REVIEW ARTICLE

Polymerase Chain Reaction (PCR): A Short Review

*MT Rahman[1], MS Uddin[2], R Sultana[2], A Moue[3], M Setu[4]

*Prof. Dr. Md. Tahminur Rahman, Professor & Head, Pathology, Anwer Khan Modern Medical College, Dhaka
[2]Muhammed Salah Uddin, Senior Research Assistant, ICDDRB, Mohakhali, Dhaka
[2]Razia Sultana, Senior Research Assistant, ICDDRB, Mohakhali, Dhaka
[3]Arumina Moue, Mhil Student(Microbiology), Stamfored University Dhaka
[4]Dr. Muntahina Setu, Asstt Profe, Dept. of Paediatric, Anwer Khan Modern Medical College, Dhaka

*Corresponding author

ABSTRACT

Diagnosis of disease now a days is mostly laboratory dependent. Due to recent advances in medical science and molecular biology, most of the diagnosis of uncommon, complicated, unusual presentation of disease has left the option of molecular diagnosis as the number one diagnostic modalities. Many molecular techniques are now being widely used throughout the world including PCR, flow cytometry, tissue microarray, different blots, and genetic diagnosis. Among these PCR is the most widely accepted, commonly used diagnostic modalities with very high specificity and sensitivity for correct diagnosis. We have reviewed the principle, application, advantages and disadvantages of PCR in laboratory diagnosis of disease.

Key words: PCR, Molecular techniques, Review

Introduction

Polymerase chain reaction (PCR) is a new, popular molecular biology technique for enzymatically replicating DNA without using a living organism, such as E. coli or yeast. The technique allows a small amount of the DNA molecule to be amplified many times, in an exponential manner. With more DNA available, analysis is made much easier. PCR is commonly used in medical and biological research labs for a variety of tasks, such as the detection of hereditary diseases, the identification of genetic fingerprints, the diagnosis of infectious diseases, the cloning of genes, paternity testing, and DNA computing[1]. The technique was developed in 1983 by Kary Mullis, PCR is now a common and important technique used in medical and biological research labs for a variety of applications. These include DNA cloning for sequencing, DNA-based phylogeny, or functional analysis of genes; the diagnosis of hereditary diseases; the identification of genetic fingerprints (used in forensic sciences and paternity testing); and the detection and diagnosis of infectious diseases. In 1993, Mullis was awarded the Nobel prize in Chemistry along with Michael Smith for his work on PCR[2]. The PCR is commonly carried out in a reaction volume of 10-200 µl in small reaction tubes (0.2-0.5 ml volumes) in a thermal cycler. The thermal cycler heats and cools the reaction tubes to achieve the temperatures required at each step of the reaction. Many modern thermal cyclers make use of the Peltier effect, which permits both heating and cooling of the block holding the PCR tubes simply by reversing the electric current. Thin-walled reaction tubes permit favorable thermal conductivity to allow for rapid thermal equilibration. Most thermal cyclers have heated lids to prevent condensation at the top of the reaction tube. Older thermocyclers lacking a heated lid require a layer of oil on top of the reaction mixture or a ball of wax inside the tube.

Uses of PCR

PCR can be used for Diagnosis of many human diseases, broad variety of experiments and analyses[3,4,5]. Some examples are discussed below.

1. Infectious diseases HIV, CMV, Mycoplasma, Pneumonia, Cancer, Syphilis, fungal & Protozoal disease, hepatitis etc.
2. Diagnosis of cancer specially leukaemia and lymphomas
3. Genetic fingerprinting, paternity test

PCR permits early diagnosis of malignant diseases such as leukemia and lymphomas, which is currently the highest-developed in cancer research and is already being used routinely. PCR assays can be performed directly on genomic DNA samples to detect translocation-specific malignant cells at a sensitivity that is at least 10,000-fold higher than that of other methods.

PCR also permits identification of non-cultivatable or slow-growing microorganisms such as mycobacteria, anaerobic bacteria, or viruses from tissue culture assays and animal models. The basis for PCR diagnostic applications in microbiology is the detection of infectious agents and the discrimination of non-pathogenic from pathogenic strains by virtue of specific genes.

PCR is used to amplify a short, well-defined part of a DNA strand. This can be a single gene, or just a part of a gene. As opposed to living organisms, the PCR process can copy only short DNA fragments; usually up to 10 kb (kb stands for kilo base pairs). Certain methods can copy fragments up to 40 kb in size, which is still much less than the chromosomal DNA of a eukaryotic cell -for example, a human cell contains about three billion base pairs.

PCR requires several basic components. These components are:

- DNA template, or cDNA which contains the region of the DNA fragment to be amplifie
- Two primers, which determine the beginning and end of the region to be amplified (see following section on primers)
- Taq polymerase, which copies the region to be amplified
- Nucleotides, from which the DNA-Polymerase for new DNA
- Buffer, which provides a suitable chemical environment for the DNA-Polymerase.

The PCR reaction is carried out in a thermal cycler. This is a machnie that heats and cools the reaction tubes within it to the precise temperature required for each step of the reaction. To prevent evaporation of the reaction mixture (typically volumes between 15-100 l per tube), a heated lid is placed on top of the reaction tubes or layer of oil is put on the surface of the reaction mixture.

Primers

The DNA fragment to be amplified determined by selecting primers. Primers are short, artificial DNA strands not more than fifty (usually 18-25 bp) nucleotides that are complementary to the beginning and end of the DNA fragment to be amplified. They anneal (adhere) to the DNA template at the starting and ending points, where the DNA-Polymerase binds and begins the synthesis of the new DNA strand

The PCR process consists of a series of twenty to thirty-five cycles. Each consists of three steps.

1. The double-stranded DNA has to be heated to 94-960C in order to separate the strands. This step is called denaturing; it breaks apart the hydrogen bonds that connect the two DNA strands. Prior to the first cycle, the DNA is often denatured for an extended time to ensure that both the template DNA and the primers have completely separated and are now single-strand only. Time 1-2 minutes up to 5 minutes. Also Taq-polymerase is activated by this step.

2. After separating the DNA strands, the temperature is lowered so the primers can attach themselves to the single DNA strands. This step is called annealing. The temperature of this stage depends on the primers and is usually 50C below their melting temperature (45-600C). A wrong temperature during the annealing step can result in primers not binding to the template DNA at all, or binding at random. Time 1-2 minutes.

3. Finally, the DNA-Polymerase has to fill in the missing strands. It starts at the annealed primer and works its way along the DNA strand. This step is called extension. The extension temperature depends on the DNA-

Polymerase. The time for this step depends both on the DNA-Polymerase itself and on the length of the DNA fragment to be amplified. As a rule-of-thumb, 1 minute per 1 kbp.

Although these resulting 'fingerprints' for example, parent-child or sibling, can be determined from two or more genetic fingerprints, which can be used for paternity tests. A variation of this technique can also be used to determine evolutionary relationships between organisms. The PCR product can be identified by its size using agarose gel electrophoresis. Agarose gel electrophoresis is a procedure that consists of injecting DNA into agarose gel and then applying an electric current to the gel. As a result, the smaller DNA strands move faster than the larger strands through the gel toward the positive current. The size of the PCR product can be determined by comparing it with a DNA ladder, which contains DNA fragments of knows size, also within the gel.

The detection of hereditary diseases in a given genome is a long and difficult process, which can be shortened significantly by using PCR. Each gene in question can easily be amplified through PCR by using the appropriate primers and then sequenced to detect mutations. Viral diseases, too, can be detected using PCR through amplification of the viral DNA. This analysis is possible right after infection, which can be from several days to several months before actual symptoms occur. Such early diagnosis gives physicians a significant lead in treatment.

Stages of PCR

The PCR process can be divided into three stages:

Exponential amplification: At every cycle, the amount of product is doubled (assuming 100% reaction efficiency). The reaction is very sensitive: only minute quantities of DNA need to be present.

Leveling off stage: The reaction slows as the DNA polymerase loses activity and as consumption of reagents such as dNTPs and primers causes them to become limiting.

Plateau: No more product accumulates due to exhaustion of reagents and enzyme.

Practical modifications to the PCR technique:

Nested PCR- Nested PCR is intended to reduce the contaminations in products due to the amplification of unexpected primer binding sites. Two sets of primers are used in two successive PCR runs, the second set intended to amplify a secondary target within the first run product. This is very successful, but requires more detailed knowledge of the sequences involved.

Inverse PCR- Inverse PCR is a method used to allow PCR when only one internal sequence is known. This is especially useful in identifying flanking sequences to various genomic inserts. This involves a series of digestion and self ligation before cutting by an endonuclease , resulting in known sequences at either end of the unknown sequence.

RT-PCR (Reverse Transcription PCR) is the method used to amplify, isolate or identify a known sequence form a cell or tissues RNA library. Essentially normal PCR preceeded by transcription by Reverse transcriptase (to convert the RNA to cDNA) this is widely used in expression mapping, determining when and where certain genes are expressed.

Asymetric PCR-Asymetric is used to preferentially amplify one strand of the original DNA more the other. It finds use in some types of sequencing and hybridization probing where having only one of the two complementary strands is ideal. PCR is carried out as usual, but with a great excess of the primers for the chosen strand. Due to the slow (arithmetic) amplification later in the reaction after the limiting primer has been sued up, extra cycles of PCR are required. A recent modification on this process, known as LInera-After-The-Exponential-PCR (LATE-PCR), uses of limiting primer with a higher melting temperture (Tm) than the excess primer to maintain reaction efficiency as the limiting primer concentration decreases mid-reaction.

Quantitative PCR-Q-PCR (Quantitative PCR) is used to rapidly measure the quantity of PCR product (preferably real-time), thus is an indirect method for quantitatively measuring starting amounts of DNA, cDNA or RNA. This is commonly used for the purpose of

determining whether a sequence is present or not, and it is present the number of copies in the sample. There are 3 main methods which vary in difficulty and detail.

Quantitative real -time PCR is often confusingly known as RT-PCR (Real Time PCR). QRT-PCR or RTQ-PCR are more appropriate contractions. RT-PCR can also refer to reverse transcription PCR, which even more confusingly, is often used in conjunction with Q-PCR. This method uses fluorescent dyes and probes to measure the amount of amplified product in real time.

Touchdown PCR-Touchdown PCR is a variant of PCR that reduces nonspecific primer annealing by lowering of annealing temperature between cycles.

Colony PCR-Bacterial clones (E.coli) can be screened for the correct ligation products. Selected colonies are picked with a sterile toothpick from a agarose plate and dabed into the master mix or sterile water. Primers (and the master mix) are added-the PCR protocol has to be started with extended time at 950c.

Allele-specific PCR: a diagnostic or cloning technique based on single nucleotide Polymorphisms (SNPs) (single-base differences in DNA). It requires prior knowledge of a DNA sequence, including differences between alleles, and uses primers whose 3' ends encompass the SNP. PCR amplification under stringent conditions is much less efficient in the presence of a mismatch between template and primer, so successful amplification with an SNP-specific primer signals presence of the specific SNP in a sequence.

Assembly PCR or Polymerase Cycling Assembly (PCA): artificial synthesis of long DNA sequences by performing PCR on a pool of long oligonucleotides with short overlapping segments. The oligonucleotides alternate between sense and antisense directions, and the overlapping segments determine the order of the PCR fragments, thereby selectively producing the final long DNA product.

Asymmetric PCR: preferentially amplifies one DNA strand in a double-stranded DNA template. It is used in sequencing and hybridization probing where amplification of only one of the two complementary strands is required. PCR is carried out as usual, but with a great excess of the primer for the strand targeted for amplification. Because of the slow (arithmetic) amplification later in the reaction after the limiting primer has been used up, extra cycles of PCR are required.A recent modification on this process, known as Linear-

After-The-Exponential-PCR (LATE-PCR): uses a limiting primer with a higher melting temperature than the excess primer to maintain reaction efficiency as the limiting primer concentration decreases mid-reaction.

Dial-out PCR: a highly parallel method for retrieving accurate DNA molecules for gene synthesis. A complex library of DNA molecules is modified with unique flanking tags before massively parallel sequencing. Tag-directed primers then enable the retrieval of molecules with desired sequences by PCR.

Helicase-dependent amplification: similar to traditional PCR, but uses a constant temperature rather than cycling through denaturation and annealing/extension cycles. DNA helicase, an enzyme that unwinds DNA, is used in place of thermal denaturation.

Hot start PCR: a technique that reduces non-specific amplification during the initial set up stages of the PCR. It may be performed manually by heating the reaction components to the denaturation temperature (e.g., 95°C) before adding the polymerase. Specialized enzyme systems have been developed that inhibit the polymerase's activity at ambient temperature, either by the binding of an antibody or by the presence of covalently bound inhibitors that dissociate only after a high-temperature activation step. Hot-start/cold-finish PCR is achieved with new hybrid polymerases that are inactive at ambient temperature and are instantly activated at elongation temperature.

Intersequence-specific PCR (ISSR): a PCR method for DNA fingerprinting that amplifies regions between simple sequence repeats to produce a unique fingerprint of amplified fragment lengths.

Inverse PCR: is commonly used to identify the flanking sequences around genomic inserts. It involves a series of DNA digestions and self

ligation, resulting in known sequences at either end of the unknown sequence.

Ligation-mediated PCR: uses small DNA linkers ligated to the DNA of interest and multiple primers annealing to the DNA linkers; it has been used for DNA sequencing, genome walking, and DNA footprinting.

Methylation-specific PCR (MSP): developed by Stephen Baylin and Jim Herman at the Johns Hopkins School of Medicine, and is used to detect methylation of CpG islands in genomic DNA. DNA is first treated with sodium bisulfite, which converts unmethylated cytosine bases to uracil, which is recognized by PCR primers as thymine. Two PCRs are then carried out on the modified DNA, using primer sets identical except at any CpG islands within the primer sequences. At these points, one primer set recognizes DNA with cytosines to amplify methylated DNA, and one set recognizes DNA with uracil or thymine to amplify unmethylated DNA. MSP using qPCR can also be performed to obtain quantitative rather than qualitative information about methylation.

Miniprimer PCR: uses a thermostable polymerase (S-Tbr) that can extend from short primers ("smalligos") as short as 9 or 10 nucleotides. This method permits PCR targeting to smaller primer binding regions, and is used to amplify conserved DNA sequences, such as the 16S (or eukaryotic 18S) rRNA gene.

Review of literatures on PCR application and diagnosis of diseases:

Detection of Mycobacterium tuberculosis from 100 suspected cases of Tuberculosis attending DOT corner of Mymensing Medical College Hospital PCR was done using primers mtb1 and mtb2 based on IS6110 sequence present in all mycobacterium tuberculosis strains using sputum sample collected from those patient's sensitivity and specificity of PCR was found to be 94.7% and 100% respectively[6]. One study showed that real time PCR assay accurately detected Klebsiella pneumonia carbapenemase genes who is responsible for multidrug resistance with high analytical sensitivity and specificity. Realtime PCR assay based on SYBR Green I was designed to amplify a 106bp product of the blaKPC gene from 159 clinical gram negative isolates resistant to several class of lactam antibiotics showed the sensitivity and specificity was cent percent when compared to those of MHT and the real time PCR detection limit was about 0.8cfu using clinical isolates[7].

PCR diagnosis was also tried in detection and differentiation of Salmonella typhi and paratyphi, Brucellosis, Malaria parasite detection, Dengue infection, H.pylori in different studies throughout the world. The target gene produced amplicons at 429bp, 330 bp and 600bp showed 100% sensitivity for detection of salmonella species, typhi and paratyphi[8]. For detection of Plasmodium vivax the positive predictive values of thick blood microscopy, nested PCR and realtime PCR were 47.8%, 56.5% and 60.9% respectively. The real time PCR was found to have a specificity of 75% and sensitivity of 100% while nested PCR showed 81.2% and97.7% respectively[9]. For diagnosis of brucellosis the most common zoonotic disease PCR detection using primers B4/B5 that amplifies gene encoding a 31kda immunogenic outer membrane protein (bcsp31) showed 51.3% accurate sensitivity specificity of 100%[10].Histopathological and urease tests in biopsy sample sometimes produce false negative results. To compare PCR assays were done in all controls positive for Hpylori showed sensitivity and specificity of 64% and 80% respectively using best combination of 16rRNA+ureA.[11] Chagas disease or Treypanosoma cruzi is a major tropical threat.accurate diagnosis and parasitological response to treatment are priorities.PCR detection were tried using serial dilutions of purified DNA stocks representing Trypanosoma cruzi discreate typing unitsI,IV,VI(set A),human blood spiked with parasite cells(SetB), and Guinadine Hydrochroloride EDTA blood sample from 32 seropositive and 10 seronegative controls(SetC) were run by different PCR methods using primers 121/122.The result showed 83.3%-94.4% sensitivity and specificity of 85-95%.This represents international validation of PCR procedures for detection of Trepanosoma cruzi in human blood samples[12].

For diagnosis of scrub typhus one study investigated performances of conventional PCR (C-PCR), nested PCR(N-PCR), real time

quantitative PCR(Q-PCR) targeting the O.tsutsugamushi specific 47kDa gene.Two template systems plasmid DNA and a genomic DNA from buffy coat sample of a single patient was used.The sensitivities of C-PCR,N-PCR and Q PCR performed with blood samples taken from patients with 4 weeks of onset of fever were 7.3%,85.4% and 82.9%(95%CI)[13]

Diarrhoea caused by Clostridium difficile is mostly diagnosed by ELISA.More recently PCR based diagnosis has been tried in one study on samples from 150 hospitalised adults and healthy volunteers.Using culture as gold standard the sensitivity and specificity of PCR were 100% and 99.2% respectively.[14]

Viral DNA can likewise be detected by PCR. The primers used need to be specific to the targeted sequences in the DNA of a virus, and the PCR can be used for diagnostic analyses or DNA sequencing of the viral genome. The high sensitivity of PCR permits virus detection soon after infection and even before the onset of disease. Such early detection may give physicians a significant lead in treatment. The amount of virus viral load") in a patient can also be quantified by PCR-based DNA quantitation techniques. Genetic fingerprinting is a forensic technique used to identify a person by comparing his or her DNA with a given sample, such as blood from a crime scene can be genetically compared to blood from a suspect[15,16,17].

Advantages of PCR

" PCR can be used for diagnosis of many human diseases,broad variety of experiments and analysis.

" PCR is very important confirmatory diagnostic aid in infectious disease like tuberculosis, HIV,CMV, Mycoplasma,Hepatitis, Syphilis, fungal& protozoal disease, cancers specially leukaemia and lymphoma Malaria, Staphylococcal bacteremia, Tuberculosis, Toxoplasma Gondic 18, 19, 2021.

" PCR is also important for genetic fingering and paternity test.

" PCR has high sensitivity(95-100%) and specificity (100%)[21,22]

Disadvantages of PCR

*Requires costly instruments like thermal cycler, agargel diffusion tray, DNA separation kit, other chemicals & reagents which not all laboratories can afford to buy.

*Requires trained, experienced, qualified manpower and technologists,

*Adequate space with aircondition, dehumidifier, laminar flow facilities,

*Limited scope for diagnosis of diseases,

*Costly and not all people can afford to do the test,

*False positive and false negative results may lower specificity & sensitivity,

Conclusions

For accurate diagnosis of some disease with more sensitivity and specificity PCR is a very common and widely accepted method now a days throughout the world and it is also gaining popularity in Bangladesh. Many advance medical centers, modern diagnostic labs and medical institutions are using PCR as routine lab diagnostic and research modalities. PCR can play an important role in diagnosing disease with diversify and atypical clinical presentation and can lead to early and definitive diagnosis which helps the clinician to start early treatment, manage better treatment plan and follow up for the patient. This leads to reduce economical and social burden the patient and the family.

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