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RESEARCH ARTICLE

APPLICABILITY OF PCR TECHNIQUE IN DENTISTRY AND RESEARCH

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ABSTRACT

Polymerase Chain Reaction (PCR) is a tool for studying the molecular constitution comprising of DNA and RNA. It utilizes the science involving the complementary base pairing and enzymatic reactions under optimal conditions of temperature, pH and ion concentration. This reaction can be used to study microbial and human genome for the purpose of pathogen identification and genetic defects in form of mutations. This article encompasses the various areas in which PCR can be utilized as a diagnostic aid and research tool.

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INTRODUCTION

Several techniques have been employed in dental sciences to diagnose and understand the pathogenesis of infectious agents which lead to maxillofacial diseases/disorders. These techniques include- microbiological culture, microscopic analysis, immunofluorescence-based and enzyme-linked immunosorbent assays. Polymerase chain reaction (PCR) is a highly specific and sensitive technique by which minute quantities of specific DNA and/or RNA segments are enzymatically amplified to detect various genetic and microbial nucleic acids in variety of disorders such as hemoglobinopathies like sickle cell anemia, β- and α-thalessemia, Duchenne muscle dystrophy, cystic fibrosis, hemophilia and B. (Dos Santos *et al*., 2004; Boehm, 1989) Clinical samples which can be used for PCR analysis include blood, sputum, hair, nails and sputum. Saliva is an important source for diagnostic testing in oral as well as systemic conditions. (Dos Santos *et al*., 2004)

The Polymerase Chain Reaction (PCR) Technique

Polymerase chain reaction technique was developed by Kary Mullis in the 1980s, who received the Nobel Prize for the same in 1994. (Valones *et al*., 2009) This technique uses multiple cycles comprising of denaturation, annealing and elongation to amplify specific DNA sequences. It is an exponential process wherein the amplified products from previous reaction cycle act as templates for next amplification cycle. Thus, after 20 to 40 cycles, abundant amplified product gets generated, which can be visualized on ethidium bromide stained gel. Various components in PCR include- a template, forward and reverse primers, reaction buffer, magnesium ($MgCl_2$), dNTP mix and a thermostable DNA polymerase enzyme, Taq polymerase. (Dos Santos *et al*., 2004; Boehm, 1989; Valones *et al*., 2009) The Template used can be purified genomic or plasmid DNA or complementary DNA obtained by converting RNA by means of 'reverse transcriptase' enzyme. The forward and reverse primers determine the length and sequence of the genetic sequence of the amplified product. Magnesium ion is required for regulating the enzymatic activity, annealing, melting temperature, dimer formation and primer-dimer fidelity. (Dos Santos *et al*., 2004; Boehm, 1989; Valones *et al*., 2009) The DNA region to be annealed is specifically identified by oligonucleotide primer sequence, which after annealing to the complementary sequence limits the DNA fragment amplification. (Dos Santos *et al*., 2004; Boehm, 1989; Valones *et al*., 2009) The polymerase enzyme copies the DNA strand from 3' to 5' direction while synthesizing a new DNA strand in 5' to 3' direction (extension). This extension process is stopped by elevating the temperature ($94^{O}C$) resulting in separation of DNA strands. Cooling of the reaction mixture to $37^{O}C$-$60^{O}C$ results in annealing of the primer with DNA. Increasing the temperature to $72^{O}C$ again, causes the Taq polymerase enzyme to copy the required DNA template. On addition of two primers

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complementary to each strand, an exponential amplification of both DNA sequences is obtained. (Dos Santos *et al*., 2004; Boehm, 1989; Valones *et al*., 2009) After the amplification process, the PCR products are size-fractioned using agarose or ethidium bromide stained polyacrylamide gel using electrophoresis. (Dos Santos *et al*., 2004; Boehm, 1989; Valones *et al*., 2009)

Applications of PCR

Polymerase chain reaction was first used by Saiti in 1985 for detection of sickle cell mutation. (Dos Santos *et al*., 2004) This technique can be utilized in Dentistry for studying microbial and other underlying factors in causation of dental caries, periodontal diseases, endodontic infections, oral potentially malignant disorders, oral cancer and maxillofacial diseases of hard and soft tissue origin.

Applicability of PCR in periodontal conditions

Parra and Slots (1996) provided evidence of viruses within the gingival crevicular fluids in advanced periodontal conditions. (Parra and Slots, 1996) Saygun (2002) confirmed human cytomegalovirus in crevicular fluid samples of periodontitis patients. They suggested a strong association between human cytomegalovirus (HCMV) and Epstein Barr Virus-1 (EBV-1) in subgingival areas. (Saygun *et al*., 2002) Gartlet (2003) examined the presence of chemokines, their receptors and cytokines in periodontal diseases. They found higher expression of macrophage inflammatory protein-1 alpha and interferon-gamma inducible protein and their receptor, CCR5 and CXCR3, respectively. Also, elevated expression of monocyte chemoattractant protein-1, and its receptor, CCR4 and IL-10 were observed. (Gartlet *et al*., 2003) Shelburne (2002) used the quantitative real time reverse transcription PCR (RT-PCR) for detection of Porphyromonas gingivalis. They observed that PCR was more effective method as compared to culturing methods to detect this organism. Sakamoto (2002) detected Treponema socranskii in higher quantities in subgingival plaque samples as compared to salivary samples. (Sakamoto *et al*., 1999) Okada, Hyashi and Nagasaka (2001) reported that Campylobacter rectus was most common pathogen found in children. (Okada *et al*., 2001) Findings by Okada and Sakamoto suggest the establishment of T. socranskii and C. rectus in earlier years of life. Takeuchi (2001) detected a higher prevalence of T. socranskii in periodontitis. (Takeuchi *et al*., 2001) Lyons, Griffen and Leys (2000) determined the quantity of Porphyromonas gingivalis in plaque samples using the TaqMan Real-time PCR using culturing techniques. (Lyons *et al*., 2000) Thus, species more commonly found associated with chronic periodontitis include- T. denticola, Eubacterium saphenum, Porphyromonas endodontalis, P. gingivalis, T. forsythensis, Filifactor alocis, Prevotella denticola, Cryptobacterium curtum, Treponema medium, T. socranski and Actinomyces naeslundii. (Saygun *et al*., 2002; Gartlet *et al*., 2003; Shelburne *et al*., 2002; Sakamoto *et al*., 1999; Okada *et al*., 2001; Takeuchi *et al*., 2001; Lyons *et al*., 2000)

Applicability of PCR in dental cariology

Dental caries is a polymicrobial disease with multifactorial etiologies comprising of genetic, environmental, microbial and behavioral factors. (Acedo *et al*., 2013) Biofilms are dynamic communities comprised of exopolysaccharides, nucleic acids and proteins covering tooth and mucosal surfaces. Additionally, cariogenic biofilm contains extracellular DNA and lipoteichoic acids. Mutans streptococcus is the main matrix producer in a cariogenic biofilm and rapidly modulates its production on availability of dietary sugars. MS-released glucosyl transferases (Gtfs) are constituents of dental pellicle which help in glucan synthesis. The glucans aid in MS colonization. The dietary sugars enhance the cDNA release into the exopolysaccharide matrix due to upregulation of lytTS and CepA during biofilm synthesis. LysTS activates the ligAB gene expression which controls autolysis and biofilm formation. gtfB expression is upregulated in biofilm formation in presence of dietary sugars. cDNA along with glucans provide enhanced binding sites for MS colonization. Thus, cDNA along with GtfB-derived glucans facilitates cariogenic bacteria accumulation in a biofilm matrix. (Klein *et al*., 2015) Saarela (1996) used arbitrarily primed PCR (AP-PCR) for differentiation between Mutans Streptococcus (MS) and S. sobrinus species. He selected two randomly selected primers, OPA-05 and OPA-13. The OPS-05 primer distinguished two patterns among the MS isolates while one pattern in S. sobrinus. The OPA-13 primer detected one main AP-PCR pattern. Thus, the AP-PCR typing revealed 33 different types among the 81 isolates, indicating a good discriminative ability in differentiating between MS clones. (Saarela *et al*., 1996) Igarashi, Yamamoto, Goto (1996) used the dex A probe and dextranase electrophoretic profiles for identification of MS species using PCR. Same investigators in 2000, devised oligonucleotide probes based on DNA fragments (1610 bp) on dexA gene. The PCR products obtained were specific for S. sobrinus while negative reaction was obtained for other members of MS group i.e., Mutans streptococcus, S. downei, S, cricetus, S. rattus, S. macacae and S. ferus. These results indicated that the prevalence of S. sobrinus is higher in oral cavity (80%) as compared to other MS subtypes. (Igarashi *et al*., 1996) Okada (2002) analyzed dental plaque samples from 77 Japanese school children using PCR technique and observed a higher incidence of dental caries was found in children harboring both MS and S. sobrinus as compared to MS alone. (Okada *et al*., 2002) Sanger sequencing of 16S rRNA genes have shown that Lactobacillus species are dominant species in rampant caries and their levels rise significantly as caries progresses. This organism is undetectable in healthy subjects' oral cavity. (Gross *et al*., 2010) Richard (2002) described a specific primer and probe to discriminate Lactobacillus rhamnosus from other Lactobacilli species. This helped in elucidating the progression of carious process.[18] Several studies have provided evidence of genetic heterogeneity of MS strains. Alaluusua has suggested greater ribotype diversity of MS in children with high sucrose consumption. (Alaluusua *et al*., 1996) Similarly, Napimoga reported that caries-active subjects have higher numbers of genotypes than caries-free subjects. (Napimoga *et al*., 2004) Preralisi (2010) using the AP-PCR fingerprinting employed primers specifically for glucosyltransferase B (gtf B) gene and showed the correlation between MS genotypic diversity and caries activity. (Kreulen *et al*., 1997) Li and Tanner using the quantitative PCR technique detected multiple micro-organisms associated with severe early childhood caries. These included-

oral Bifidobacterium species, Scardovia wiggsiae, S. mutans and S. sobrinus. (Li and Tanner, 2015) Amelogenesis is a genetically regulated process responsible for enamel formation, regulation of shape/size of a tooth as well as caries susceptibility. These genetic malformations may be transmitted from parents to off-springs. For example, mutation in KLK4 gene results in an enamel of quality which does not respond to etching and bonding procedures. (Simmer, 2001)

Applicability of PCR in understanding endodontic pathologies: Machado de Olivera (2000) evaluated the existence of Porphyromonas endodontalis in both symptomatic and asymptomatic endodontic infections using 16S rRNA based PCR techniques. He observed that the organism can be found in both symptomatic as well as asymptomatic endodontic infections. (Machado de Oliveira *et al*., 2000) Fungal organisms also have been reportedly associated with infected root canal systems. Baumgartner, Watts, Xia (2000) reported the presence of Candida albicans in endodontic samples. (Baumgartner *et al*., 2000) 16S rRNA is a RNA type associated with protein synthesis. It is localized in the major groove in the upper portion of rRNA. This rRNA segment consists of highly conserved regions among various biological species. (Geevarghese *et al*., 2012) 16S rRNA gene sequencing is a more accurate and objective method of microbial identification and taxonomic definability among bacteria. (Petti *et al*., 2005) Using the 16S rRNA-directed PCR technique, Siquieria (2001) assessed the role of black pigmented anaerobic rod, Treponema denticola in pathogenesis of periradicular lesions of endodontic origin. (Siqueira Junior *et al*., 2001b) However, Al-Ahmad *et al* (2006) demonstrated that employing 16S rRNA gene for PCR analysis gives false-positive results for Mutans streptococcus. (Al-Ahmad *et al*., 2006) A. actinomycetemcomitans and P. gingivalis are pathogens found in endodontic infections and are detectable by PCR method than by traditional culture techniques. (Pereira *et al*., 2010) Root canal microflora in failed endodontically treated teeth is different from microflora isolated from untreated teeth. Necrotic pulpal teeth are polymicrobially infected with a predominance of anaerobic microorganisms. Endodontically treated teeth with apical periodontitis contain Enterococcal species, Streptococci, Lactobacilli, facultative and anaerobic bacteria. E. faecalis is isolated commonly from endodontically treated root canals with 12-90% prevalence. Candida albicans is also a common pathogen in apical lesions. (Dumani *et al*., 2012)

Applicability of PCR in understanding oral cancer pathogenesis

The International Agency for Research on Cancer (IARC) published a report on cancer prevalence in 2014. As per this report, there are 14.1 million new cancer cases, 8.2 million deaths due to cancer and 32.6 million people leading their lives with any form of cancer living around the globe. (Banerjee *et al*., 2015) Oral cancer diagnosis and its prognostication can be studied using the PCR technique. Chromosomal aberrations such as deletions and translocations are responsible for development of carcinogenicity. TaqMan Real-time PCR technique is a 5' fluorogenic, nuclease-based, real-time polymerase chain reaction used for quantitative microbial evaluation. (Banerjee *et al*., 2015) The TaqMan probe is a fluorescent DNA probe based on 5' to 3' exonuclease activity of Taq polymerase. This oligonucleotide probe has a reporter fluorescent dye attached to the 5' end and a quencher dye attached to the 3' end. The probe hybridizes to the target gene. During the amplification process, the quencher dye is cleaved by the 5' nuclease activity of Taq polymerase, which results in reporter accumulation dye accumulation. Release of the fluorescent dye allows DNA quantification and rapid detection. (Suzuki *et al*., 2005) Detection of 22q11.2 deletion using the TaqMan PCR-based gene analysis has shown 99.7% statistical correlation with fluorescent in situ hybridization based detection. (Kariyazono *et al*., 2001) Role of telomerase enzyme in oral carcinogenesis has been studied by investigating its three major subunits- hTR, hTP1 and gTERT expressions. Lee (2001) reported that hTERT expression is useful biomarker for early squamous cell carcinoma detection and distinguishes healthy tissues from carcinomatous cells. (Lee *et al*., 2001) Pathogenic microbes present within oral cavity can contribute to carcinogenesis. (Banerjee *et al*., 2015) Salivary micro-organisms can also be used as diagnostic markers for oral cancers. Metagenomic investigation of microbiome within oral cavity using can be done using 454 pyrosequencing and next generation pyrosequencing. (Banerjee *et al*., 2015) Saliva obtained from oral squamous cell carcinoma patients has been found to comprise of Firnicutes, Bacteroides, unclassified bacteria and Mycoplasma species using V4-V5 16S rRNA-based 454 parallel DNA sequencing. (Banerjee *et al*., 2015; Chang and Parsonnet, 2010)

Applicability of PCR in Forensic Dentistry

PCR can be used in forensics to identify molecular markers which can include a gene, a restricted DNA site or genetic variations on a particular locus. Some of these markers are specific to some populations and are termed as 'ancestor indicators'. (Da Silva *et al*., 2006) Gender determination can be done by utilizing the tooth enamel for detection of Amelogenin gene on X chromosome. This particular gene can be amplified by up to 90% and is one of the main biomarkers for sex determination. (Da Silva *et al*., 2006) Genetic relations from post-mortem samples can also be detected using the PCR technique, specially in mass disasters, paternity suits and victim identification. (Da Silva *et al*., 2006)

Detection of blood-borne pathogens

NASBA (Nucleic Acid Sequence Based Amplification), a RNA-dependent nucleic acid sequence-based amplification and molecular beacon can be used to detect bacteria and fungi utilizing 16S rRNA ans 28S rRNA targets, respectively. NASBA is an isothermal process involving selective RNA amplification even in presence of genomic DNA. This reaction results in 10^{12} amplified copies of the template in a 30 minute assay. This reaction gives comparable results with nested PCR technique. This technique detects both live and dead cells. The amplified single-stranded RNA can be detected real time by molecular beacon probes which are small, self-reporting, single-stranded nucleic acid 'hairpin probes' which fluoresce on target binding. This real-time technique is used for detecting RNA viruses like Human Immunodeficiency Virus, pathogens

like- Legionella species, Vibrio cholera, Candida species and Aspergillus species. (Zhao *et al*., 2009) H. pylori was first reported in 1983 within the human antral mucosa. The IARC has designated it as a group I carcinogen due to its carcinogenic potential. (Sayed *et al*., 2014) The Polymerase Chain Reaction has been used for H. pylori detection from recurrent aphthous lesional swabs. Culture studies have shown negative results for H. pylori infection within the oral cavity, however, it has been suggested that this organism might manifest itself on co-infective conditions with Herpes Simplex Virus (HSV) infection. (Afghari *et al*., 2011) On the other hand, Al-Asqah (2009) observed that 60% patients affected with periodontitis harbored gastric H. pylori as compared to subjects with healthy periodontal condition. (Al Asqah *et al*., 2009) A significant correlation has been observed between CD8+ T cell activation levels and total bacterial 16S RNA gene levels in HIV-infected subjects. Li *et al*. (2014) demonstrated positive association between CD8+ T cell counts and S. mutans colonization levels in HIV-infected individuals. (Li *et al*., 2014)

Conclusion

Polymerase chain reaction is an enzyme-based technique used for synthesizing million copies of a desired DNA or RNA sequence. It comprises a series of temperature-dependent steps. This technique can be used for a variety of molecular analysis for genetic screening, microbial and forensic studies. It is extremely useful for understanding the etiopathogenesis, prediction of disease risk and predictability of various diseases such as oral cancer, developmental disorders, dental caries, periodontal pathosis and so forth. Continuous research in this field of research is helping the physicians, dentists and researchers in understanding the underlying genetic and microbial contributors so as to design and implement effective therapeutic means for treatment and disease management.

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