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ORIGINAL ARTICLE

POLYMERASE CHAIN REACTION: THEORY, PRACTICE AND APPLICATION: A REVIEW

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ABSTRACT

Polymerase Chain Reaction (PCR) is a rapid procedure for in vitro enzymatic amplification of specific DNA sequences using two oligonucleotide primers that hybridize to opposite strands and flank the region of interest in the target DNA. Repetitive cycles involving template denaturation, primer annealing and the extension of the annealed primers by DNA polymerase, result in the exponential accumulation of a specific fragment whose termini are defined by 5' end of the primers. The primer extension products synthesized in one cycle can serve as a template in the next. Hence the number of target DNA copies approximately doubles at every cycle. Since its inception, PCR has had an enormous impact in both basic and diagnostic aspects of molecular biology. Like the PCR itself, the number of applications has been accumulating exponentially. It is therefore recommended that relevant scientists and laboratories in developing countries like Nigeria should acquire this simple and relatively inexpensive, but rather robust technology.

Key words: *Polymerase Chain Reaction, Disease Diagnosis*

INTRODUCTION

When a living cell divides, each progeny must contain identical genetic information, that is, each deoxyribonucleic acid (DNA) molecule must become two identical molecules (Figure 1) each carrying the information that was contained in the parent molecule. This duplication process is called *replication*. DNA replication involves the initial conversion of DNA to double stranded molecules, which then serves as a template for synthesis of single strand identical to the parent molecule by addition of nucleotide monomers under the influence of the enzyme, DNA - dependent DNA-polymerase. This enzyme was first isolated and characterised from extracts of *E. coli* by Arthur Kornberg in 1957.

Thus the duplication of living cells basically involves a template (DNA) primer, an enzyme (DNA polymerase) and a favourable physiological environment.[1,2]

Despite the existence of the background knowledge concerning the ingredients and processes for natural multiplication of cells in the late 1950s and 1960s, no one before Kary Mullis considered the possibility of duplicating cell (DNA) under *in vitro* condition.

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Hence, it was only in 1983 that Kary Mullis synthesized this existing information into one of the most powerful tools of molecular biology – called the Polymerase Chain Reaction (PCR) or '*in vitro* cloning [3], PCR is a method of making unlimited copies of DNA using a test tube, a heat source and few simple reagents [template, primers, DNA polymerase, deoxyribonucleoside triphosphate (dNTPs) and a buffer]. In technical terms however, PCR may be defined as a rapid procedure for *in vitro* enzymatic amplification of specific DNA sequences (in pure form or complex mixtures) using two oligonucleotide primers that hybridize to opposite strands, and flank the region of interest in the target DNA.

A repetitive series of cycles involving template denaturation, primer annealing and extension of the annealed primers by DNA polymerase results in the exponential accumulation of a specific fragment whose termini are defined by 5' end of the primers[4] Beacause the primer extension products synthesized in one cycle can serve as a template in the next, the number of target DNA copies approximately doubles at every cycle. Hence, it is estimated that beginning from a single fragment of DNA, the PCR can generate 100 billion similar copies.[5,] The application of the PCR is so robust and versatile, that since the submission of the manuscript in 2001, several other reviews and

scientific publications have appeared on the application of the technique in several areas of scientific research [7-10], vaccine development [11-14] and disease diagnosis [15-17].

The DNA Molecule

The DNA is a delicate chain consisting of two strands of linked nucleotides: deoxyadenylates (A's), deoxythymidylates (T's), deoxyguanylates (G's) and deoxycytidylates (C's). The sequence of nucleotides in one strand is complementary to that in the other strand – the A's are always opposite T's, and the G's are opposite C's and this complementarity binds the strands together (Figure1). Each strand has a three prime (3') and a five prime (5') end. Their orientations oppose one another and the strands are therefore said to be *antiparallel* (Figure. 2)

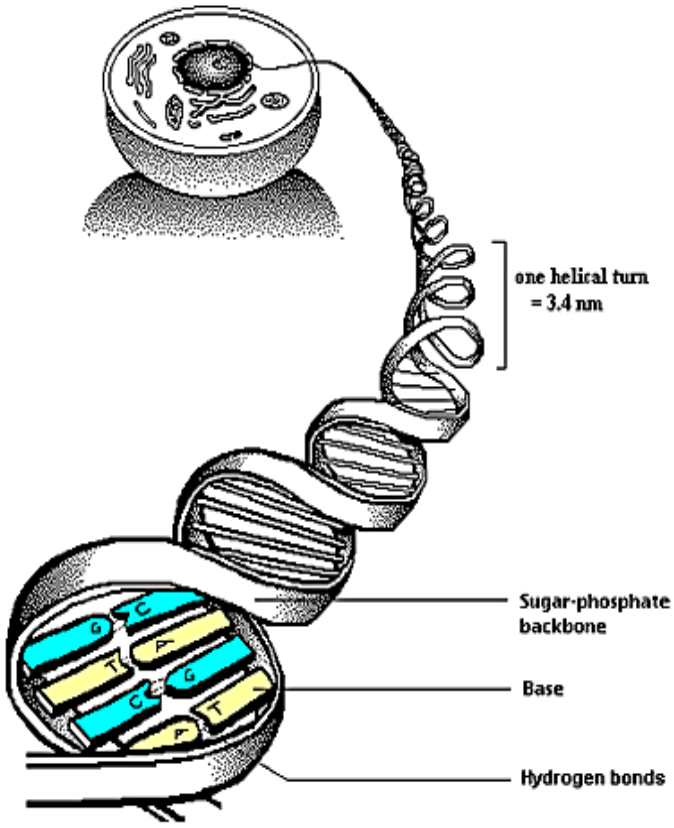

Figure 1: Structure of DNA

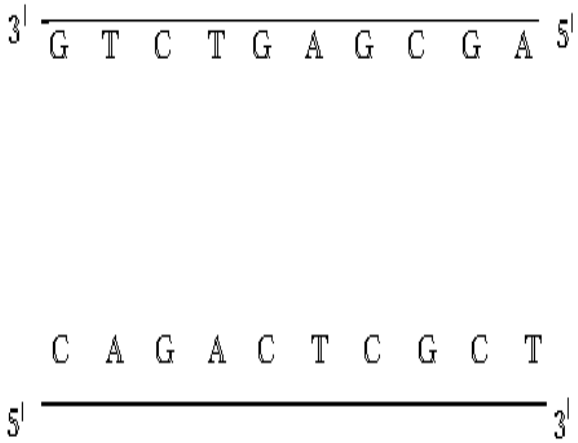

Fig. 2: Complementarity of base pairs in DNA

The sequence of these bases encodes the genetic information. Hence, the genetic code is the information relationship between nucleotides and amino acids which are synthesized according to a pattern of non-overlapping contiguous triplet codons. Each amino acid is specified by one or more codon which is the term used for a set of adjacent nucleotides [1, 2, 5, 18, 19]

The Basic Protocol of PCR

The first step of PCR simply entails mixing the template DNA, two appropriate oligonucleotide primers, DNA polymerase, deoxyribo - nucleoside triphosphates (dNTPs) and a buffer. Once assembled, the mixture is cycled many times, usually 30 times through temperatures that permit denaturation, annealing and synthesis. The product is then displayed on an appropriate gel and examined for yield and specificity [20]. Hence, the basic PCR protocol may be outlined as follows:

1. Optimize $MgCl_2$ concentration.
2. Prepare a 10X amplification buffer containing $MgCl_2$ at 10-fold the optimal concentration.
3. Test the optimised 10X amplification buffer by preparing a reaction cocktail for a single PCR. If PCR is successful, prepare more reaction cocktail for storage at -20^0C.
4. Add 68µl 15µg/ml template DNA and 9µl of H_2O.
5. Add 1µl 2.5U/µl Taq DNA polymerase.
6. Overlay the reaction mixtures with 100µl mineral oil to prevent evaporation.

7. Heat samples for 90secs at 94^0C (in a bath or an automated thermal cycler) to denature the DNA.
8. Incubate at 55^0C for 2 min to anneal.
9. Incubate the sample at 72^0C for 3 min to extend.
10. Repeat steps 7 to 9 for another 29 cycles.
11. Electrophorese 10µl from each sample on an agarose non-denaturing polyacrylamide gel.
12. Stain the gels with ethidium bromide.
13. Examine the stained gel.

The basic reagents and equipments for PCR outlined below.

Optimisation of PCR

Despite it's apparent simplicity, the PCR is a relatively complicated and, as yet, incompletely understood biochemical brew, where constantly changing kinetic interaction among the several components determine the quality of the products obtained.[21] Although the results will be good in most cases, there are a number of parameters that can be explored, if better results are required, or if the reaction fails altogether. These parameters are examined below:

Primer Selection

There is no set of rules that will ensure the synthesis of an effective primer pair. Yet more than anything else, it is the primers that determine the success or failure of an amplification reaction. However, the chances of success may be increased by

i. Selecting primers with random base distribution and with a GC content similar to that of the fragment being amplified.
ii. Avoid sequences with significant secondary structure (the use of a computer programme is strongly recommended for this).
iii. Reducing incidence "Primer dimmer" by avoiding primer complementarity.

Primer dimmer is an amplification artefact, usually a double stranded fragment whose length is very close to the sum of the two primers, and appears to occur when one primer is extended by the polymerase over the other primer. Most primers will be between 20 and 30 bases in length and the optimal amount to use in amplification will vary. Sequences not complementary to the template can be added to the 5^1 end of the primer. These exogenous sequences become incorporated into the double stranded PCR product and provide a means of introducing restriction sites or regulatory elements (e.g. promoters at the ends of the amplified target sequence)[22]

The Buffer

Changes to the PCR reaction buffer will usually affect the outcome of the amplification. In particular, the concentration of $MgCl_2$ can have a profound effect on the specificity and yield of amplification. A concentration of about 1.5 milliMoles (mM) is usually optimal (with 200µm each dNTP), but in some circumstances, different amounts of Mg^{2+} may prove to be necessary. Generally, excess Mg^{2+} will result in the accumulation of non-specific amplification products, and insufficient Mg^{2+} will reduce the yield. Therefore, for any particular application, the optimal concentration of Mg^{2+} required for the PCR protocol must be established.

DNA Polymerase

Initially the PCR used the Klenow fragment of *E. coli* DNA polymerase to extend the annealed primers in a rather tedious procedure. This enzyme was inactivated by the high temperature required to separate the two DNA strands at the outset of each PCR cycle, hence necessitating the addition of this enzyme at the end of every cycle. The introduction of the thermostable DNA polymerase (Taq polymerase) isolated from the hot spring bacteria, *Thermus aquaticus* transformed the PCR into a simple and robust reaction, which could now be automated by a thermal cycling device.[4]

The concentration of the enzyme typically used in PCR is about 2.5 units per 100µl reaction. For amplification reactions involving DNA samples with high sequence complexity such as genomic DNA, there is an optimum concentration of Taq polymerase, usually 1 - 4 units per 100µl. increasing the amount of enzyme beyond this level can result in greater production of non-specific PCR products and reduced yield of the desired target fragment.

Deoxynucleotide Triphosphates

The deoxynucleotide triphosphates (dATP, dCTP, dGTP and dTTP) are usually present at 50 to 200µl each. Higher concentration may tend to promote misincorporations by the polymerase (thymodynamic infidelity). At 50 and 200µl, there are sufficient precursors to synthesize approximately 6.5 and 25µg of DNA respectively.

As deoxynucleotide triphosphate appear to quantitatively bind Mg^{2+}, the amount of dNTPs present in a reaction will determine the amount of free magnesium available. In the standard reaction, all four triphosphates are added to a final concentration of 0.8mM, this leaves 0.7mM of the original 1.5mM $MgCl_2$ not complexed with dNTP. Consequently, if the dNTP concentration is changed significantly, a compensatory change in $MgCl_2$ may be necessary.

Cycling Parameters

In a typical PCR reaction, the double stranded DNA is denatured by briefly heating the sample to 90 - 95^{0}C, the primers are allowed to anneal to their complementary sequence by briefly cooling to 40 - 60^{0}C followed by heating to 70 - 75^{0}C to extend the annealed primers with the Taq polymerase[7-15]. The time of incubation at 70 - 75^{0}C varies according to the length of target being amplified: allowing 1 min for each kilobase of sequence is almost certainly expensive, but it is a good place to begin. Shorter times should be tried once the other amplification conditions have been established.

The ramp time, or time taken to change from one temperature to another is not critical, but care must be taken to ensure that the samples reach the intended temperatures. The number of cycles necessary depends on both the efficiency of the reaction and the amount of template DNA in the reaction. Starting with as little as 100ng of mammalian genomic DNA (10^4 cell equivalents) after 30 cycles, 10% of the reaction should be readily visible on an ethidium-bromide stained gel as a single predominant band (with more templates, fewer cycles may suffice) [8-12]

APPLICATIONS OF POLYMERASE CHAIN REACTION (PCR)

Broadly, the applications of PCR in the biological sciences may be divided into:

a) Medical applications
b) Research applications

Medical Application of PCR

Polymerase chain reaction has helped in the realization of the potential of clinical DNA-based diagnoses by producing enough of the target sequence, so that simple, rapid and robust methods for identifying it could be employed. Specific applications of PCR in the medical sciences include:

Diagnosis of Monogenic Diseases

Monogenic diseases are diseases resulting from single gene disorder e.g. sickle cell anaemia[23] β – thalassemia [24]and, some cases of hemophilia[25.] Before now, the pre-natal diagnosis and carrier detection of these diseases relied upon the disorder using mixed DNA polymorphisms and family studies, and the procedures involved can take several weeks. However, with PCR, it is now possible to diagnose this disease directly and, indeed within a few days [26]

Mullis and Faloona first reported PCR analysis of DNA polymorphisms for sickle cell anaemia[6], while Kogan and co-workers reported a PCR-based technique for haemophilia A diagnosis [27] . Similarly, PCR techniques for polymorphism analysis have been reported for Pre-natal diagnosis of cystic fibrosis [28] , and for pre-symptomatic and pre-natal diagnosis of Huntington chorea, an autosomal dominant disorder. The possibility of testing for positive genetic traits in the pre-implanting embryo also arises because of the availability of PCR. One or two cells could be removed from the embryo at the blastomere stage, and using PCR techniques, a genetic diagnosis could be made on the DNA of these cells. The embryo could undergo further growth *in vitro* and be later implanted into the uterus. In some advanced countries/hospitals, the PCR technique is fast replacing the use of southern blotting in the diagnosis of monogenic diseases [26] .

Diagnosis of Mutation Diseases

Duchenne muscular dystrophy (DMD) and the Lesch-Nyhan (LN) mutation syndrome are genetic diseases arising from heterogeneous mutation at the loci for dystrophin and the Hypoxanthine

phosphoribosyltransferase (HPRT) genes respectively.[29] The genes that are deficient in the two diseases are located on the X-chromosomes, but have many other different features. Dystrophin is expressed in the muscle and the brain, and is not detectable in other tissues, while the HPRT gene product is ubiquitously expressed.[30] Southern blotting analysis of LN and DMD genes reveal that the spectrum of mutation in the two disorder are different.

The diverse genetic organisation represented by the two loci provides for an excellent model for mutation detection that can be applied to most of the other genetic diseases. The development of a unified strategy for simultaneous identification of molecular lesions are varied as gross DNA rearrangements and single DNA base substitution, represents a considerable challenge that would have seemed unattainable until the advent of the direct DNA sequence analysis of mutant HPRT alleles, and simplified detection of DNA variations of known sequence at both loci have been described.[30] The polymerase chain reaction offers advantage for the diagnosis of dystrophin gene abnormalities due to its ease of application, speed and sensitivity, and has permitted the development of a rapid method of detecting 80-90% of all dystrophin gene deletions.[31] The basis of the method relies on the observation that although deletion of the dystrophin gene arises in a heterogeneous manner, they are generally large (typically several hundred Kb), and are concentrated around specific regions of the gene. Thus, the vast majority of DMD deletion is detectable by assaying for the presence or absence of only a small percentage of the total *exons* in the gene. Since the average *intron* size in this gene in approximately 35kb, each *exon* must be amplified on a separate DNA fragment.

The general strategy for molecular diagnosis of newly arising human disease mutations is to use multiplex DNA amplification as a primary screen to detect DNA deletions. Cases not possible to diagnose by deletion studies can be further studied via DNA analysis, or if the target fragments are small enough, by direct sequence analysis of the mutations. The mutant sequence information can then be used in simplified assays to diagnose future disease cases in the family or detect carrier state of the disease.

DNA Typing, Evolutionary Trends and Disease Susceptibility Studies.

The capability of the PCR to amplify a specific segment of genomic DNA has made it an invaluable tool in the study of polymorphism and evolution, as well as in the analysis of genetic susceptibility to diseases. In all of these areas, a particular gene must be examined in a variety of individuals, either within a species, in different but closely related species, or in-patient and in healthy control populations. Based on this principle, the polymorphism of HLA Class II gene has been described[32.] The detection of HLA class II polymorphism is valuable in the area of individual identification, tissue typing for transplantation and genetic susceptibility to specific autoimmune diseases. Similarly, in molecular toxicology, PCR is used in the genotyping of carcinogen metabolism polymorphisms, since human genetic polymorphisms in metabolic activation and detoxification pathways appear to be important sources of inter-individual variation in susceptibility to cancer 33 and diseases [34, 35]. Thus, individuals who inherit the “at risk” alleles of genes for enzymes such as glutathione S-transferases (GST) and N-acetyltransferases (NAT) may not be protected against carcinogens in cigarette smoke, diet industrial processes, and environmental pollution [33 – 35] .

PCR and Forensic Science

The ultimate goal of forensic DNA analysis is to obtain a positive identification of the donor of a biological evidence sample. The ability to detect DNA polymorphisms in biological evidence samples, have revolutionized forensic biology. Whereas, methods like restriction fragment length polymorphism (RFLP) analysis and individual – specific DNA “fingerprints” requires 50-100ng of DNA, which is frequently not available from forensic evidence, the PCR technique requires much less quantity of DNA for a successful detection outcome. A number of highly informative PCR-based typing systems which should make RCR analysis more useful in individual identification have

been developed [36]. Thus, it has been demonstrated that DNA typing results can be obtained from semen stains, bloodstains, single hair and epithelial cells [37, 38]. All of these had been used in the unequivocal settlement of criminal cases.

A treasured property of marker systems for forensic PCR analysis is the high degree of sequence variability, since this determines the discriminating power of the assay. Fortunately, there are many polymorphic regions of DNA that can be exploited by PCR methods to improve the discriminating power of forensic DNA analysis. The marker systems that have been used in forensic PCR analysis include sequence polymorphism of the HLA complex[39] and the D-loop region of human mitochondrial DNA.[40]

Detection of Human Infectious Diseases

The PCR has revolutionized the detection of bacterial [41], fungal [42] and viral pathogens [43]. The targeted amplification of nucleic acid sequences provides not only dramatic increases in the number of copies to be detected, but concormitantly provides a nearly equivalent reduction in the complexity of the nucleic acid to be probed. Either DNA or RNA (following the production of complementary DNA using reverse transcriptase) can be used as a template for amplification. Further, since PCR is a target rather than a signal amplification system, the benefit of the variety of procedure developed over the years to amplify signal can be exploited coincidentally.[44] Combined, these aspects of PCR allow ready detection of a single pathogenic organism or virus particle, such an accomplishment could be provided earlier, only by procedures employing *in vitro* propagation of such pathogens.

Several laboratories have reported the detection of as few as three hepatitis B virus (HBV) genome (or about 300 virus particles per ml of serum) which represent a 10,000 fold increase in sensitivity over standard procedures [45] . Similarly, the use of PCR technique has brought great improvement in the clinical sensitivity and specificity of methods for detecting human immunodeficiency virus I and II [46], human papiloma viruses[47], nitrobacter population in soil[148], Clostridial organisms[49] and a host of other organisms.

Detection of ras Oncogenes

Cancer is thought to be mainly due to alteration in the cellular genome that affects the expression or function of genes controlling cell growth and differentiation. Present day cancer research aims at identifying the alterations responsible for the development of tumours, at characterizing the genes involved and at determining the consequences of the genes alterations for the control of cell growth and differentiation, and for the process of carcinogenesis using a variety of approaches. The ras family has been the most thoroughly investigated gene associated with carcinogenesis[50].

Although the precise role of ras oncogene in human tumors remain sbscure, the *ras* family consisting of three related genes, H*ras*, K*ras* and N*ras*, have been characterized as potential transforming genes by their presence in certain acutely transforming retrovirus, and in transformed NIH-3T3 cells transfected with DNA isolated from a variety of tumors[51]. The PCR procedure has greatly simplified the analysis of *ras* mutations in human tumors, so that it has been possible to survey a large number of different tumours types at a sensitivity that could not be achieved by other techniques.

PCR and DNA Vaccine Production

Genetic, DNA or nucleic acid-based immunization refers to the induction of an immune response to protein antigen, which is synthesized and expressed *in vivo* within the cells of the mammalian recipient of the DNA vaccine, subsequent to the administration of a DNA sequence or gene coding for an antigenic polypeptide. The sequence of the gene(s) inoculated contain a suitable eukaryotic transcription and translation control signals for the correct and efficient *in vivo* synthesis, conformation and post-translational processing of the proteins.[5,52,53] PCR plays a key role in the production of DNA vaccines. It is a much simpler, efficient and rapid procedure for the amplification (multiplication) of the desired sequences of the gene(s), once selected.

Research Applications of PCR

The ability to synthesize large amount of a specific DNA fragment from a complex template

has significantly facilitated subsequent analysis. The nucleotide sequence of amplified DNA fragments can be determined directly without molecular cloning and preparation of template by growth of the host and biochemical purification of the vector.

Most examples of applications of PCR in scientific research may be summarized as follows:

i. Direct sequencing of *in vitro* amplified DNA[54].
ii. Engineering DNA to meet specific needs[55].
iii. Detection of mutation[56].
iv. Detection of gene expression[44].
v. Specific amplification of a DNA specie[57]
vi. Geometric amplification of unknown DNA sequence through inverse PCR [58]
vii. Analysis of DNA sequences in individual gametes[59].
viii. Evolutionary analysis[60].

CONCLUSION

The PCR has made an enormous impact in both basic and diagnostic aspects of molecular biology since the few years since its discovery. Like the PCR, the number of applications has been accumulating exponentially and will most probably continue to do so in the near future. It may not be an over statement to say that all aspects of biomedical sciences will have a good "touch" of the PCR technology in this millennium. Therefore, it is our recommendation that the scientific communities of the developing world should not be left behind in acquiring this simple and relatively inexpensive, but rather robust technology.

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