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Review Article

PCR- Revolutionizing the Diagnostic Approach and Applications in Dentistry: A Review

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ABSTRACT

The polymerase chain reaction (PCR) is a scientific technique in molecular biology to amplify a single or a few copies of a piece of DNA across several orders of magnitude, generating thousands to millions of copies of a particular DNA sequence. PCR is now a common and often indispensable technique used in medical and biological research labs for a variety of applications. Three major steps involved in the PCR technique: denaturation, annealing, and extension. Qualitative PCR can be used to detect not only human genes but also genes of bacteria and viruses, thus useful in the investigation and diagnosis of a growing number of diseases. Various types of PCR are applicable in dental diagnostic procedures. Articles from the esteemed journals are referred to review principles, applications, advantages and disadvantages of PCR in laboratory diagnosis of disease in this article.

Key words: Polymerase Chain Reaction, Molecular techniques, DNA amplification.

INTRODUCTION

In acute care settings, it is always critical to provide timely, rapid and accurate detection of pathogens. To give an example of importance of rapid diagnosis for early decision making, the recent anthrax related bioterrorist event and outbreak of severe acute respiratory syndrome (SARS) were the conditions in which prompt infection control, provision of timely treatment and vaccination would have scored the most life and death consequences of patients.

Would not a rapid reliable diagnostic assay, allowing accurate identification of infected patients & informed early therapeutic intervention be invaluable for emergency critical care?

The traditional culture methods have inherent advantages, but have shortcoming regarding bacterial vitality preservation, lower limit of microbial detection. While microbiological tests such as dark field microscopy are not able to detect non-motile periodontal pathogen. Immunodiagnostic methods and enzymatic assays can produce false positive results and cross-reactions.

So to overcome these, a new molecular method known as "diagnostic tool for new millennium" was discovered. This was found to be most promising in its assessments and applications. [1]

Historical perspective of PCR (Polymerase Chain Reaction)

Era of human genetic began when Johnan Friedrich Miescher first isolated DNA in 1869. [2] In 1975 Southern blotting technology was used for genetic analysis. Later in 1980 Ray White developed RFLP (Restriction Fragment Length Polymorphism) technique in adaptation to it. [3] The revolutionary 'PCR' technique was introduced by the American biochemist, Kary Mullis in 1983, for which he received

Nobel Prize in 1993. [4] Though PCR was utilized for identification of pathogen in various fields, in Dentistry DNA identification from human tooth pulp was done in 1992. [5] From then till now PCR has been used for genome mapping of entire bacterial spectrum including rapid semi-quantitative determination of 10 periodontal pathogen through DNA microarray analysis. [6]

Basic Concepts of PCR

It is a technique to amplify a single or few copies of a piece of DNA across several orders of magnitude, generating thousands to million copies of a particular DNA sequence.

As the name implies, it is a chain reaction: One DNA molecule is used to produce two, four, eight copies and so forth. This continuous doubling procedure is accomplished by specific proteins known as polymerases, enzymes that are able to string together individual DNA building blocks to form long molecular strands. Polymerases require supply of DNA building blocks, i.e. nucleotides consisting of four bases adenine (A), Thymine (T), cytosine (C) and guanine (G). Now these require a small fragment of DNA, known as primer, to which nucleotides get attached along with longer DNA molecule to serve as a template for constructing a new strand. [7]

1. Thermo stable polymerases / Tag

Brock and Freeze [8] isolated a new species of thermophilic bacterium. Thermus equaticus from which 'Taq polymerases' was isolated in 1976. [9] Taq polymerases can sustain its activity at high temperature. It copies the region to be amplified. Usually 1-1.5 U is required in 50 µl of reaction mix. If inhibitors are present (ex. Low purity of DNA template) 2-3 U may be required to obtain better field of amplified products.

2. Template DNA

Contains the region of DNA fragment to be amplified. DNA should be pure without trace amounts of phenol, EDTA or proteinase k etc, as it strongly inhibits Tag polymerases action. 0.01-1ng of DNA template is required in 50µl of reaction mix.

3. Primers

Primer designing is the most important consideration for amplification of target site within a region of the genome. Forward and reverse primers determine the beginning and end of the region to be amplified. Size of the primer is determined by the genome size. [10]

4. dNTPs

The concentration of each dNTP is about 4µl in 50ul reaction mix.

Steps in PCR Cycle (Cycle)

- Initial denaturation occurs where two strands of DNA get separated at 90-95°c for 3-5 minutes.
- It is followed by annealing step at 50-65°c for 30-55sec allowing binding of the complimentary forward and reverse primers to the 3' flaking regions.
- Extension step starts by the action of dNTPs over the new strands, it occurs at 72°c for 30-35sec.
- After the last cycle i.e. 30-35 cycles, at 72°c the samples are incubated for 5-15 minutes to fill in the protruding ends of newly synthesized PCR products.
- Storage of PCR product is done at 4°c till further assay.

Genetic variation exists due to insertion or deletion of nucleotides. Depending upon whether single nucleotide or multiple nucleotide sequence alteration there could be Single Nucleotide Polymorphism /Multiple Nucleotide Polymorphism (SNP/MNP). Where MNPs can again be of two types- Multiple Nucleotide Polymorphism (MNPs) and Triple Nucleotide Polymorphism (TNPs). [11] PCR- Restriction Fragment Length Polymorphism (RFLP) is an advanced method of genotyping, wherein after amplification of DNA fragment the variable region is processed with appropriate restriction enzyme. Size of fragments depends upon the position of restriction enzyme recognized site. The obtained allele

fragment is identified by Electrophoresis. [12] Terminal (T)-RFLP enables identification of species diversity. (REF) In this pairs of primer at both ends are labeled with a fluorescent dye. Advantages of T-RFLP over RFLP are extensive analysis of multiple species through primer use especially for profiling multiple microbial communities. [13]

Representation of SNPs according to Rosenfeld et al [14]

5'-3'CATAGATATCACGAGGAACTCCGATT
SNP G/C
5'-3'CATAGATATCACGACGAACTCCGATT

5'-3'CATAGATATCACGAGGAACTCCGATT
DNP A/T, G/C
5'-3'CATAGATATCACGTCGAACTCCGATT

5'-3'CATAGATATCACGAGGAACTCCGATT
TNP A/T, G/C, G/A
5'-3' CATAGATATCACGTCAAACTCCGATT

Types of PCR

Variants of Standard PCR
Reverse Transcription –PCR (RT-PCR)
Real Time- PCR or Quantitative PCR (qPCR)
RT- PCR/qPCR combined

Certain modification in the basic PCR tech led to the development of variants in PCR.

1. Allele Specific PCR

It requires prior knowledge of the target DNA sequence such as difference between alleles and utilizes the primer with 3' mismatch ends encompassing the single nucleotide variations. [15] Generally two PCR reactions are needed with 2 allele specific primers for election of both alleles at SNP. [16]

2. Asymmetric PCR

This tech is used specifically amplify only one strand of the target DNA molecule using unequal primer concentrations. [17]

3. Inverse PCR

It allows amplification of only one known sequence. It requires a sequence of restriction digestions and ligation resulting in the formation of a looped DNA fragment, which is further primed from a section of known DNA sequence for PCR. [18]

4. Colony PCR

This technique is commonly used in bacterial genomic studies. It has several advantages over traditional method, such as it can quickly screen the plasmid inserts, its size and orientation in the vector, helps in generating sufficient amount of desired PCR product for sequencing and it avoids sequencing of false positive clones.

5. Degenerate PCR

This tech employs degenerate primers to amplify unknown sequences of DNA, related to known DNA sequence. It allows for identification of new members of gene family or orthologus genes from different organisms. [19]

6. Hot Star PCR

To avoid non specific amplification at lower temperature in this tech Taq polymerase is added after DNA melting temperature. [20]

7. Mini Primer PCR

In this PCR, Taq polymerase and 10 nucleotides long 'miniprimers' are used. This is done since conventional Taq polymerases have less efficiency of DNA synthesis as they require longer primers (20-30 nucleotides). Miniprimers can be used for identification of conserved DNA sequences such as 16 S rRNA (eukargotic 18 S rRNA) which are not possible with standard primers. [21]

8. Multiplex PCR

Advantage of this variant is, it targets multiple genes at once in single test run, incorporating multiple primer sets within a single PCR mixture to produce amplicons of varying sizes, specific for different sequences of genes.

This tech has been applied successfully to analyze deletions, polymorphisms, mutations, SNPs and microsatellites. [22]

9. Nested PCR

It involves 2 sets of primers, in two successive runs of PCR reaction to minimize the amplification of non specific and spurious PCR products. As the second set of primers binds to a secondary target within a sequence amplified by first set of

primers, thus avoiding primer binding at unwanted sites similar to the target DNA. [23]

10. Touchdown PCR

This tech allows annealing of specific primers at high temperature which is least permissive for now specific binding, by using early steps of PCR cycles at high temp and with subsequent cycle, the annealing temp is decreased in increments. [24]

11. Reverse Transcription – PCR (RT-PCR)

The retroviral reverse transcriptase, (AMV) Avian Myeloblastosis Virus and (MMLV) Moloney murine leukemia virus are the innovation of this technique which are most characterized in the field of molecular biology. RT-PCR enables quantitative detection of levels of RNA expression by creating complimentary DNA (cDNA) from RNA with the help of reverse transcriptase, followed by its further amplification by standard PCR. [24]

14. Real Time- PCR or Quantitative PCR (qPCR)

This is advantageous over standard PCR, since qPCR combines the amplification and detection into a single step by eliminating the need for any post amplification processing of samples. The intensity of fluorescent dye, such as SYBR Green I correlates the amount of DNA amplification at each cycle of PCR. Other advantages being its sensitivity speed of analysis, precision in measurement and real time detection of reaction progress. [25]

15. RT-PCR / qPCR Combined

RT-PCR tech is used through conversion of RNA template to cDNA for qualitative detection of RNA expression, whereas both RT-PCR and qPCR are merged for quantitative detection of RNA expression called as RT-qPCR. [26]

Principle and applications of PCR

1. In mycology and parasitology

Early identification of disease causing agents is fundamental into all aspects of fungal epidemiology and diagnosis. Due to advances in molecular diagnostic techniques it has become easy diagnosing fungi through PCR since it is highly specific, fast, detects trace amounts of fungal DNA from samples thereby implementing early disease control. [27]

Detection of pneumonia causing fungus- Pneumocystis jirovecii is restricted to microscopy of specimens in respiratory tract. It generally involves use of stains, but it is more expensive and requires specialized equipments. PCR is however more sensitive, can therefore be of considerable use. [28]

Species Aspergillus found in patients with neutropenia is notoriously difficult to diagnose due to poor sensitivity of culture methods and due to low platelet counts it is rarely found in histopathological specimens. RT-PCR thus can be used even to quantify the pathogens. [29]

Many parasites are not cultivable in laboratory. Microscopy is supportive to the diagnosis of malaria but PCR is more sensitive technique in comparison to it. [30]

2. In Microbiology

Conventional PCR assays have been developed for Bacillus anthracis, the Anthrax agent [31] and Variola Major [32] but clinical validation of these assays is limited due to the unavailability of human specimens.

3. In Bacteriology

Anaerobic bacteria which are involved in broad range of infections are poorly characterized due to the inadequacy of conventional anerobic bacteriological methods and phenotype of tests. [33] Recently the growing trust in genotyping for microbial characterization is due to the powerful means of these methods in clarifying taxonomic positions of these pathogens. RT-PCR has been used widely which provides qualitative data with greater sensitivity. Molecular detection methods have been beneficial for identification of Mycobacterium tuberculosis, Chlamydia Trachomatis, Neisseria gonorrhoeae and Bordetella pertussis which are of public health concern. Not only this, but PCR has a

wide range of detection and sensitivity for sexually transmitted and genital bacteria. [34]

4. In virology

With implementation of molecular techniques, diagnosis of many viruses and monitoring antiviral therapy has shown tremendous progress, especially for HIV-1 (Human Immunodeficiency Virus -1) [35] HBV (Hepatitis B virus) and HCV (Human Cytomegalovirus) Amplification assays have also been developed on HSV-1 and HSV-2 (Herpes simplex Virus 1 and 2). [35] Where the routine viral culture is not available, molecular methods are found to be advantageous with implementation of anatomic extraction and detection of virus. [36] At one hand where culture process are of low sensitivity and slow development of viruses in artificial media hampers their identification requiring more time, PCR technology on the other hand facilitates early detection of viral variants and syndromes and also contributes to epidemiological studies by quantifying nucleic acids and discriminating multiple viral genotypes in single reaction. [37]

Principles and Applications in Dentistry

With the evolution of diagnostic aids and techniques to better understand and diagnose infectious agents leading to maxillofacial infections in dentistry, a number of methods have been employed. Which includes microscopy, cultures, immunufluoroscenes assays, DNA probes and more recently PCR. Many studies have been carried out to find an association between systemic diseases and oral conditions for which blood, serum, saliva or GCF samples are analyzed. Of them saliva has been considered to be having potential source of genetic material since it contains biologically active proteins and exogenous substances. [38] The varied applications of molecular techniques in dentistry are thus enlisted below.

I. In Periodontology

In identification of microbial pathogens

The precise and sensitive methods such as Q-PCR or Real Time PCR with species specific primers provide accurate quantification of individual microbial species and total bacterial count is plaque samples thus accounting as a useful tool for studies on etiology of periodontal diseases.

GCF has been considered an important marker for identification of various forms of periodontal diseases. Para Slotts [39] through GCF determined the prevalence of human cytomegalovirus (HCMV), Epstein Bar Virus Type 1&2, HSV, HPV and HIV prevalence with various forms of periodontal disease. Ssygun et al [40] confirmed the frequent presence of HCMV in GCF samples of Chronic periodontitis lesions and suggested a strong correlation between HCMV & RBV-1 in subgingival areas. Number of studies had been performed by various authors using subgingival plaque samples for detection of putative pathogens such as Porphyromonas gingivalis (Pg), Actinobacillus actinomycetemcomitans (Aa), Tannerella forsythus (Tf), Prevotella intermedia (Pi), Prevotella nigricans, Campylobacter rectus (Cr), Capnocytophaga sputigena, Capnocytophaga gingivalis and Parvimonas micra (Pm) etc through PCR & RT-PCR. [41]

Tekenori Nozari [42] compared effectiveness of PCR and Conventional Indirect Immunofluorescence Assay (IIF). Wherefrom the subgingival plaque samples containing fewer than 100 P gingivalis cells were reproducibly detected by PCR-Hybridization assay and it was found to be 100 times more sensitive than IIF. Association of Treponema socranskii with periodontal disease through subgingival plaque sample was identified by Sakamoto et al [43,44] by application of PCR. In year 2011 he compared conventional PCR, RT-PCR and culture method in the detection and quantification of Aa, B forsythus, Pg, T socranskii and T denticola in both saliva and subgingival plaque samples. Use of RT-PCR found to optimally simplify the process and determined periodontopathogenic

bacterial amount within an hour. [45] PCR technique has also been used to study the association of systemic disease such as Kidney disease, Coronary heart disease, Osteoporosis, Pregnancy complications and respiratory disease with periodontitis by evaluating the periodontal pathogen levels in various tissue samples such as plaque, thrombi, carotid endarterectomy, placenta, maxillary sinus wash samples etc. [46]

PCR as a diagnostic tool in peri-implantitis

This technique is efficient in detecting bacterial infections causing peri-implantitis before implant placement to prevent further risk. Various pathogens such as Aa, Pg, Td, Tf have been found in the foci of peri implantitis. Also some uncultured phyla such as chloroflexi, synergisters, Peptostreptococcus stomatis and Solobacterium moorei associated with peri implantitis including fungal species such as Candida were identified using PCR. [47]

Site suggestive of peri implantitis are most often seen to be infected by E faecalis for which routine decontamination or removal of prosthesis is advised. However, if these pathogens are identified earlier by PCR or Q-PCR implant site infection can be minimized.

Identification of Inflammatory markers

Tissue breakdown is a result of release of pro inflammatory cytokines and host derived enzymes triggered by subgingival biofilm. PCR has got a capability of cytokine, enamel matrix proteins and microbial antigens detection along with its mainstay of protein detection. Application of Q-PCR has been mentioned by Shelburne CE in 2002 [48] in the analysis of Pg gene expression in vivo for detection of virulent factors. Garlet GP found through Q-PCR, the correlation between MMPs, RANKL and IL-IB, TNF-a, Tf-gamma expression and alveolar bone loss. [49] When a study was carried out to find effects of different setting of diode laser on the mRNA expression of growth factors and type I collagen on human gingival fibroblast, RT-PCR could identify along with mRNA expression of TLR (Toll Like Receptors), signaling mediators CD14, MYD88 and TIR- domain containing adaptor inducing interferon beta and NOD2 (Nucleotide binding oligomerization domain containing protein 2) [50]

Application in genetic polymorphism

Genetic factors also contribute to the individual's susceptibility to periodontitis with or without systemic and environmental factors. Genetic polymorphism effects periodontal disease with number of SNPs occurring in gene coding for cytokines, receptors and immune cells and thus PCR provides the most promising application in determination of association between genetic polymorphism and susceptible groups of patients.

IL-1 polymorphism, most prominently IL-1A (-889), IL-1A (-4845) & IL-1B (+3954) have been associated with chronic periodontitis in whites. [51] Il-1 gene polymorphism, IL-1A -4845, IL-1B +3954 and composite genotype was found to be associated with severe chronic periodontitis by PCR and RFLP. [52] Resistin gene which is seen to be associated with most of the inflammatory and metabolic disorders were found in T2DM and obese individuals through the use of PCR and was effectively seen to be showing SNPs at -420, +299 positions in these patient groups as detected by RFLP. [53]

II. Identification of Dental caries

Different methods have been used to identify caries causing species/ or cariogenic bacteria for example Streptococcus cricetus, S. ratti, S. mutans, S. sobrinus etc are frequently isolated from oral cavity. Where biochemical immunological methods could not show reliability to its max due to their longer time requirement, species characterization based on DNA testing such as PCR has shown satisfactory results. [54] Saarela et al investigated the discriminative power of

PCR for S. mutans and S. sobrinus. [54] As reported by Rupf et al [55] competitive PCR is a method which can determine exactly even the unknown bacterial quantities quickly thereby evaluating risk of caries in patients and providing as well as monitoring preventive and therapeutic measures. To compare the relationship between number of S. mutans and the prevalence of dental caries, PCR method was employed for saliva samples using Sm479 F/R & 16S RNA primers. These assays were found to be useful for assessment of individuals risk of dental caries. [56]

III. Diagnosing endodontic infections

Molecular methods such as PCR are more sensitive and specific than conventional culture methods, which is based on identification through phenotypic characteristics of each microbial species. For years microbial species were remained unidentified infecting the root canals by means of conventional culture procedures. Treponema denticola, Dialister pneumosintes, Filifactor alocis, Tf, T. socranskii are some of the important endodontic pathogens which got identifies by molecular methods. [57] Not only bacteria but fungi have also been found to be the etiological agents in pulpal disease. PCR was considered to be extremely sensitive method by Baumgartner et al [58] since it could assess the presence of Candida albicans in the contents of infected root canals, cellulitis aspirations as well as abscesses of endodontic origin. Thus molecular techniques have revolutionized medical microbiology through its wider patterns of detection and higher sensitivity.

IV. Detection of oral cancers

One of the uses of PCR in dentistry is the detection of markers in the diagnosis and prognosis of some types of oral cancers. Premalignant lesions of head and neck have been employed in tumor progression in humans based on data from patients with cancer. [59] Different techniques such as PCR, RT-PCR, in situ hybridization etc have been employed to determine the occurrence of viral infection and role of HPV in carcinogenesis so as to find out new strategies for prevention and early detection of squamous cell carcinoma cases. Through RT-PCR S. anginosus can be detected with greater sensitivity & specificity which is mostly isolated from Squamous cell carcinoma of head and neck. [60]

Advantages of PCR

1. PCR has high sensitivity (95-100%) & specificity (100%)
2. Generation of large amount of probes is possible.
3. Diagnosis of monogenic diseases.
4. Analyze DNA from microscope slides of tissue preserved years before.
5. Crucial forensic evidence may be present in small quantities e.g. Body fluid (blood, serum, saliva) one human hair.

Disadvantages

1. Requires costly instruments like thermal cycler, agar-gel diffusion tray, DNA separation reagents etc which not all laboratories can afford to buy.
2. Requires thorough knowledge of molecular techniques, experienced and qualified manpower.
3. False positive and false negative results may lower the sensitivity & specificity due to contamination from extraneous DNA.
4. In conventional PCR non specific building of primers and primer dimer formation are reasons for unexpected results.

CONCLUSION

The advancements in science have transformed the lives of human beings in ways that would have been unpredictable just a half century ago. The invention of PCR has been a boon to the modern science with its applicability in clinical diagnostics, DNA fingerprinting, DNA profiling, recombinant DNA technology along with the additive advantages due to modifications in the basic PCR technique, such as RT-PCR, Q-PCR and combined RT-PCR/qPCR.

PCR has completely metamorphosed the detection of RNA and DNA viruses. It is a rapid test with high sensitivity & specificity.

PCR thus helps the clinician to start early treatment and manage better treatment plan. This leads to reduce economical and social burden of the patients. Though it is a sophisticated technique, requires infrastructure support, expensive, nevertheless one cannot discount its utilitarian advantages which are many compared the existing conventional diagnostic methods.

Conflict of Interest: The authors report no conflicts of interest related to this study.

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