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Review Article

Molecular Diagnostic Methods in Endodontics

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Abstract

The endodontic disease is fundamentally a microbial disease initiated by one or several bacterial or fungal species. The disease process is initiated and propagated by a complex community of microorganisms that are common commensals of oral microflora. The gold standard for decades was to culture microorganism from infected root canals or periapical abseccess. Although the culturing techniques remains the technique of choice for studying the phenotypic characteristics of bacteria and susceptibility to antimicrobials, Its known that only half of the bacteria is cultivable. The modern molecular methods helps to identify the un cultivable microorganisms. The article mentions the importance of molecular methods for identification of microorganisms.

Keywords: Biofilm; Microbial Culture; Microscopic Methods; Molecular Biology Methods; Polymerase Chain Reaction; DNA Hybridization

Introduction

Primary endodontic infections are polymicrobial. The microflora in root canal is initially colonized by aerobes and facultative anaerobes. The unique environment of root canal system due to changes in oxygen tension when root canals are opened up for treatment, effect of irrigating agents and due to introduction of various materials into root canal system changes the p H in the root canal. Due to these factors the ecology of root canal microflora changes as the disease advances [1,2].

- **Primary endodontic infections:** Primary endodontic infections are polymicrobial. The predomonent bacteria are Bacteroides, Prophyromonas, Prevotella, Fusobacterium, Treponema, Peptostreptococcos, Eubacterium, and Camphylobacter species [3,4]. In invading the root canal space] all bacteria within the oral cavity share the same opportunities, however only a restricted group of species have been identified in root canals that were infected. The root canal environment is unique in that the biological selection that drives the type and course of infection due to the disproportionate ratio between potential and actual number of species. The various factors are an anaerobic milieu, availability of nutrition, interaction between microbial factors are contributing factors that define the composition of the microbial flora [5].
- **Secondary endodontic infection:** are post-treatment disease, in the secondary infection the microflora is dominated mainly by the facultatively anaerobic gram-positive cocci and rods such as Streptococcus, Enterococcus, Peptostreptococcus and Actinomyces speciesIt appears, however, that certain organisms viz. Gram-positive facultatives including enteroccoci, which often have a suppressed representation in primary endodontic infections, anaerobes.

Biofilms

The *in vivo* microbial organization that initiates the root canal infection occurs not as separate colonies, but grow within an extracellular matrix in interconnected communities as a bacterial biofilm. The ability of the microbes to form biofilms is also a potential virulence factor. The accurate depiction of ultastructural biofilm appearance in the infected root canal was first reported by Nair, who described them as coaggregating communities with a palisade structure [6]. When compared with planktonic forms, the characteristic growth pattern of a biofilm is that bacteria are relatively protected within the coaggregated community, the coaggregated biofilm community are more resistant to antimicrobial treatment measures [7-9]. In multispecies infections, microbes ability to find synergistic partners for positive interactions with other microbes. The ability to tolerate or evade host responses, the release of bacterial mediators such as lipopolysaccharides (LPS). The synthesis of host tissue damaging enzymes have an influence on microbial survival (Figure1).

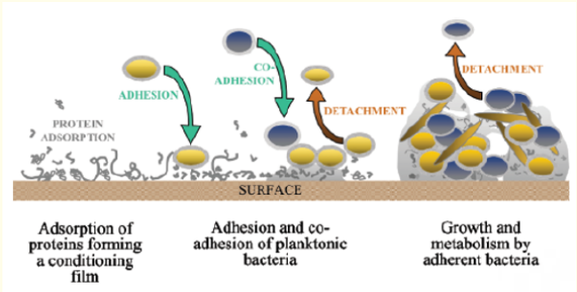

Figure 1: Stages of biofilm formation.

It is known that adhesion of microorganisms to surfaces triggers altered expressions of a large number of genes, which result in phenotypical changes. These genes may be transferred and shared by different species in a biofilm community. The higher density of bacteria were found in the infected root canals of the teeth with larger lesions when compared to those teeth with small lesions. Studies on the dynamics of root canal infections have shown that the relative proportions of anaerobic microorganisms and bacterial cells increase with time in infected root canals, with dominance of facultatively anaerobic bacteria outnumbered when canals were infected for more than three months or more [10]. The selective habitat of endodontic milieu supports the development of specific proportions of the anaerobic microflora. The presence of oxygen and oxygen products has an important role as ecological determinants in the development of specific proportions of the root canal microflora [11-13]. The development of a low reduction oxidation potential by the pioneer species along with consumption of oxygen and production of carbon dioxide and hydrogen favour the growth of anaerobic bacteria. The possible role for fungi and viruses along with bacteria are involved in endodontic infections. Fungi are eukaryotic microorganisms that have been detected by culture in endodontic infections. Studies show the presence of Candida albicans in 2-5% in primary endodontic infections and 9% in case of failed endodontic treatment.

Methods of studying biofilms in endodontics

Culture

Microscopy

Immunological methods

Molecular biology methods

DNA-DNA hybridization

Nowadays, biofilms are studied in two ways: in one, the microorganisms community as a single unit and, in the second, effects and relationship between the species [14]. The advances in technology and computational biology enables to study gene and protein expression in such communities,thus helps in relieving the role that each species has in that specific community [15]. Since only few techniques are capable of re- creating both the extra cellular matrix as well as the microorganism in the biofilm, identification of the exact intracanal biofilm nature is a real challenge.

- **Microbial Cultivation:** With improvement in the culturing techniques and advent of molecular methods of detection of microbes, the diversity of the bacteria implicated in the root canal infection has expanded largely. Though most of the bacteria arise from the normal oral flora, not all species can survive in the root canals and cause infection.

Based on Moller's work, to perform MRS properly

- The operative field needs to be prepared by scaling of hard and soft deposits and be effectively isolated with a rubber dam.
- The operative field is to be sterilized with 30% hydrogen peroxide and followed by 5% tincture of iodine.
- The sterility of the operative field has to be monitored by sampling tooth surfaces using a charcoal-impregnated pellet.
- This pellet to be cultured, if the results are positive to bacteria, any evident microbiological data obtained from that particular tooth has be discarded, due of the risk of contamination.
- The root canal is filled with sampling fluid; a sample is taken with charcoal-impregnated points that are transferred to a transport medium.
- The medium is then serially diluted and to be inoculated into an suitable nutrient broth and/or agar plates. The medium has to be aerobically and anaerobically incubated for a period of time sufficient enough to allow even slowly growing species to form colonies.
- Results are microbiologically analyzed using growth/no growth determination or identification of isolated microorganisms based on colony morphology, micromorphology, and both physical and biochemical tests
- Molecular techniques such as polymerase- chain-reaction-based methods (PCR) may not give available information on the abundance of microorganisms.

Polymerase chain reaction

With the advent of 'molecular biology' microbiologists are assistance of another avenue to pursue with respect to understanding the microbiology of root canal infections. Kary Mullis described polymerase chain reaction technique (PCR), for which he received Nobel prize in 1993. PCR is an elegant tool for amplifying a nucleic acid sequence of DNA or RNA, provided there is enough nucleic acid to detect and identify the organism. The advantage of PCR over direct detection with molecular probes (hybridization) because PCR allows amplification of. The DNA or RNA target sequences [16]. The target sequence is multiplied millions of times without having to culture the microbe. The microbe need not be viable, as long as the DNA is intact for PCR to detect the microorganisms.. PCR is sensitive, so that it can detect the presence of less than 10

bacteria in a sample.

Primer pairs, both sense and antisense, are used for bacterial PCR.

The primers are designed from the variable sequences of the 16S rRNA gene that is specific species of interest. A primer is a small piece of synthetic DNA, an oligonucleotide, which is known to complement the DNA sequence of the particular microbes gene being assayed. The 16S rRNA gene sequence for most bacteria can be downloaded from GenBank, National Center for Biotechnology Information and aligned using Geneworks software (version 2.5, Intelligenetics, Campbell, CA, USA). PCR mixtures containing the extracted DNA from the clinical sample, the species-specific primers, polymerase, deoxynuclotide triphosphates (dNTPs), MgCl2, and buffers are placed in a thermal cycler [17]. The heat during each cycle causes the DNA to denature into single-stranded DNA. The single stranded DNA can anneal with the complementary dNTPs and form another amplicon with each cycle. With each 1-min thermal cycle, the number of DNA amplicons between the primers is doubled. In each minute with the doubling of DNA amplicons, a million amplicons are produced in 20 cycles and billions of amplicons in 30 cycles. This instead of trying to find a genetic needle in a haystack, a haystack of needles (amplicons) is produced. By knowing the number of bp in a species-specific amplicon, it is possible to perform gel electrophoresis and by comparing the amplicon to a 100bp ladder helps to determine if the species of interest is present.

Nested PCR was developed to increase both the sensitivity and the specificity of PCR. For nested PCR, there are two pairs of amplification primers and two rounds of PCR. The amplicon products of the first round are subjected to a second round of amplification. The help of a second set of primers that anneal to an internal sequence of the first amplicon. Nested PCR has been used to detect treponemes in endodontic infections. [18-22].

RT-PCR has been developed to amplify RNA targets. In this process complementary DNA (cDNA) is first produced from the RNA target by reverse transcription. The cDNA is then amplified by PCR. There were initial problems with the technique because the RT enzyme was not heat stable. This problem was solved with the development of a thermostable DNA polymerase derived from Thermus thermophilus that functions properly as both an RT and a DNA polymerase. This technique is used to detect the cytmegalovirus and Epstein-Barr virus in periapical lesions of teeth with intact crowns [23,24]. Standard PCR is qualitative in that it can only detect or identify a gene sequence. Now quantitative PCR (QPCR) is available [25]. Q-PCR will allow enumeration of bacteria, determination of viral titer, and measure gene expression.

DNA-DNA hybridization

Once the DNA from a microbe has been sequenced, a single-stranded molecular probe of complementary nucleotide bases can be designed. The molecular probe is unique for that target DNA of the microbe of interest. The molecular probe is usually labeled with a fluorescent molecule, an enzyme or a radioactive label that can be detected. The patient sample may be cultured to increase the number of microbes of interest. The culture sample then treated to lyse the microorganisms and release the DNA. Single-stranded DNA is produced by alkaline denaturation of the double-stranded DNA. The denatured single-stranded DNA is immobilized on a nitrocellulose membrane for hybridization to the labeled probe. The membrane is washed to remove any unbound probe. Following the extent of hybridization is measured by the amount of probe remaining on the membrane [26-30]. Notable alternative technique is checkerboard DNA-DNA hybridization. This technique involves deposition of bacterial DNA from clinical samples obtained from root canal, plaque etc in parallel (vertical) lines on a nylon membrane. Digoxigenin - labelled whole genomic DNA probes are run at right angles to the samples (horizontal). Following washing the bound probe is detected and quantified.

Traditional vs. molecular methods: Because of the spectacular nature of molecular techniques, some investigators have suggested that they will soon completely replace the need for microscopy, culturing, and immunological assays [31]. A review of the literature demonstrates the importance of each of these investigative techniques. For example, in the field of medicine the diagnosis of leprosy caused by Mycobacteriam leprae and Lyme's Disease caused by Borrelia burgdorferi can be made microscopically. In identifying microorganisms in field of endodontics, light microscopy, dark-field microscopy, electron microscopy, and the confocal microscope continue to be important investigative tools [32-37]. The use of microscopes and specifically confocal microscope is used to study coaggregation. The confocal microscope provide significant insight into microbial pathogenicity in endodontic infections.

Immunological assays have provided indirect evidence of the microbes responsible for producing endodontic infections [38-42]. Molecular methods have improved our detection of organisms that are in low numbers and those with fastidious growth requirements. Molecular methods may not be better than culturing for those organisms with well defined growth conditions already in common use [43-46].

Conclusion

The elimination of microbes from root canal system by proper disinfection of root canal system requires the knowledge of root canal microbial ecosystem. The identification of microbial biofilm by culture methods, microscopes, immunological assays and mo-

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