However, that can be accomplished if a K file is inserted using

a reaming motion to the working length and only the apical 2 mm are sectioned, as suggested by Tavares et al. (45).

Collection of exudate samples

If it is a study of exudates, the lesion can be aspirated via a sterile 16-gauge needle to syringe after disinfection of the oral mucosa with 2% chlorohexidine gel and before surgical drainage. If the aspiration is unsuccessful (no pus being collected), sampling can be conducted by swabbing the lesion after incision and discharge of the pus. The microbial pus specimens sampled are then immediately placed into a test tube containing RTF. Use of the aspiration technique may help prevent sample contamination with residual oral flora.

Collection of post-obturation samples

Sunde et al. (46) reported that the high percentage of endodontic treatment failure in teeth with periapical lesions have been related to circumstances of microbial origin. In fact, refractory cases and postoperative pain (interappointment flare-ups) are often related to an ongoing overgrowth of anaerobic bacteria in the periapical area (46). Thus, it may be helpful to identify treatment resistant bacteria, and this can only be achieved by laboratory studies of post-obturation samples.

It is obviously critical that aseptic techniques are strictly followed throughout endodontic sampling. If there is a post, it is desirable to remove it by ultrasonic vibration, a method less invasive than the use of burs.

After removal of coronal restoration and localization of the root canal orifice, the filling material is removed, manually (with type K and/or H files) or with an appropriate mechanical system under irrigation with sterile physiological saline solution, as avoidance of chemical solvents minimizes disruption of the bacterial milieu (30). The retrieved material can be transferred to microtubes containing TE buffer (10 mmol/L tris-HCl, 1 mmol/L EDTA, pH 7.6), according to Rôças et al. (47), if Nucleic Acid (NA) studies are the only aim, or RTF, if samples are to be anaerobically cultured irrespective of the performance of NA studies. Radiographs are an easy way to verify that all filling material had been removed. Pulp space is humidified prior to the collection of the sample with paper points inserted to a level approximately 1 mm short of the root apex, based on diagnostic radiographs. This material must be aseptically transferred to the tubes containing the mentioned solutions. No irrigant is used until the initial sampling is complete.

Preceding sampling, some investigators like to establish canal patency with a file ISO 15 in order to produce minimal instrumentation, running the risk of removing some target material. If this is the case, sample should also include the file, but it should have its head removed to minimize potential external contamination.

Investigation on apical periodontitis of treated root canals

Apical lesions assessed after apicetomy

The protocol explained by Subramanian and Mickel (48) initiates with disinfection of the surgical site should be obtained by an one minute long oral rinse with 0.12% chlorhexidine digluconate followed by swabbing the surgical area with the same solution. A full-thickness mucoperiosteal flap is then reflected, using both a submarginal or intrasulcular incision after local anaesthesia, and the root end is accessed with a surgical bur cooled with sterile water. The periradicular tissue is removed by curettage and stored in tubes containing 1 ml sterile water. Two to three millimetres of root ends are resected after curettage and similarly stored. All samples collected are stored at -20˚C until processed in the laboratory.

A portion of the periradicular tissue sample is sent for histopathological examination.

It is desirable that in randomly selected cases, immediately after flap reflection, periosteal tissue samples are collected from areas adjacent to the surgical sites using curettes and paper points to test for bacterial contamination of the surgical site (48)

Apical lesions assessed in extracted teeth

Immediately after extraction, each tooth is profusely rinsed with sterile saline solution, and an ISO 15 sterile scalpel is used to remove all attached soft tissue, including the apical periodontitis lesion from

the root. The cleansing of external root surfaces is made with 3% hydrogen peroxide and disinfected with 2.5% sodium hypochlorite; the latter is inactivated by sterile 5% sodium thiosulphate. The solutions are scrubbed onto the root surfaces using sterile cotton applicators. After disinfection, a sterility control sample is obtained from the external root surfaces using an ISO 80 sterile paper point dampened with TE buffer or RFT. After decoronation with a diamond disc under saline cooling, the root can be cut into two halves horizontally (coronal and apical) with the use of another diamond disc. Apical segments are transferred to tubes containing 1 ml TE buffer and immediately frozen at -20°C (9, 47).

Problems during collection

Sample exchange

As with any clinical sample, collection tubes must be correctly labelled with the pre-established designation of each sample, desirably at the very beginning of the appointment, and always before any collection procedure.

Maintenance of paper point shape throughout sampling

As previously explained, once wet, the microbiological content of the root canal is collected with paper points. These present the problem of not maintaining the original shape as they become impregnated with solution. A further difficulty arises when dealing with narrow root canals, as paper points become very difficult to handle properly, making sampling of the apical third a difficult accomplishment. The last paper point is the most important because it will absorb the liquid from the most peripheral areas of the apical region (10). This is especially true if the preceding paper cones become easily soaked and, therefore, can hardly be collected as valid samples of those root canal regions.

Wide canals do not usually present major challenges. In these canals, even a n. 30 paper point will easily fit into the termini of the pulp space. Thus, samplings in post-instrumented root canals do not usually present any kind of problems to insert and collect paper points. This is clearly not the situation for thin root canals before debridement. To overcome this problem, one can use sterile small files (ISO 008, 010, 015), but these will create a different dilemma: if the files are conducted by hand, the head must be cut off before insertion into the microtube in order to prevent introduction of external contaminants. This can be accomplished with a sterilized orthodontic plier. Alternatively, files can already have no cable and be individually sterilized. Handling of these files should be performed with sterile tweezers, although it complicates travelling through the root canal anatomy.

The Question of False Negatives/Positives

The major issue of any test is its validity: does it measure what it claims? (18).

The strict conditions under which MRS must be performed emphasize how potentially error-prone it can be (30). Indeed, false positive and false negative results may adversely affect the performance of MRS. However, despite these risks, adopters comfortable with the clinical protocols appeared to produce valid samples most often (94); irrespective of the detection method used, the sampling method resulted in an appropriate collection of the microorganisms present in that particular root canal.

False Negatives

Inaccessible Areas

False negative results can occur if there are bacteria located in inaccessible areas for MRS as alerted by Siqueira (1), Heling and Shapira (49) and Wu et al. (50). In practice, these bacteria can repopulate root canals after the first MRS showed a negative result.

In histological observations of Ricucci and Siqueira (51), bacteria have been found in inaccessible inter-canal isthmuses, dentinal tubules, irregularities and accessory canals or even in some untouched areas of the main canal often in the form of biofilms. Low-level ultrasonic agitation has been used by Nguyen et al. (52) in microbiological research to segregate clumped bacteria organized in biofilms without injuring cells and a similar approach could probably be applied in root canal sampling.

Its ability to dislodge bacteria from inaccessible locations especially deep within dentinal tubules is however unknown (30).

In vitro studies, as the one of Rôças et al. (47), have shown the usefulness of cryogenically ground samples. In this technique, all tooth is destroyed allowing recovery not only of the pulp space microorganisms, but also of the anatomically hidden ones. Samples are cryogenically pulverized with the use of a freezer mill. The powdered root segments are frozen at -80 ˚C in 5 ml UV-treated RNA stabilization reagent (RNAlater Qiagen, Hilden, Germany). According to Alves et al. (53), this procedure can also be useful if the intention is to compare the microbiota between the coronal and apical part of the root canal system.

The legacy of drugs

At the end of the chemomechanical preparation, and after the use of dressings, it is mandatory to do MRS only after neutralization of the chemicals used. This is because both classes of chemicals cause bacterial latency (10) and thus prevent bacterial growth (30). This fact justifies the usage of inhibitors such as 5% sodium thiosulfate for halogen-containing antiseptics (iodine or chlorine) or the combination of 3% Tween 80 and 0.3% L-alpha-lecithin (L-*a*-phosphatidylcholine) when chlorohexidine was the chosen therapeutic. Furthermore, if nucleic acid testing is to be used in lab procedures, alcohol could be avoided and be substituted by 5% sodium thiosulfate, as the former increases the likelihood of free nucleic acid precipitation at the time of specimen collection.

Sample transport and storage

Special concern must be taken regarding transport medium, as it not only needs to keep the viability of all microorganisms, but also be bacteriostatic in the sense that no cell division should take place (10). Moreover, desirably it ought to inactivate therapeutic substances used in clinical endodontic procedures, which otherwise prevent bacterial cells from growing in the laboratory media as well as contain reducing substances (as cysteine) to keep the medium from being oxygenized (10).

Procedures in the laboratory

The overwhelming majority of isolates from infected root canals were found to be anaerobic bacteria, having Sato et al. (54) suggested that the environment in root canals is mostly anaerobic and therefore supports their growth.

Culture is time-consuming and has important limitations, including low sensitivity and misidentification because of the inability to grow many oral species under laboratory artificial conditions (55). This is especially true as we may be dealing with anaerobe fastidious bacteria that have stringent environment and nutritional requirements, as noticed by Sathorn et al. (30) and Wade (56). Since these methods depend heavily on the viability of the Culture and on phenotype-based species identification, results may be far from in vivo reality.

In the Laboratory, the lack of appropriate culture media for the bacteria in the sample may also result in a false negative result. This is especially important, since as noted above, the endodontic infection is usually polymicrobial in nature, forcing multiple selective growth broads to be used.

Finally, the false-negative samples are especially difficult to avoid when taking samples at revision of a previously root-filled tooth. They may be in a stressed situation, after mechanical removal of gutta-percha and sealer, which may not allow in vitro growth (1).

False Positives

False positive results are usually the result of sample contamination. Thus, to accurately perform MRS, special care should be taken to remove any source of contamination material from the handling area.

Indeed, it seems that false positive results, as long as endodontic sampling relies on evidence--based principles, are well controlled. Generally, they are limited to contaminants of the operative field. Facultative anaerobic species like polysaccharide-producing streptococci (S. mutans, S. sanguis, S. oralis, and S. salivarius), Corynebacterium spp., Neisseria spp. and Haemophilus spp. are oral bacteria that are empirically known not to establish in the anaerobic and non-saccharolytic environment of the root canal and, thereby, strongly indicate

leakage (10). Equally, micrococci, coagulase-negative staphylococci, spore-forming bacteria (Bacillus spp.) and enteric rods are most likely contaminants from the surroundings by careless handling of the samples in the office or laboratory (10).

Proper control of external contaminants

Use of Sterilized Material

Prior to the procedure, all plaque debris and caries should be removed and existing restorations should be checked. In addition, the procedure field should be prepared, first by mouth rinsing with chlorohexidine solution, and then by the use of a sterilized rubber dam tightly adjusted to the cervical part of the crown. Furthermore, only sterilized clinical material should be used. Despite all these precautionary measures, the endodontic field might not be entirely sterile or immune from saliva leakage and air contamination. As a result, false positives can still occur, although the mentioned procedures certainly keep these to a minimum.

The performance of the pre-endodontic restoration (if necessary) is well proved: defined as a restoration made mainly with glass ionomer or composite, before the beginning of endodontic treatment. Jensen et al. (57) pointed out that it is decisive in providing better conditions to the application of the rubber dam, thus increasing its efficiency, enhances the irrigants action inside the root canal space as it reduces "extrusion" to adjacent areas and promotes the stability of the temporary restoration between visits.

Decontamination protocol of the operative field

Also critical in the exclusion of contaminants is a decontamination protocol for the entire clinical field (clamp, rubber dam and tooth surface) after it is fully applied. This decontamination commonly uses sterile tweezers and cotton rolls or pellets impregnated first with 3% hydrogen peroxide (one should wait until bubbling is finished), followed by 3% sodium hypochlorite or 10% iodine tincture. Only after this first disinfection, the root canal can be accessed by the opening of an access cavity, removal either of temporary fillings, caries, existing restorations or, if it is the target, root fillings. Subsequently, a novel disinfection (performed as described) is made to guarantee the absence of contaminants at the operative field. The next step is to perform a drug inactivation disinfection with 5% sodium thiosulphate fluid using the same procedure. This inactivation is crucial to avoid false negative samples, due to viable but non-cultivable bacteria (that is to say bacteriostatically affected by iodine or sodium hypochlorite) (10).

After the inactivation step, a swab impregnated with sterile physiologic serum is scrubbed into the operative field and external tooth surface, specially the cavo-superficial angle, and immediately transferred to a transport medium in order to check the sterility: if Culture positive results are observed, all samples collected must be regarded as contaminated and excluded from the study.

Proper management of each paper point

Extreme care must be taken when handling the paper cones: any contact with any external surface of the root canal space (even the access cavity, especially at the cavo-superficial angle) dramatically increases the chance for false positive results.

Normally, the paper points are inside a paper package that has an appropriate site to open it: this must be performed using both hands and taking extremely caution to avoid touching the sterilized coins. This means that when pulling the back part of box it is recommendable not to until it is completely apart, so that the paper points stay protected from contamination during sampling collection.

It is also crucial for obtaining a valid sample to touch only one cone at a time, leaving the untouched ones inside the paper package.

Intracanal Dressing

Another issue that can also give rise to false positive results is the removal of intracanal dressing before sampling: the remnants left in the canals can be equally collected and become part of the sample, altering the true results of the clinical protocol. For example, the residual calcium hydroxide may affect the viability of bacteria on the paper point.

On the other hand, bacteria can re-enter the pulp space between appointments through coronal leakage of the temporary restoration and/or

marginal deficiency, cracks and exposed dentinal tubules as demonstrated by Fors et al. (58). In those cases, despite the efficiency of endodontic procedures, cultures will be positive since the hermetic seal was not achieved.

Sample processing

Culture

Traditionally, infections of the oral cavity have been studied by classical microbiological methods, as no real alternatives were available (10, 31, 59).

Briefly, the culture of microorganisms starts with sample dilutions in Phosphate Buffered Saline (PBS) of the transport medium. Carlsson and Sundqvist (60) suggested inoculating them into appropriate enriched medium under conditions that prevent oxygen diffusion so that toxic intermediates of oxygen do not accumulate and interfere with viability of anaerobic bacteria. Plates are then aerobically, micro-aerobically and anaerobically incubated for a period (long enough to allow even slowly growing species to form colonies; not less than 2 weeks in the case of strict anaerobic bacteria). Gomes et al. (24) and Sunde et al. (46) affirmed that the use of all these conditions is important as former results indicate that 60-70% of the bacterial isolates are found to be either strict anaerobes or microaerophiles and Zielke et al. (61) observed that an aerobic culturing technique alone is not sufficient to reflect the microbiologic status of the root canal system.

After detection of bacterial growth, the procedure includes the isolation of the representative Colony Forming Unit (CFU) in order to obtain pure cultures. After this, it is possible to apply morphological and biochemical tests to identify the bacterial species.

Thus, the growth determination or identification of isolated microorganisms is based on colony morphology, cell morphology and both physical and biochemical tests (10) and so, a Culture test may be used not only to confirm the decrease of bacterial load at each dental visit, but also to identify the bacteria present in the root canal system.

It is thought by several authors, such as Wade (56) and Olsen et al. (62), that about 50% of oral bacteria have not yet been cultivated. Authors such as Siqueira (63) go even far beyond this statement and propose an emerging concept stating that bacterial species involved in endodontic infections are generally uncultivable. Thus, bacteria numbers may be severely underestimated (8) by Culture results. Despite this, dentists can obtain useful information with this culture-dependent method. If the Petri's Plaques are plenty of several kinds of CFUs, it means that the bacterial load is large and reveals the polymicrobial nature of the infection. If the CFUs on the plate are few or spotted, bacteria may be decreasing. If only some scattered colonies are detected, it means that a bacteria-free condition in the root canal system is likely to be achieved soon (12).

Despite a rather time-consuming, labour-intensive and expensive undertaking, the major limiting factor has been the lack—until recently—of microbial techniques that are specific and rapid enough to allow the assessment of the large numbers of samples needed for meaningful in vivo studies (8).

Nucleic Acid based approaches

As the Molecular Biology approach (now globally referred as Metagenomics), firstly applied in Endodontics in 1997 by Conrads et al. (64) and since then widely used in this area, relies on principles developed to overcome the boundaries of the classic Culture, it has been used in order to provide additional valuable information regarding the identification and understanding of the causative factors associated with Endodontic diseases.

Molecular methods have become available, which have helped clinical management and improved our understanding of endodontic infections. The use of several techniques such as conventional Polymerase Chain Reaction (PCR), Quantitative Real-time PCR (qPCR), microarrays, clonal analysis and 16S ribosomal RNA (rRNA) gene sequencing have emerged as valuable tools for bacterial detection and identification, enabling more accurate taxonomic assignments (6).

PCR methods are designed to detect microbial DNA rather than living microorganisms (30). It must, however, be stressed that most molecular biology protocols do not discriminate between live and dead bacteria (as both have amplifiable DNA). Accordingly, the results may prove themselves difficult to interpret, since they may not reflect the living endodontic flora, but rather a historical record

of the microorganisms that entered the canal regardless of their capacity to survive there (30).

One possibility to overcome this limitation has been recently described by Loozen et al. (65). It relies in the chemical inactivation of free DNA and of DNA from damaged cells by a light activated Propidium Monoazide driven chemical reaction. These sources of DNA are rendered non-amplifiable by PCR whereas DNA from intact cells is protected and thus can be amplified and detected.

Culture and the complementarity of Metagenomics

Both Culture and Metagenomics approaches have advantages and disadvantages as published by Figdor and Gulabilava (66).

The identification and characterization of infectious agents can be more reliably performed with genotypic than with phenotypic markers, although, in accordance with Nair (59) and Relman (67), the latter cannot be disregarded. Effectively, often both types of methods are used because the available data supports a cumulative benefit of the two approaches.

Since its origins in the late 19th century, Bacteriology has largely been based on the ability to culture organisms of interest usually under *in vitro* Laboratory conditions. Indeed, it may be argued that the historical success of Bacteriology has been a direct result of bacteriological culture, as well as its widespread adoption throughout the world. Today the ability to culture bacteria *in vitro* remains the cornerstone of this discipline. However, Millar et al. (68) stated that there are several situations where molecular approaches should be considered as conventional Culture fails to identify the causal organism due to one or more of the following reasons):

- The organism is fastidious in nature;
- The organism is slow growing, e.g. *Mycobacterium spp*.;
- Specialized cell culture techniques are required, e.g. *Chlamydia* spp. and *Coxiella burnetti*.

Spratt (69) evoked that the resolution of these problems may take profit from the rapid advances in Molecular Biology over the last 20 years, which provides us with a bewildering array of techniques aimed at teasing apart numerous aspects of Biology. Accordingly, several human diseases and associated microbial pathogens were first identified directly from clinical specimens by using molecular approaches (55). From a microbiological point of view, it must however be regarded that Culture and NA techniques are expected to show different results, and some species are detected by both and others identified only by one of them. Neither approach is perfect, as both are poised with different strengths and limitations. Thus, more work is needed to satisfactorily reconcile, clarify and resolve the reasons for different results from these two approaches (66).

Despite its drawbacks, Culture still has a place in Microbiology studies. It must be noted that contrary to NA tests, conventional cultures allow phenotypic studies and preserve the specimen under investigation. Thus, in cases of organism identification in unusual clinical conditions and in situations where pure cultures are needed for additional analysis, culture still is an important resource (8). Thus, Culture will still be, for numerous years, the gold standard for most clinicians, while new techniques are further developed, disclosing new knowledge and complementing the present information in endodontic microbiology (10).

The last years have witnessed an awakened interest in the biomedical community for the study of biofilms since biofilm communities are responsible for the majority of bacterial infectious human diseases. This is a unique opportunity to meld in vitro, in vivo and translational research, but it is critical that these three approaches be carried out in parallel with collaborative interactions. There is a great fear that the research community and research administrators have become enraptured with "Metagenomics", "Proteomics" and *in vitro* systems (8), and disregard the important role that conventional cheaper technologies can still play. In fact, genotypic studies alone may have little relevance to the in vivo ecosystem of interest. The challenge for the near future will be to balance those three approaches so that major advances can be made in our understanding of biofilm ecosystems (8).

MRS as surrogate marker of endodontic outcome

Positive cultures and the prognosis of endodontic treatment

MRS is useful for the development of clinical protocols and ultimately could be used as a predictor (a surrogate endpoint) for the success rate of endodontic treatment (30). However, a judicious look at the published data gives a more complex picture, that is to say that MRS results and clinical outcomes do not show a simple all-or-none relationship limiting the predictive value of MRS (30).

It has been questioned by several authors including Heling and Shapira (49), Zeldow and Ingle (70), Ergström et al. (71), Bender et al. (72), Oliet and Sorin (73) and Eggink (74) whether the results of the endodontic treatment are improved by the observation of a negative culture before filling. In fact, authors such as Bender et al. (72) and Morse (75) even claimed that culturing was no longer necessary, stating that the results of endodontic treatment were not based on culture findings. In fact, some studies, such as the ones published by Mollander et al. (29) and by Sundqvist et al. (76), argued that a substantial number of canals with positive culture can heal and a number of "diseased" cases yielded negative cultures. This controversy has remained to the present day with some studies showing that complete periapical healing occurs in 94% of the cases that yielded a negative culture, whereas in samples with positive cultures prior to root filling, treatment success rate dropped to 68%, concomitantly to same year studies indicating that 32–56% of endodontic treated teeth with persistent apical lesions (failed cases) show negative bacterial cultures (29, 76). Molecular biology studies, as the one of Kaufman et al. (77), with its improved sensitivity have, in some cases, added to the controversy showing that residual bacteria can be detected in a high proportion (77%) even in successful cases.

However, this conflicting data can, at least to some point, result from use of limited value or even inadequate microbiological techniques (no anaerobic culturing, for example). Also, some of the studies, not entirely supporting MRS usefulness, conducted by Siqueira and Rôças (16), Sathorn et al. (30), Sjögren et al. (34), Sundqvist et al. (76), Byström et al. (78), Waltimo et al. (79) and Fabricius et al. (80), show, nevertheless, a small outcome difference (10-15%) between MRS positive and negative teeth, thus still supporting the notion that, at the time of obturation, a positive culture leads to lower success rates. Thus, as stated by Matsumoto et al. (81), testing for lack of endodontic bacteria before filling the root canal can be regarded as an important measure to the prevention of refractory periapical lesions. This has even been recognized by some MRS opponents such as Morse (82), who, in disagreement with previous own statements, wrote: "Anaerobic microbes are becoming clinically important and with the adoption of simplified anaerobic culture techniques, it may once again become important to take cultures".

Later, Sjögren et al. (34) emphasized the idea that "a single factor produces a single effect" is probably too simplistic and does not fit the complex relationship between host and disease. The host response and/or the quality of coronal restoration were factors, as related by Ray and Trope (83), Tronstad et al. (84), Hommez et al. (85), Dugas et al. (86) and Siqueira et al. (87), that could also influence endodontic clinical outcomes. It is unrealistic to expect that a culture taken immediately before obturation could be a perfect predictor of outcome (30).

Even when the endodontic treatment does not succeed to completely eradicate the infection, the truth is that the huge majority of bacteria are eliminated and the environment is markedly disturbed. To survive and therefore be detected in post-treatment samples, bacteria have to resist or escape intracanal disinfection procedures and rapidly adapt to the drastically altered environment caused by treatment procedures. Hence, some authors suggested that, if cultivable bacteria persist in the canal, the healing of lesions may be delayed (79) and long term outcome may depend on the quality of the root canal filling (5, 80). Therefore, a good treatment outcome may not be solely associated with bacteria-free root canals, but the importance of bacterial elimination from the infected site should not be ignored (12). The radiographic success may still miss histological inflammation, but several studies have demonstrated that the clinical protocols that ensure a significant decrease on bacterial load during endodontic procedures tend to achieve a very high success rate (19). Furthermore, Nair (88) wrote that causes other than intraradicular infections

might be responsible for persistent lesions, such as true cyst, extraradicular infection, foreign body reaction and scar tissue, which cannot be managed by orthograde endodontic treatment.

Culture results and the healing of apical lesions

The probability for apical lesion of teeth with negative cultures to heal completely after treatment is 6.8 times greater than those with positive cultures, yet, this apparently strong association might not be particularly high from an epidemiological perspective (30). Thus, although a relation to clinical outcome does exist, intraradicular bacterial status might not be a strong predictor of apical lesion clinical outcome.

Single- or Multiple-visit Endodontics

In the meta-analysis study of Sathorn et al. (89), no significant difference was found between clinical outcomes for Single- and Multiple-visit Endodontics. Calcium hydroxide intracanal dressing has been associated with enhanced bacterial elimination, as demonstrated by Law and Messer (90) through the reduction in the number of positive root canal cultures. However, this theoretically predicted advantage of multiple sessions does not translate to higher healing rates, as documented by three randomized controlled clinical trials performed by Trope at al. (91), Weiger et al. (92), Peters and Wesselink (93) and the meta-analysis of such trials mentioned before (89).

Result reversals

When MRS measures an increase of bacterial colonies relative to the first bacterial status, the result will be interpreted as a reversal. Higher numbers of culture reversals are usually interpreted as a signal of lower MRS accuracies (30). However, bacteria detected in post-instrumentation samples may be remnants of the initial infection that resisted instruments and irrigation or were introduced in the root canal because of a breach in the aseptic chain (16). Another possibility is that if bacteria numbers are low, they may not be detected in the initial sampling due to sampling difficulties; however, after chemomechanical preparation, enlargement of the canal may benefit sampling quality and the efficiency of the render sampling. In these cases, although MRS has measured bacterial status correctly, the use of the mentioned rational would disregard them as clinically incorrect (30). Thus, result reversals frequency may not be an appropriate measure of MRS accuracy.

Influence of MRS on Endodontic Performance

The ideal clinical question to be answered can be framed in terms of a PICO question (P=problem, I=intervention, C=comparison and O=outcome) as follows (90): with healing or prevention of apical periodontitis by endodontic treatment as target, does MRS improve clinical protocols and success rates?

As mentioned before, MRS is a passive sample of the main root canal space, which does not include inaccessible areas such as accessory canals, fins and dentinal tubules, nor adherent biofilms. The technical handling is simple and comprehensible but can be perceived as not so straightforward to be applied: this is the main reason, affirmed Molander et al. (94), for practitioners' rejection of MRS.

It is also relevant to observe that since endodontic treatment frequently relieves patients of their symptoms, the benefits of MRS may appear very limited from a strict symptomatic point of view. Thus, it can be claimed that MRS benefits are only related to a decreased probability of some undesirable future event (failed periapical healing), which seems quite poor. This may be the supporting argument for those defending that MRS could be useful specifically in selected cases such as teeth of high strategic prosthodontics, where one must guarantee optimal results (94).

Even nowadays, the benefit of using MRS is not immediately observed in agreement with the statements of Molander et al. (94): besides economic incentives, MRS would possibly attract a higher adoption rate if it was associated with the provision of high-status dentistry and positive health effects.

Obviously, despite the divergence linked to the diffusion of the technology, there are issues such as the cost, the learning curve, the extra time-consuming

procedure requested to sample and the articulation with the laboratory that must be considered. Thus, using MRS in all endodontic cases may be difficult.

From another perspective, the chairside anaerobic culture tested by Yoneda et al. (12) has an educational effect for inexperienced dentists such as trainees. Educating trainee dentists and dental school students about the importance of correct dental treatment from the beginning of their dental careers is certainly a valid work. The results of the chairside anaerobic culture test can help them understand that root canals with apical periodontitis are seriously contaminated, and they should try to eliminate bacteria under the rubber dam and make good restorations (12).

Undoubtedly, MRS implies an optimal control of the cross infection during endodontic procedures as we just want to collect bacteria from the inside of the root canal system. If we have in mind the role of intracanal bacteria in causing apical periodontitis and the need to achieve bacteria control during endodontic treatment, then it can be easily argued that MRS is a valid mean of checking the asepsis of the procedures and those operative conditions that favor the success of the therapeutic action should be, positively, the way to work: the standard behavior.

A well-instrumented and well-filled root canal has greater chance to be free of microorganisms. Careful antiseptic measures in all treatment steps cannot be underestimated. In other words, MRS is the very foundation of clinical Endodontics (30).

Conclusion

The infected root canal system acts as a reservoir of microbial cells, virulence products and antigens, which collectively evoke and maintain apical periodontitis (31). Conceivably, we must remove from the root canal system those etiological factors.

The current knowledge in endodontic microbiology is based on the application of several methods such as conventional histology and electron microscopy, scanning confocal laser microscopy, microbial culturing and biochemical and molecular techniques (59).

MRS is a sensitive procedure in a hostile setting. Bacterial culture and identification is not an end in itself (30). Its main clinical purpose is to test different endodontic protocols and, if possible, to document an association between cultures status and healing.

In our search of a more reliable approach, results from sampling studies are recommended as surrogate endpoints for long-term clinical outcome studies, despite the well-recognized strict conditions of the sample collection (30).

Future longitudinal studies are necessary to evaluate if the persistence of some specific species or remaining uncultivable microorganisms (10) are of significant importance for endodontic treatment failure (i.e., if any given species persisting in the root canal are a risk factor for post treatment apical periodontitis). To pursuit these intentions, methods for the recovery of all microorganisms are critical and must be a target concern. Spångberg (95) advertised that it is crucial to establish and agree on the best methods of sampling for molecular techniques and culturing methods.

With the insight that we still do not have the complete picture of the microflora and that new techniques will complement or even change our future opinions on the natural history and complexity of the root canal flora (10), we do not have an alternative approach to study this enthralling subject: sample the pulpal space supported by evidence-based guidelines presented throughout this article.

Future improvements in root canal treatment will likely come from a deeper biological insight into the microbial pathogenicity and the factors regulating community behavior (23). The two approaches described (Culture and Molecular Biology studies) are not exclusive to each other and should be used together by endodontic microbiologists in an informed polyphasic manner to understand the complex nature of root canal infections (69).

While culturing may not be mandatory for clinical practice, the practitioner should choose a treatment protocol that has been shown in controlled studies to result in a predictably low microbial count before filling the root canal (10). Nevertheless, to become closer to the real endodontic microflora and the way to eradicate them, MRS is undoubtedly the best option yet.

Consequently, sampling is in the mood.

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