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THE POLYMERASE CHAIN REACTION AND ITS ROLE IN PATHOLOGY

MING TEH, MD, MRCPath, FRCPA

Department of Pathology, National University of Singapore.

One of the problems in molecular biology is the fact that very often the particular stretch of nucleic acid being studied constitutes only a very small fraction of the overall nucleic acid sample. To overcome this, laborious techniques for cloning, sequencing and detecting interesting nucleic acid molecules have been devised, each with its own limitations. It can therefore be appreciated that a simple and easily reproducible method of selectively amplifying a particular nucleic acid sequence, which comprises only an extremely minute fraction of the total nucleic acid in a sample, would constitute a powerful revolutionary advance in molecular biology.

GENERAL PRINCIPLES

The main principle behind the Polymerase Chain Reaction (PCR) is disarmingly simple, and that is to make each new strand of the specific DNA sequence that has just been synthesized the template for the next round of DNA synthesis. In this way, the number of DNA strands synthesized will increase in an exponential manner since the templates are likewise being formed exponentially. Usually 25 – 40 cycles are performed since too many cycles will lead to an unacceptable increase in the amount and complexity of nonspecific background products. Also, there is a plateau effect in that the exponential rate of product synthesis becomes increasingly attenuated in the late PCR cycles.[1] In practice, the presence of an upper limit in the number of cycles that can be run in a PCR reaction usually does not pose a serious problem since 30 cycles are sufficient to increase the target molecule approximately a million times.

Earlier attempts used the Klenow fragment (a DNA polymerase isolated from E. coli) to catalyse the reaction. Each cycle of the reaction comprised three steps (Fig. 1):–

(1) Denaturing of the double-stranded DNA.
(2) Re-annealing of the single-stranded DNA with a primer. This is a synthetic oligomer, usually of length 18–28 bp, complementary to the DNA sequence flanking the segment to be synthesized.
(3) Elongation of the primer with the help of the polymerase.

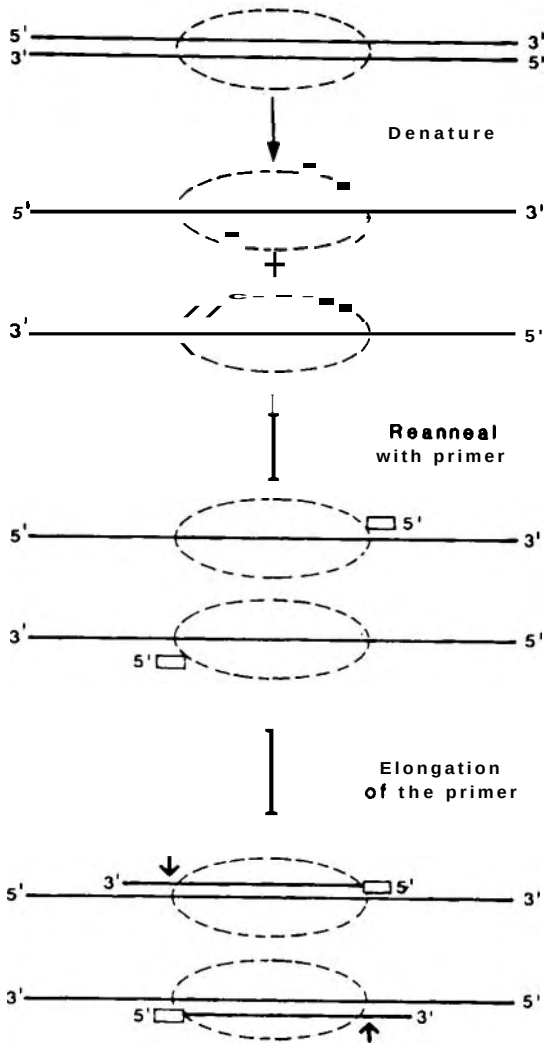

FIG. 1: A diagrammatic representation of the polymerase chain reaction. The DNA segment of interest has been circled by a dotted line. Note that the primers have to flank the dotted circle. Also, for the newly synthesized strand to act as a template in the next round of synthesis, the primer must elongate sufficiently so that it includes the flanking sequence for the next round of primers to anneal to (short arrow).

Address for reprint requests: Dr. Ming Teh, Department of Pathology, National University of Singapore, National University Hospital, Singapore 0513.

This was a major drawback since the melting temperature (Tm) for DNA in commonly used buffers ***in vitro*** is about 90°C. Hence denaturing the double-standed DNA, as is necessary at tne beginning of each cycle, also resulted in the concomitant inactivation of the Klenow enzyme. A dramatic improvement came about when the Klenow fragment was substituted by Taq polymerase.[2] This novel DNA polymerase was discovered in the bacteria ***Thermus acquaticus,*** and functions best in the temperature range of 70–75°C. Furthermore, it is not inactivated at the melting temperature for DNA, making it unnecessary to add fresh aliquots of DNA polymerase for each cycle. The rest of the reaction can thus be carried out under more stringent temperature conditions. As a result, the PCR process can now be fully automated.

The design of efficient primers is crucial to the success of the PCR reaction.' The sites of attachment of the two primers have also to be reasonably near, since each primer must elongate until it incorporates the flanking sequence on the other side for it to act as a template in the next cycle of synthesis (Fig 1). There is therefore a limit to the length of DNA sequence that can be amplified before the process becomes inefficient, the upper limit being approximately 2–3 kbp. As long as the above conditions are fulfilled, PCR can be used to synthesize almost any segment of nucleic acid. For the amplification of mRNA, the use of reverse transcriptase to convert it to cDNA is required initially.[3]

APPLICATIONS OF PCR

The ability of the PCR to amplify unique gene sequences has led to dramatic improvements in a number of fields, many of which have a bearing on our practice as pathologists. For example, forensic science has already been widely revolutionised by the ability to "DNA fingerprint" a person using a standard set of probes like A. Jeffrey's minisatellite probes. Basically this works on the principle that there are highly polymorphic stretches along the human genome that uniquely characterize an individual. PCR has vastly increased the occasions when such fingerprinting can be used since it can amplify relevant DNA stretches from extremely minute amounts of material.[4]

In the field of microbiology, PCR has allowed pathogens to be detected in very early stages of infection because of its sensitivity. A target sequence present only once in a sample of 10^5–10^6 cells can be amplified.[2] As a result PCR is ideally suited to detect viral diseases, particularly those for which culture or serology is impractical, and for epidemiological studies where asymptomatic carrier individuals have to be identified. For example, the use of PCR to detect HPV (human papilloma virus) infection in cervical epithelial cells have revealed a far higher incidence of infection than previously suspected.[5,6] In fact, this technique is so powerful that it is possible to screen for viral infections using only specimens such as exfoliated cells from a urine sample or cervical lavage.[7,8] Obviously, such a degree of sensitivity can easily lead to falsely positive results. To minimise this, strict experimental conditions with scrupulous cleaning of all equipment are mandatory since the most common source of error is contamination from a previous assay.[9]

One other major use of PCR lies in the detection of genetic mutations. Not only can we now work with minute samples of material, as has been emphasized above but certain inherent properties of the PCR method has made the work of the medical geneticist easier. For example, a novel way of detecting a point mutation is to construct primers in which the 3' ends correspond to the site of the point mutation (amplification refractory mutation system)[10] This takes advantage of the fact that under appropriate experimental conditions, elongation of the primer (Fig. 1) is markedly attenuated if the terminal 3'-nucleotide is not complementary to the template strand. Hence point mutations can be detected simply by observing whether the PCR is normal or refractory on using "normal" and "mutated" primers. On the other hand. we can tail the 5' end of the primer with short nucleotide seauences of our choice without appreciably affecting the specificity of the hybridization between the primer and template. Tailing it with a G+C rich sequence (GC-clamp) greatly increases the sensitivity of denaturing gradient gel electrophoresis (DGGE) and allows the separation of single-base mutations by DGGE that otherwise cannot be separated. This technique has been used successfully to detect haemoglobin sickle (HbS) and haemoglobin C (HbC) mutations in the human beta-globin gene. The huge amplification of genomic fragments by PCR also permits signals to be directly visualized on the

agarose gel making radioactive probes unnecessary.[11] The PCR technique can also be modified to sequence the DNA segment directly after synthesis. This has permitted highly polymorphic stretches of the human genorne, such as the HLA loci. to be studied at the nucleotide level with relative ease.[12]

For histopathologists. one of the biggest advantage is that PCR can be used to analyse DNA from paraffin-embedded formalin-fixed tissue.[13,14] This makes available a vast quantity of archival tissue blocks for retrospective analysis. PCR can also detect somatic mutations in malignancies. It is obvious that PCR is able to detect proto-oncogenes that have been activated as a result of point mutations in specific codons e g. ras oncogenes.[15] What is less obvious is that it can detect oncogene amplifications as well. This is done by co-amplifying the target gene jointly in the same reaction with a single copy reference genc. The above method has been successfully tried on the neu and the epidermal growth factor receptor gene using DNA isolated from as few as 100 breast carcinoma cells or single sections of formalin-fixed paraffin embedded material.[16] PCR has also becn shown to be an elegant method for detecting somatic mutations that are the result of chromosomal translocations. As a general rule, both primers must flank each side of the DNA segment for amplification to occur (Fig. 1). For a fused gene like **bcl-2/J_H, t(14;18)**, one of the primers will have to bind to a sequence in chromosome 14 and another in chromosome 18. However. if there has becn no translocation these two primers will be on different chromosomes and amplification will not occur. We can take advantage of this principle to detect for the presence of residual disease, even if minimal, in B-cell lymphomas where this cytogenetic abnormality is present.[17] This is done by isolating DNA from appropriate specimens, e.g. lymph node, bone marrow, etc., and running the DNA sample with the above primers. The presence of residual disease is implied if gene amplification is present (visualized as a specific hand on agarose gel electrophoresis).

As can be seen from these examples, the usefulness of PCR is limited only by the ingenuity of the user and continually more novel applications of PCR are being devised.' It is therefore safe to predict that PCR will soon gain widespread acceptance in pathology laboratories and prove to be every bit as revolutionary as monoclonal antibodies have becn.

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