Review Paper:

Advances in Molecular Biology Techniques for the Diagnosis of Dental caries: A Mini Review

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Abstract

The early and accurate detection of dental caries is very important in preventing the irreversible loss of tooth, reducing treatment costs and minimising invasive techniques for restoration of tooth. Conventional diagnosis of dental caries is restricted to visual inspection, tactile sensation and radiographs. Although they are satisfactory in the detection of carious lesions, they are inadequate in early detection of dental lesions. Because of these deficiencies, new molecular detection methods have been developed to aid better and early diagnosis.

In the present review, various molecular biology techniques for early detection of dental caries are explored. The advantages of each molecular method are discussed in detail. The use of molecular techniques adjunct to clinical visual examination for caries diagnosis will facilitate preventive care in dentistry and lower the treatment cost as well.

Keywords: Dental caries, Molecular techniques, Preventive dentistry.

Introduction

Dental caries, also known as tooth decay, is the damage of the tooth's surface by acids that are produced by decay causing bacteria when it grows on the sugar and starch from food and drinks[13,27,37]. The enamel gets demineralised and hence gets weakened and destroyed due to the chronic attack of these acids. This results in a small hole in the tooth called a cavity. The symptoms start appearing after the tooth decay advances. It may include toothache, sensitivity and in severe cases, pus formation, facial swelling and fever may also be observed. The microflora of the dental caries includes both aerobic and anaerobic microbes. Bacteria is predominantly present in the biofilm, yeast, protozoa, *Archaea* and virus may also be present[19].

The most common bacterial strains found in dental caries are *Streptococcus mutans,* various species of the genera are *Lactobacillus, Atopobium, Propionibacterium* and *Actinomyces*[1]. The conventional way of diagnosis is either by the direct visualisation of the cavity or detection by the dental radiographs or X-rays, transillumination for caries that are less visible and go deeper into the tooth. Lasers are also used for the diagnosis of dental caries in the interproximal decay as a method free of ionising radiation[11].

Detection of lesions in the early stage than in the cavity stage of the caries is essential for treatment in preventing further decay. Early infections can be easily and accurately detected by using molecular diagnostics as it is a much more sensitive method compared to the conventional methods and detects even minute amounts of infectious agents.

During the past decade, remarkable progress has been made in understanding the molecular basis of one of the most common oral diseases, dental caries.

Newer molecular methods and technologies in detection allow for early caries detection, allowing better intervention at an earlier stage, thus preserving tooth structure as well as lowering the treatment costs. The advantages of molecular techniques over conventional techniques in detection of dental caries are summarised (Table 1).

Table 1
Advantages of the molecular techniques over conventional methods in detection of dental caries

	Limitations of conventional methods	Advantages of molecular techniques
1.	Visual method has low sensitivity and fails to detect early signs of caries[2,21,33].	Molecular techniques are highly sensitive and can detect the risk factors associated with caries[20,30,38].
2.	Tooth separation is done for the examination of caries progression[3,33].	qPCR can be used to examine the caries progression[10,17,25].
3.	Sharp probes cause transportation of cariogenic bacteria to unaffected areas and mechanical damage to the enamel[33].	The analysis by molecular techniques requires mostly salivary samples[12,14,34].
4.	Radiographs are not reliable for the detection of occlusal enamel lesions. It also uses a potentially ionizing radiation[7,33].	Various molecular techniques have been used in the diagnosis and management of occlusal caries[26,29].

Molecular methods in the diagnosis of dental caries

PCR: Polymerase chain reaction (PCR) is a molecular technique used for the amplification of certain parts of the DNA by using respective forward and reverse primers for it. In the diagnosis of dental caries, PCR can be used for detection of microbial populations. The presence of *Lactobacillus* was investigated by performing PCR and using genus specific primers[26]. These primers amplified the intragenic spacer between the 16S and 23S fragments of rRNA. By using this method, they were able to detect *Lactobacillus* in the isolates.

In another study, using PCR and MALDI-TOF (MS), microflora in both caries active and caries free Indian children were detected. *Lactobacilli* and *Streptococcus* were found in higher numbers in caries active samples and gram-negative *Bacilli* like *Acinetobacter, Enterococcus, E. coli* were found to be lower in caries active than caries free samples[5].

In a study done by Kouidhi et al[18], 17 microbial species using PCR assay in Tunisian children were analysed. In this study, it was found that *S. mutans, S. salivarus, Candida spp., L. acidophilus* and *L. plantarum* were associated with caries formation. *Candida spp.* and *P. Gingivalis* were incident at a higher rate in caries active samples compared to the control. The determination of *Scardoviawiggsiae* in Indian adolescent population having caries risk was carried out by another study[23]. PCR was used to detect and amplify particular sequence of the 16S rRNA gene present in *Scardoviawiggsiae.*

It was found that *Scardoviawiggsiae* was present largely in patients with dental caries and also those having high risk of caries. It was however absent in those samples having low risk of caries. Hence it can be interpreted that *Scardoviawiggsiae* is one of the predominant microbes for initiating the caries and also in its progression[32].

Quantitative PCR: Quantitative polymerase chain reaction or qPCR not only amplifies the target DNA, but also measures the amount of DNA present in the given sample. It is also called as real-time PCR since the results are obtained simultaneously with the amplification of DNA. In this method most of the time, if not always, an accurate count of target sequences is obtained. This method is extremely important in the diagnosis of dental caries, as it can show the intensity and the progression of decay in the patient. In an earlier study, the quantity of the microbes in dental caries was measured by qPCR based on the target sequences selected for each species[24].

It was found that *S. mutans* was present in higher amount in inactive than in active lesions. The quantification also showed that *Bifidobacterium spp.* and *L. Casei* group were detected in higher level and may be associated with dentin lesion activity. In another study done by Klein et al[17], the total bacterial and *S. mutans* content was quantified by employing qPCR. Reverse transcription real-time PCR assay was carried out for the analysis of the transcription products in parallel and determined the presence of gtfB and gtfC genes responsible for the virulence of *S. mutans.*

Hence, in this study, it was shown that the quantification of the microbial population as well as the gene expression can be analysed simultaneously. This further helps doctors in understanding the progress of caries and also helps in determining the virulence of *S. mutans.* In another study carried out by Mun et al[22], the infection rate of bacteria causing dental caries was determined.

Here, real-time PCR was used for quantitative study of *Streptococcus mitis, Streptococcus mutans, Streptococcus sobrinus* and *Lactobacillus casei.* It was found that *Streptococcus mitis* and *S. mutans* were present in 99.3% and 63% of the samples respectively. It was also found that there was a significant variation in the number of *S. mutans* and *S. mitis* with the age of patients.

AP-PCR: Arbitrarily primed PCR (AP-PCR) is a type of PCR that is used as a technique for DNA fingerprinting and also called as "Random Amplified Polymorphic DNA" (RAPD). A study was done by Rodriguez et al[30]to determine the relationship between the genotypic diversity of *Streptococcus mutans* and the risk of caries in children. After amplifying the DNA of *S. mutans,* AP-PCR was carried out by using the OPA-13 primer.

It was seen that 66.67% of the caries free children had only one genotype and 77.78% of the caries active children have 2 or more phenotypes of *S. mutans.* Hence it can be inferred that there is a positive relationship between numbers of genotypes of *S. mutans* present with the risk of caries experience.

Terminal restriction fragment length polymorphism(T-RFLP): T-RFLP is a culture independent RFLP, which is a molecular technique used for the profiling of highly complex microbial communities based on variations in 16S rRNA. In this study done by Schmidt et al[31], the intact cell derived and cell free bacterial DNA from carious samples are characterized by the use of the molecular technique T-RFLP. T-RFLP was able to differentiate almost all the microbes present in these carious samples mainly *Lactobacillus spp., Actinomyces spp., Streptococcus spp.* and *Bifidobacterium spp.*

Pyrosequencing: Pyrosequencing is a method of DNA sequencing that relies on light, which is produced when pyrophosphate is released when an appropriate nucleotide is added. In a study done by Simón-Soro et al[32], metatranscriptomics was done to reveal overall bacterial composition in caries lesions. Here pyrosequencing is used to obtain sequences which help in identification of about 700 metabolically active bacterial species wherein *Streptococcus mutans* was the dominant bacteria. In another study,

pyrosequencing was used to amplify the V3 regions[8]. *Streptococcus* and *Neisseria* species were predominantly present in patients with dental caries and periodontal disease.

In another study 16s pyrosequencing was done to reveal the bacterial diversity in supragingival plaques in adults with dental caries[38]. High quality sequences were obtained and data showed about 453 independent species of bacteria predominantly containing *Capnocytophaga, Prevotella, Actinomyces, Corynebacterium, Neisseria, Streptococcus, Rothia* and *Leptotrichia.* Therefore, it can be seen that pyrosequencing is a very efficient and one of the most useful tools in the detection of various microbes in dental caries.

Illumina: Illumina is high throughput DNA sequencing method based on reversible dye terminators that enable the identification of incorporation of single nucleotides. It can be used in the characterization of microorganisms in dental caries. A comprehensive study was done on the microbiomes present in dental caries of elderly people[15]. The DNA present in saliva was amplified by PCR and sequenced by IlluminaMiSeq high-throughput sequencing. Illumina was used to detect 305 species of bacteria present in dental caries. Illumina was also used in another study for the analysis of pathogenic microbes in dental caries in different Chinese ethnic people[39].

The main species of pathogens in dental caries included *Veillonella, Aggregatibacter, Leptotrichia, Bacteroides, Granulicatella, Streptococcus* and *Prevotellai*. The advantage of Ilumina is that it gives results in only a few hours irrespective of the genome size and has also proven to have accurate base calling with only 0.1% deviation. Moreover, it provides unbiased and uniform coverage across difficult regions like repetitive or GC-rich regions**.**

Single molecule real time sequencing: Single molecule real time sequencing (SMRT) is a single molecule DNA sequencing method by using zero-mode waveguide (ZMW). This method is a rapid approach in the detection of microorganisms in dental caries. In a study done by Wang et al[36], characterization of the oral microbiota in childhood caries based on single molecule real time sequencing was done. Purified amplicons were sequenced using SMRT technology on a PacBio RS II sequencer.

This approach is useful for estimating the details of the oral microbiota at a more in-depth level due to the production of 702,304 high quality sequences as seen in the study. From this, various species like *Streptococcus spp., Neisseria spp., Lactobacillus spp., Proteobacteria spp.* and *Veillonella spp.*, were found predominantly in caries affected children.

Capillary sequencing: Capillary sequencing is a type of Sanger sequencing using a capillary tube across an electric field without the use of gels. This technique was used in a study done by Dame-Teixeira et al[9] to check the presence of archaea in dental caries biofilm. By this method, they were able to detect presence of archaea of phylum Thaumarchaeota in low abundance, in addition to the commonly reported methanogens. Hence by such molecular methods, low abundant archaea can also be detected in dental caries.

Denaturing Gradient Gel Electrophoresis: Denaturing Gradient Gel Electrophoresis (DGGE) is a type of gel electrophoresis in which a double stranded DNA strand migrates into a gradient of linearly increasing denaturing conditions**.** In a study carried out by Vieira et al[35], DGGE was used to find the protein profile present in saliva. Histatin was not detected in caries free children and had one less band in gel when compared to the caries affected children and therefore can be used as a biomarker in dental caries.

In another study, DGGE was used to study microbial diversity in saliva and plaque samples from caries free and caries affected children wherein DGGE formed different bands, which distinguished samples on the basis intensity of band and other visual features[28]. This method can be further used as a pattern recognition tool for the detection of specific types of bacteria in the sample obtained from dental caries.

ELISA: Enzyme-Linked Immunosorbent Assay (ELISA) is an assay technique used commonly for the detection of antibodies and other proteins present in a given sample. This technique detects biomarkers associated with dental caries. In a study, ELISA was used to evaluate the level of salivary sHLA-G in children aged 3-5 years with or without dental caries[6].

From this study we find that sHLA-G gene plays a significant role as a determining factor of dental caries in different individuals, thus providing an early assessment of dental caries in affected patients.

In a study done by Bakir and Ali[4], ELISA was used to compare the microorganisms present in a throat infection Pharyngotonsillitis. These microorganisms usually are seen to be from the *Streptococcus* species, *Neisseria* species and *Staphylococcus* species.

The detection of biomarkers plays a vital role in the diagnosis of dental caries and this is easily carried out by molecular techniques. The various biomarkers of dental caries that are detected by molecular techniques include microorganisms and proteins (Table 2).

With this information on different biomarkers, the early diagnosis of dental caries can be done. By comparing various studies, it was seen that microorganisms present in the oral cavity are different for different geographical inhabitants.

In Tunisian people, *S.oralis* and *S.salivaris;* in Chinese ethnic groups *Veillonella, Leptotrichia* and *Bacteroides* species; and in Indians *Scardoviawiggsiae* species were predominantly present.

Table 2
Biomarkers detected by various molecular techniques in dental caries

Biomarker (Microorganism/ Protein)	Molecular method used for detection of biomarker
Streptococcus species	(i) PCR[5,18] (ii) qPCR[17,22,24] (iii) AP-PCR[30] (iv) Pyrosequencing[8,32] (v) Illumina[15,39] (vi) SMRT[36]
Lactobacillus species	(i) qPCR[22,24] (ii) SMRT[36] (iii) PCR[5,18,26] (iv) T-RFLP[31]
Neisseria species	(i) SMRT[36] (ii) Pyrosequencing[8] (iii) Illumina[15]
Proteobacteria species	(i) Illumina[15] (ii) T- RFLP[31] (iii) SMRT[36]
S oralis, S.salivarius(Tunisian group)	PCR[18]
*Veillonella*species (Chinese group)	(i) Illumina[39] (ii) SMRT[36]
Scardoviawiggsiae(Indian group)	PCR[23]
Bacteroides species	(i) Illumina[39] (ii) T-RFLP[31]
Thaumarchaeota (not in abundance)	Capillary sequencing[9]
Histatin	DGGE[35]
sHLA-G	ELISA[6]

Hence it is important to research the predominant dental caries causing microorganisms in people of different ethnicity. Moreover, it was seen that the number of bacterial populations increased with age, hence this will aid in the appropriate doses of treatment required according to the age of the patients.

From the review, it can be concluded that detection of various biomarkers of dental caries by qPCR and ELISA is one of the most efficient molecular techniques. The main advantage of qPCR is that it gives the results in real time. Additionally, it is an inexpensive method. It is also extremely convenient for high throughput detection and quantification of the target sequences. Although there is not much research done on the use of ELISA for the diagnosis of dental caries, it has a plethora of advantages when compared to other techniques.

It is cost effective, gives instantaneous results, has high specificity and sensitivity and easy to perform. Currently, ELISA is used for the detection of biofilms producers in throat infections. In the same way, ELISA can also be used to detect the microorganisms present in dental caries. Therefore, more research can be done for the application of ELISA in the detection of microorganisms in dental caries in the future.

Presently, molecular techniques are still not used for the diagnosis of dental caries because of a few limitations. The use of standard genomic purification techniques, using highly pure sample, high-quality materials and optimisation of tests plays a significant role in obtaining fast and accurate results from all the molecular techniques. It is however very advantageous when compared to the conventional methods of dental caries diagnosis.

Conclusion

Dental caries is a disease that is better prevented than treated, as it is an infectious disease. Molecular techniques play a major role in detecting the above-mentioned markers and risk factors before caries can progress to the cavity stage. It will also help in understanding if the microorganisms are resistant to the conventionally used antibiotics. It will give an insight to the progression of the infection based on both the qualitative and quantitative analysis of bacterial strains. Identifying the risk factors of caries will help in accurate diagnosis as well as help in the further treatment by helping in selecting the appropriate antibiotics.

Conventional methods of detecting the microflora in dental caries sample are by cultivating it in the laboratory. However, this is extremely difficult and by this method it is not possible to culture some of the anaerobes. The molecular

techniques that can be used in the detection of caries causing bacteria including anaerobes are: PCR and its types, high throughput sequencing techniques like pyrosequencing, Illumina and so on. ELISA and DGGE can be used for detection of certain proteins associated with caries. T-RFLP and RAPD play an extremely important role in profiling the bacterial populations in dental caries. In addition, molecular techniques have high sensitivity and can detect the organisms, which are present in low numbers.

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