MOLECULAR GENETIC IDENTIFICATION METHODS OF MICROORGANISMS IN ROOT CANALS

Alma Konjhodzic*[1], Lajla Hasic Brankovic[1], Irmina Tahmiscija[1], Aida Dzankovic[1], Samra Korac[1], Mirna Pasic[1], Adna Begovic[1], Madzida Halilovic Mehinovic[1], Lana Salihefendic[2]

[1] Department of Restorative Dentistry with Endodontics, University of Sarajevo Faculty of Dentistry with Dental Clinical Center, Sarajevo, Bosnia and Herzegovina

[2] ALEA Genetic Center, Sarajevo, Bosnia and Herzegovina

***Corresponding author**

Alma Konjhodzic, DDM, PhD, Associate Professor, Department of Restorative Dentistry with Endodontics, University of Sarajevo Faculty of Dentistry with Dental Clinical Center, Sarajevo, Bosnia and Herzegovina;
Email: alma01konjhodzic@gmail.com

ABSTRACT

Oral cavity is the habitat of a large number of microorganisms. In endodontic microbiology, research and identification of microorganisms was primarily based on cultivation methods. These methods have certain limitations, especially considering the diversity among endodontic microbiota, as well as the fact that certain percentage of bacteria found in root canals are uncultivable. With the introduction of molecular genetic technologies, many limitations of cultivation methods have been overcome. In molecular microbiology, 16S rRNA represents a gene, a part of DNA molecule that is used for determining the genetic diversity among bacteria species via sequencing methods. There are numerous advantages of these methods, especially emphasizing their speed, sensitivity and accuracy. By using these methods, a big step forward can be made in terms of evidence-based antimicrobial endodontic therapy. Through the identification and detailed studious analysis of obtained results, we can conduct further research and possibly create new therapeutic guidelines or revise existing therapeutic protocols, thereby increase the success of treatment and prevent the development of reinfections.

Keywords: microorganisms, root canal, next generation sequencing, Illumina, PCR.

Introduction

Several hundred bacterial species inhabit the oral cavity and coexist in multispecies communities forming the unique ecosystem. However, dental pulp, the internal tooth tissue is physiologically sterile and any bacterial invasion to this connective tissue is a pathological sign (1). If the normal oral flora gets in touch with this sterile environment, bacteria would become an opportunistic pathogen causing damage to these tissues. They may reach dental pulp either through coronal leakage, caries exposure, faulty restorations, tooth cracks, traumatic injury, anachoresis or periodontal pathways (2). Bacteria that initially invades and colonizes dental pulp primarily causes intrapulpal infection. Some of these bacteria are involved in earlier stages of pulpal invasion to the further necrosis, or they may be latecomers invading canal after pulp necrosis by taking advantage of environmental condition in root canal. Bacterial consortium in primary intrapulpal infections contain 10^3 to 10^8 bacterial cells and 10 to 30 bacterial species per canal (3). Culture based methods and sophisticated molecular biology techniques have established the polymicrobial nature of primary endodontic infections where obligatory anaerobic bacterial species are dominant (4). The most frequent microorganisms in primary endodontic infections appeared to be anaerobic gram-negative bacteria whose species from several genera have been regularly found in infections associated with apical periodontitis and abscesses (3,5). These genera include *Fusobacterium* (e.g., *F.nucleatum),* assacharolytic species as *Dialister* (e.g., *D.pneumosintes, D.invisus*) and *Porphyromonas* (e.g., *P.gingivalis, P. endodontalis*), saccharolytic species- *Prevotella* (e.g., *P. nigrescens, P. intermedia, P. baroniae, P. tannerae*), spirochetes- *Treponema* (e.g., *T.socranski*, *T.denticola*) and *Tannerella* (e.g., *T. forsythia*). Even though gram-negative bacteria have been appeared the most common microorganisms in endodontic consortium, several gram-positive bacteria in prevalence are as frequent as gram-negative species. Gram-positive bacterial genera often found in primary endodontic infections include *Actinomyces* (e.g., *A.israelii*), *Olsenella* (e.g., *O.uli*), *Filifactor* (e.g., *F.alocis), Peptostreptococcus* (e.g., *P.stomatis, P. anaerobius), Streptococcus spp. (e.g., S. anginosus, S.sanguinis, S.mitisi), Enterococcus faecalis,Parvimonas* (e.g., *P.micra*), *Propionibacterium* (e.g., *P.acnes,P.propionicum*) and *Pseudoramibacter* (e.g., *P. alactolyticus*) (6,7,8).

Interactions between endodontic pathogens can be synergistic or antagonistic. Microbial synergism is phenomenon in which all microbe population support each other's proliferation and growth by providing nutrition to each other, inhibiting phagocytosis, secreting enzymes and growth factors, decreasing pH and oxygen concentration in the root canal (9). Antagonistic interaction between oral microbes implicates their competition for food sources and territory by suppressing or destructing bacterial species that are less dominant in endodontic infection (10).

Pioneer research in endodontic microbiology is based on cultivating root canal bacteria. As endodontic microbiota is diverse and 40% of bacteria found in root canal are uncultivable, different scientific studies establish that molecular diagnostic methods are more effective to discover new endodontic pathogens. Antagonistic interaction between bacteria can cause death of some species or they might be suppressed by dominant bacteria being not present in culture methods. Knowing that, development of molecular microbiology research has resulted with spectacular discovery in technology known as next generation sequencing methods. NGS technology use 16S ribosomal RNA (16S rRNA) gene to analyze bacterial diversity (11). Compared with other techniques, next generation sequencing is revolution in molecular technology in order to empower antimicrobial effectiveness and success of endodontic treatment.

Polymerase-chain reaction (PCR)

Culture procedures have been traditionally used as the reference in the assessment of the oral microbiota associated with infections of endodontic origin. However, there are certain limitations following this method (12).

First of all, the culture method routinely uses agar-plates as a medium in highest sample dilution causes an inadequate representation of the collected sample. In addition to the drawbacks of dilution, identification of microorganisms by their phenotypic traits has some other serious pitfalls, considering

that phenotypical traits are difficult to quantify, they are generally ambiguous and some species may even show a phenotypically convergent or divergent behavior. Furthermore, non-viable, uncultivable and in certain cases even fastidious or delicate microbes cannot be isolated using culture methods. The main reason for that is the difficulty in simulating the required environmental conditions for cultivation, for example the lack of the essential nutrients for bacterial growth or disruption of bacterial intercommunication systems. Production of substances inhibitory to the target microorganism by other present species might also be the reason for bacterial uncultivability (12, 13, 14, 15). Finally, the procedure itself lacks sensitivity, is costly and laborious and it can take several days to weeks for the cultivation and identification results to be completed (12, 14).

Because of all these reasons, this method has been recently questioned as the "gold standard" (the reference method) for bacterial identification (12, 16).

Many limitations of culture method have been successfully overcome by the induction of molecular technologies. Studies on DNA, RNA and proteins have introduced new methods of infectious agents' identification detecting microbial DNA rather than the microorganisms themselves in both research and clinical cases. These procedures have revolutionized our knowledge of infectious diseases, allowing us an effective and rapid diagnosis of many clinical conditions and they have definitely showed that the root canal microbial composition is much more complex than previously thought (12, 13, 17).

Molecular genetic diagnostics methods are more accurate, sensitive, specific and rapid than the culture technique. They do not demand carefully controlled anaerobic setting during sampling and transportation being a great advantage since some of the fragile microorganisms, especially anaerobic bacteria, can lose viability during transit. When higher number of samples is surveyed in epidemiological studies, multiple samples can be gathered and analyzed all at once (13,14).

There is a wide range of molecular genetic methods and the choice of a particular approach for the microbial identification depends on the question being addressed.

The most widespread advance in diagnostics technology has come from the application of polymerase-chain reaction (PCR) for detection of microbial pathogens. The PCR method was invented in 1983 and ever since has revolutionized the field of molecular diagnostics by being able to amplify as few as one copy of a particular DNA sequence into billions of copies of that sequence (13,14).

The PCR process relies on the in-vitro DNA replication via repetitive cycles of simple reactions. Basically, each ensuing cycle uses previously synthesized products as templates for the subsequent reactions. The outcome is the exponential amplification of DNA products. A typical PCR procedure includes 25–40 cycles of amplification (12, 14). One of the great benefits of the PCR method is that it does not require particularly pure or plentiful target DNA for the process to be completed (18). Amplification in theory is possible even if the sample contains a single molecule of DNA making this technique one of the most sensitive methods for detection of microbial DNA. Furthermore, under optimized amplification conditions, PCR shows no cross-reactivity (12, 19).

A single round of PCR is a standard and most often used method for microbial identification purposes. Nevertheless, since its inception, there have been developed numerous derivates of the method, such as reverse-transcriptase PCR (RT-PCR), Multiplex PCR, Broad-range PCR and Real-time PCR. These variations have also been used to directly survey clinical samples for the presence of bacteria (12,14).

Reverse Transcriptase PCR has been developed as a method through which a DNA can be generated from RNA, using the reverse transcriptase enzyme. Once the double-stranded DNA is synthesized, it can be used as a template for further amplification, just as in standard PCR (12, 14, 20).

Multiplex PCR enables the simultaneous detection of different microorganisms by using primers specific for different targets considering that more than one target sequences can be amplified at the same time. Practically, as multiplex PCR can detect a few different species at a time, this method minimizes time used for the analysis and the number of templates and reagents needed for detection. Careful design of the primers used in multiplex assays is an imperative, considering that similar annealing temperatures and no complementarity of the primers is required in order to avoid inaccuracy (12, 13, 14).

Broad-range PCR technology can be used to detect the entire microbial diversity in a given environment. This variation of standard PCR uses primers complementary to conserved regions. Conserved regions are parts of a particular gene shared by a group of microbes. What gives this method a certain superiority is the relative absence of selectivity, meaning that any kind of species present in a sample can be detected and later identified, even the unexpected ones. Broad-range primers bring a high risk for microbial contamination of the DNA and increased level of precautions is necessary (13, 14).

Real-time PCR enables monitoring of the amplification of a targeted DNA molecule during the PCR in real-time, using florescent dyes. What makes this method the exception from the most conventional PCR assays is the additional ability of quantification. In conclusion, real-time PCR allows the quantitation of target species as well as a total number of bacteria in clinical samples. This method saves time, since it can detect bacteria in a short period of time. The contamination of DNA is reduced because there is no postamplification manipulation (13, 14).

The use of PCR technology enabled us to detect some species of Gram-negative bacteria that are difficult to grow by culture method, such as the Fusobacterium spp., Porphyromonas spp., Treponema spp., Prevotella spp. and Tannerella spp. Additionally, their prevalence was much more emphasized with the use of PCR, differently from those studies based on culture techniques only. PCR has unrivaled sensitivity, being approximately 10 to 100 times more sensitive than any other identification method (12, 14, 21).

Although the PCR method has enabled us to detect many microorganisms in the root canal and identify them rapidly, there are still certain limitations. For example, a PCR cannot determine whether target DNA comes from live or dead bacteria (22).

In conclusion, PCR represents the basis for any other kind of analysis because of its ability to amplify target DNA extracted from the given sample. From there on, by sequencing, we collect information about the origin of the sequenced DNA as well as complete taxonomic data.

Next-generation sequencing

Sequencing is the process of determining the correct order of nucleotides in the exact DNA molecule. In molecular microbiology, 16S rRNA represents a gene, a part of DNA molecule that is used for determining the genetic diversity among bacteria species via sequencing methods. More than a few methods have been developed for this process. In 1977 was developed the first method of DNA sequencing named Sanger sequencing method (23). Since then, there have been quite a few innovations (such as development of fluorescent dyes and replacement of gel electrophoresis with capillary electrophoresis, as well as software improvements) which contributed to high success results rate of this methodology (24). In fact, this high rate of accuracy of the results leads to the main drawback of this method because it implies performing one reaction at a time. Also, because the price of this sequencing method is very high, Sangers sequencing method is mostly used for analyzing specific genes, for example, in human rare diseases (25). After the Sanger sequencing, the rise of technological development provided a next step in the DNA sequencing technology and has led to next generation of sequencing (NGS) (24). The main aim of using NGS in endodontic microbiology is to replace the characterization of pathogens according to their morphology, staining methods and metabolic characteristics with their genomic definition. The genomes of pathogens can give information about drug sensitivity, their relationship with other pathogens and also give more detailed information about infection out brakes which are in this case considered very important in expecting outcomes of endodontic treatment (26). A relatively new field named *Pharmacogenomics* uses NGS data to determine which drugs can result in the most successful outcomes in correlation to certain diseases, infections. In cooperation with this pharmaceutical branch, endodontists may be able to make significant strides in the improvement of endodontic therapy.

With NGS the disadvantages of Sanger sequencing were successfully eliminated, since in this case a large number, millions of sequencing reactions take place in parallel, simultaneously. Hence the name, massively parallel or deep sequencing. From endodontic point of view, this method of processing

root canal samples can represent a big step forward in terms of success of endodontic therapy. NGS identifies microorganisms (species) contained in the given sample. Based on the enormous amount of data received by NGS (the complete genome of certain isolated species) research on genetic diversity can be conducted. Consequently, with the addition of literature research on the sensitivity of certain species to specific drugs/chemicals, by comparing genomes, the existence of identical regions can be detected and therefore mutual characteristics defined, in this case sensitivity to drugs. Although these methods may seem expensive, the situation is different: an enormous number of reactions take place in parallel. This also overcomes disadvantages in terms of price, compared to the Sanger method (23).

If only a certain fragment is to be sequenced with a significantly smaller number of base pairs, Sanger sequencing is a method of choice due to a higher price rate of NGS. In general, this technology is able to produce enormous amount of data at very economic cost

Deficiency here is the obvious need for sophisticated software in order to analyze and interpret obtained results.

Illumina

The leading role in NGS represents *Illumina.* Illumina NGS platforms are capable of paired end sequencing (sequencing that occurs from both ends of a DNA fragment) generating high-quality sequence data with in depth coverage and high numbers of reads (27, 28).

There are several Illumina sequencing platforms, and as a matter of fact, Illumina is still producing newer and upgraded versions of already existing ones. The most popular, used and recognized Illumina platforms are: MiSeq, HiSeq, NextSeq series etc. Within these platforms there are also certain kits. The choice of appropriate Illumina platform and kit depends upon the subject of the research (29).

Users must be aware that there are some limitations and occurring errors when using each of these platforms. To avoid these possibilities, the application of different technologies is often used (23).

The attached Illumina results (Figure 1-4) are an example of laboratory analysis from the original research, performed using MiSeq platform and the 16s Metagenomics kit. It shows a complete taxonomic analysis of given samples, starting from the kingdom to the species classification of the identified bacteria. These results demonstrate the superiority and necessity of Illumina and NGS.

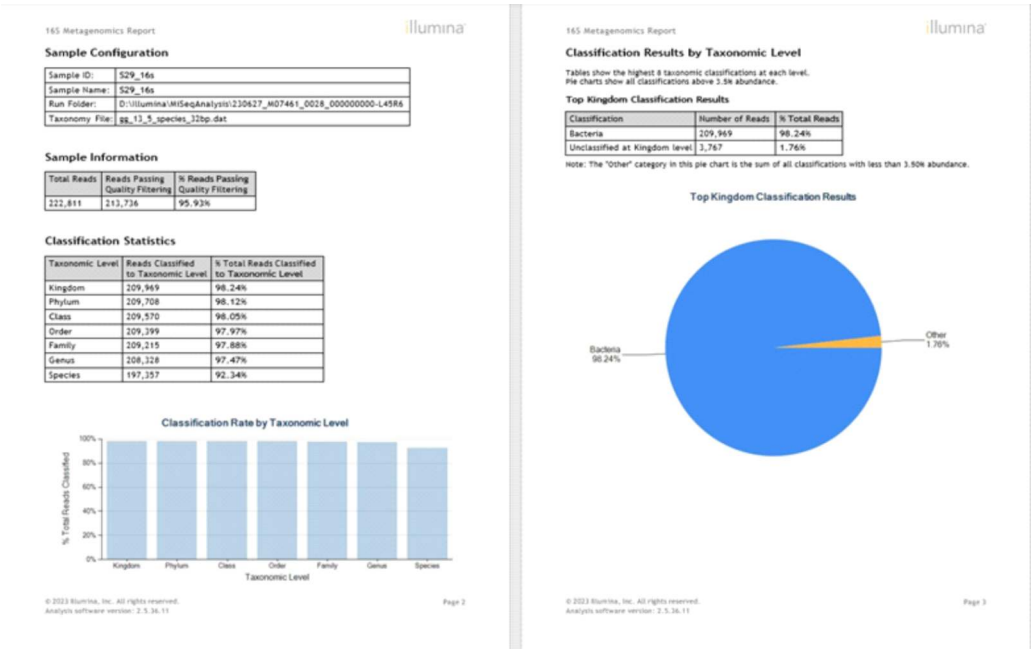
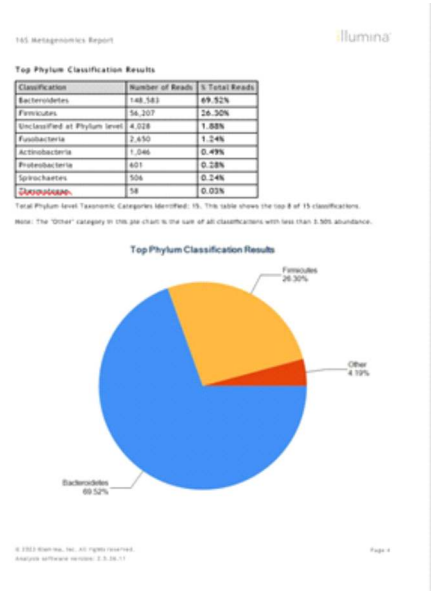


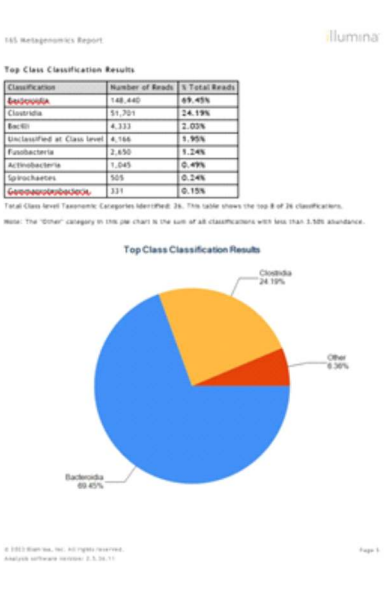
Figure 1.
Results of the original research on Molecular genetic identification methods of microorganisms in root canals



Top Phylum Classification Results

Classification	Number of Reads	% Total Reads
Bacteroidetes	148,583	69.52%
Firmicutes	56,207	26.30%
Unclassified at Phylum level	4,028	1.88%
Fusobacteria	2,650	1.24%
Actinobacteria	1,046	0.49%
Proteobacteria	601	0.28%
Spirochaetes	506	0.24%
[illegible]	58	0.03%

Top Phylum Classification Results

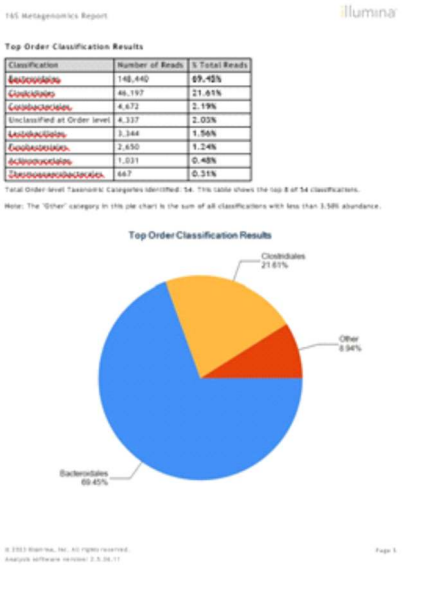


Top Class Classification Results

Classification	Number of Reads	% Total Reads
[illegible]	148,440	69.45%
Clostridia	51,701	24.19%
Bacilli	4,333	2.03%
Unclassified at Class level	4,166	1.95%
Fusobacteria	2,650	1.24%
Actinobacteria	1,045	0.49%
Spirochaetes	505	0.24%
[illegible]	331	0.15%

Top Class Classification Results

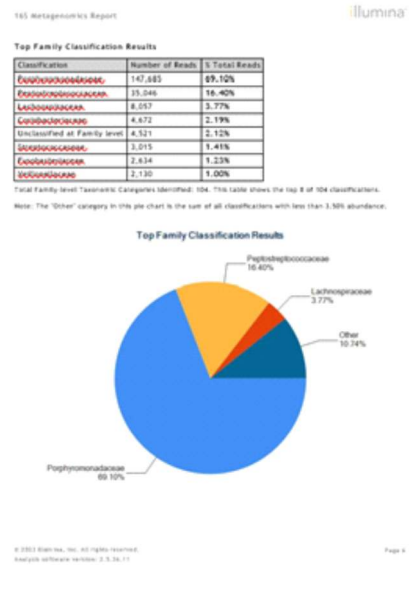
Figure 2.
Results of the original research on Molecular genetic identification methods of microorganisms in root canals. Phylum and class level taxonomic category.



Top Order Classification Results

Classification	Number of Reads	% Total Reads
[illegible]	148,440	69.45%
[illegible]	46,197	21.61%
[illegible]	4,672	2.19%
Unclassified at Order level	4,337	2.03%
[illegible]	3,344	1.56%
[illegible]	2,650	1.24%
[illegible]	1,031	0.48%
[illegible]	667	0.31%

Top Order Classification Results

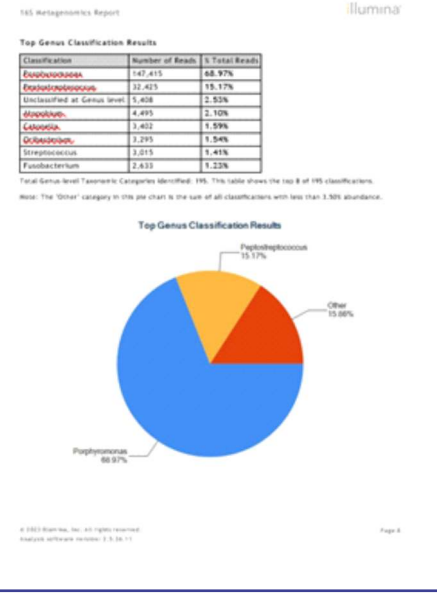


Top Family Classification Results

Classification	Number of Reads	% Total Reads
[illegible]	147,685	69.10%
[illegible]	35,046	16.40%
[illegible]	8,057	3.77%
[illegible]	4,672	2.19%
Unclassified at Family level	4,521	2.12%
[illegible]	3,015	1.41%
[illegible]	2,634	1.23%
[illegible]	2,130	1.00%

Top Family Classification Results

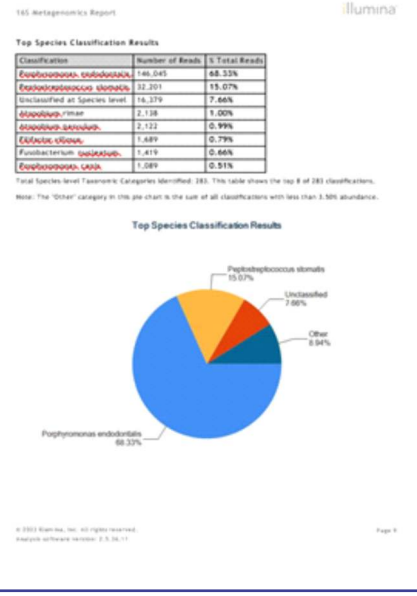
Figure 3.
Results of the original research on Molecular genetic identification methods of microorganisms in root canals. Order and family level taxonomic category.



Top Genus Classification Results

Classification	Number of Reads	% Total Reads
[illegible]	147,415	68.97%
[illegible]	32,425	15.17%
Unclassified at Genus level	5,408	2.53%
[illegible]	4,495	2.10%
[illegible]	3,402	1.59%
[illegible]	3,295	1.54%
Streptococcus	3,015	1.41%
Fusobacterium	2,633	1.23%

Top Genus Classification Results



Top Species Classification Results

Classification	Number of Reads	% Total Reads
[illegible]	146,045	68.33%
[illegible]	32,201	15.07%
Unclassified at Species level	16,379	7.66%
[illegible]	2,138	1.00%
[illegible]	2,122	0.99%
[illegible]	1,689	0.79%
Fusobacterium [illegible]	1,419	0.66%
[illegible]	1,089	0.51%

Top Species Classification Results

Figure 4.
Results of the original research on Molecular genetic identification methods of microorganisms in root canals. Genus and species level taxonomic category.

Conclusion

Oral cavity inhabits one of the highest accumulations of different microorganisms in the body, with bacteria being by far the most prevalent ones. Application of molecular genetic technologies in analyzing bacterial diversity has revealed broader and more diverse spectrum of oral bacteria compared to cultivation methods. Undoubtedly, with the well-directed use of molecular genetic methods, valuable information regarding the identification of the etiological factors associated with endodontic diseases will be provided. Moreover, these methods have the potential to make a diagnosis more rapid and to make directed evidence-based antimicrobial treatment a reality. Further efforts should be directed towards finding a connection of the predominant taxa with symptoms and other clinical conditions, unraveling the practical role of the detected species in the mixed endodontic consortium and disclosing their susceptibility to antimicrobial substances and therapeutic procedures.

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