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THE POLYMERASE CHAIN REACTION (PCR): A VALUABLE METHOD FOR RETROVIRAL DETECTION

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ABSTRACT

Although the detection of antibodies to a specific pathogen is used initially as the assay of choice, direct detection of human retroviruses is difficult. First, only a small fraction of cells are infected in the peripheral blood and lymphatic tissue may serve as a reservoir for infection. Second, infected cells may harbor only a small number of copies of the viral sequences. Third, a latent infection marked by transcriptional dormancy is often established thereby obviating the use of proteins or RNA to detect the viruses. Fourth, closely related but distinct members of the onco- and lenti-virus families may complicate specific detection of a particular virus. An additional hurdle is viral heterogeneity. HIV variants, for example, have been identified within and among individuals harboring this virus. Accordingly, sensitive and specific detection of the human retroviruses seemingly requires specific amplification of viral DNA sequences prior to detection. In this regard, an in vitro *DNA amplification procedure using DNA polymerase and termed the polymerase chain reaction (PCR) initially applied to human genetic diseases has been successfully applied to human retroviruses. A PCR-based assay has demonstrated utility for detecting infection: (1) prior to the generation of detectable antibodies, (2) in individuals with ambiguous or indeterminate serological status, (3) for neonatal screening, (4) by a specific type or multiple viruses, and (5) in therapeutic trials to allow the monitoring of infected cell load and viremia. It is also unlikely that the viruses identified to date represent all of the retroviruses responsible for human disease. Lymphatic disorders, in general, and immunodeficiencies, in particular, merit closer scrutiny for a retroviral etiologic agent. Since related viruses share short regions of homology, PCR may be useful for detecting uncharacterized viruses. PCR targeted to conserved regions of the pol gene of retroviruses has allowed the detection of the feline (FIV) and bovine (BIV) immunodeficiency viruses without prior knowledge of their genomic sequence.*

The *in vitro* nucleic acid amplification system termed the polymerase chain reaction (PCR) (1,2) has had a profound effect on numerous fields and, in particular, promises to revolutionize the detection and identification of various pathogens. The procedure is cyclical and provides for an exponential amplification of the targeted nucleic acid. Each cycle consists of three steps: denaturation or strand separation of the double helix (typically carried out at 94°C-98°C), annealing of synthetic oligonucleotides that serve as primers for the reaction (typically carried out at 37°C-55°C), and extension (typically carried out at 72°C when using a thermoresistant DNA polymerase). The extension step exploits the primer-dependent synthesis by a DNA polymerase and the template-specific pairing of adenine with thymidine and guanosine with cytidine (Fig. 1). Although successful, the

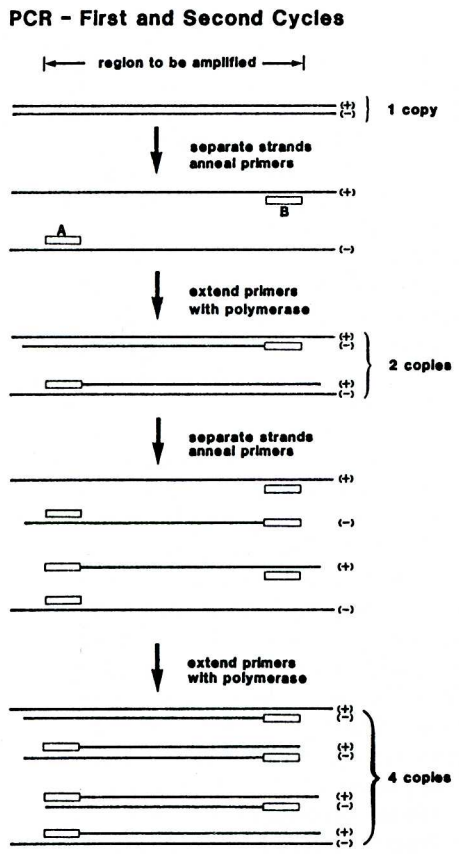

Fig. 1. Schematic representation of the polymerase chain reaction (PCR) (Reprinted from Luciw, PA, KS Steimer (Eds.): HIV Detection by Genetic Engineering Methods, *Marcel Dekker, Inc., New York, 1989, p. 247 by courtesy of Marcel Dekker, Inc.).*

initial use of *E. coli* DNA polymerase has been superseded by a superior thermoresistant DNA polymerase isolated from *T. aquaticus (Taq)* (3). The thermoresistant enzyme provides several advantages. First, the *Taq* DNA polymerase provides for more specific primer-directed synthesis thereby leading to greater sensitivity. Second, *Taq* DNA polymerase accommodates simplified automation since the enzyme need only be added at the beginning of the reaction. Third, this enzyme provides for the synthesis of longer products. Since each cycle is 2-4 minutes, multi-millionfold amplification is accomplished is 3-4 hours. The elapsed time for some amplifications can be cut by a third by using a two step procedure. Complete strand copying can be accomplished in the time elapsed in cycling between the annealing and denaturation temperature.

The procedure relies on a complex array of interactions to accomplish this remarkable task (4-6). Each component of the reaction and additional compounds effect various aspects of the process differently. For example, if PCR proceeds with a mean efficiency of 70% for 20 cycles, optimization leading to a 90% mean efficiency will lead to nearly a ten-fold increase in the amount of PCR product. Even greater discrepancies may occur between two levels of mean efficiency because the synthesis of non-specific products may further compromise the overall synthetic capacity of a PCR reaction. The overall effect on PCR product of a particular change is difficult to predict. Theoretical predictions and empirical experimentation have led to reported guidelines for optimizing a PCR reaction. Optimizing the parameters suggested below should allow one to establish efficiencies of 75-95%.

Although most primer pairs operate efficiently using what might be considered "standard" conditions (e.g., 2.5 units *Taq* DNA polymerase, 2.5mM magnesium chloride and 1mM primers) each primer pair system benefits from optimization. The reaction parameters that have in the past proven productive to examine include: 1) annealing temperature and time, 2) denaturation temperature and time, 3) enzyme concentration, 4) magnesium chloride concentration, and 5) primer concentration.

While numerous criteria for the design of optimal primers for PCR have been proposed, empirical testing remains the final and perhaps sole criteria required. An exhaustive survey of the effects of primer sequence, size, and base composition has not been carried out. However, guidelines for the design of optimal primers include: 1) 20-30 bases in size, 2) 50% or the average GC content of the target nucleic acid, 3) lack of computer-predicted secondary structure, and 4) minimal complementarity to known

non-target sequences. It should, however, be noted that primers not fulfilling these criteria have served efficiently in PCR, and primers fulfilling all of these criteria have not proven to initiate efficient PCR. Specific primer modifications have been suggested for various unique applications. Early in PCR development, 5' extensions not complementary to the template to facilitate cloning were shown not to affect efficient amplification. In fact, 5' extensions enhanced PCR efficiency if the complementary sequence of the primer was restricted to approximately 12-16 bases. In addition, short oligonucleotides containing multiple bases at one or more positions (degenerate primers) or bases theoretically capable of pairing with multiple bases (e.g. inosine) have been used effectively.

Primers have been shown to amplify templates that lack full complementarity. The effect of internal mismatches appears to depend on the number, nature, and location within the primer-template duplex. A growing number of reports documenting the effect of 3' terminal mismatches between primer and template as well as a comprehensive study using an HIV-1 model system have been reported. In that study, primers with a 3' terminal T, even though mismatched with C, G, or T in the template allowed efficient amplification to occur. An A:A mismatch moderately suppressed synthesis while the A:G and C:C mismatches dramatically reduced amplification efficiency. A decrease in the deoxyribonucleoside triphosphate concentration restored sequence-specific extension (with the G:T mismatch being the only exception).

A spectrum of prepared nucleic acid templates from highly purified to minimally prepared (by simply boiling) cells or virus particles have been used in PCR. The robustness of the subsequent PCR reaction is to a large degree dependent on the procedures used to prepare the nucleic acid. Further, different procedures have led to dramatic variability on the efficiency of the PCR reaction. To name but a few, the contribution of metal cations (both type and concentration), metal chelators, and salt concentration should be considered. A considerable amount of data has been accumulated using a proteinase K/non-ionic detergent procedure for obtaining DNA from peripheral blood mononuclear cells. This procedure employs initially the separation of the mononuclear from the polymorphonuclear cells, the cells are incubated with non-ionic detergents and finally treated with proteinase K. A 95°C heat treatment is used to inactivate the proteinase K, thereby circumventing the proteolysis of the *Taq* DNA polymerase.

Single- and double-stranded DNA serve as effective templates for amplification. With the addition of an intervening reverse transcription step, single- or double-stranded RNA can also serve as a template for PCR. The reverse transcription step has employed the eventual PCR primers, homo-oligonucleotides such as oligo dT, and hexanucleotides of random sequence. The use of the random hexamers may be advantageous since they, at least theoretically, circumvent the possible inefficient pairing of some unique sequence primers to regions folded in secondary structures such as hairpin loops not relaxed by the conditions of reverse transcription. The avian myeloblastosis and murine leukemia virus reverse transcriptases as well as the *E. coli*-expressed murine leukemia virus reverse transcriptase have been used successfully for cDNA synthesis. Temperatures ranging from 37°C-55°C for reverse transcription have been used. Examination of the reverse transcription of RNA templates in some systems indicates that cDNA synthesis is very efficient.

The PCR has sometimes been said to be too sensitive for practical diagnostic applications. Investigators should bear in mind that since the degree of amplification can be determined by adjusting the number of cycles carried out, each investigator can adjust the level of sensitivity desired. Prior to embarking on the study of the diagnostic sensitivity and specificity of a particular primer pair-probe system, optimization and the effects of parameter "windows" should be carried out on a

titration series of a known template of accurate copy number. For example, it is valuable to use (as a template for preliminary studies) a plasmid harboring the sequence being targeted for amplification whose concentration has been accurately determined. If RNA is the desired template, plasmid vectors capable of *in vitro* production of transcribed RNA are useful.

The exquisite sensitivity of PCR, namely the ability to detect a single molecule, merits further comment. The use of solutions that should contain a single copy of the template per aliquot used will, because of sampling variability predicted by Poisson distribution, only be positive a fraction of the time. For example, only 63% of reactions may be positive if the volume of the aliquot tested contains on the average a single molecule.

Regardless of the analytical sensitivity established for a primer pair-probe system, the diagnostic sensitivity of the system must be determined. Further, since the PCR is a procedure rather than a defined kit, the diagnostic sensitivity and specificity of each laboratory performing the assay may vary. The establishment of proficiency panels and protocols to be used among laboratories to ensure reproducible results is recommended.

The inability for PCR to detect pathogen in some samples from individuals that are infected can be explained in several ways. First, the appropriate type of sample must be analyzed. Although detection of the human retroviruses could have required lymph node biopsies or lymphatic fluid, testing over the last four years indicates that there is sufficient proviral DNA in the peripheral blood to result in what is expected to be nearly 100% diagnostic sensitivity. Second, the analytical sensitivity of the assay is such that the number of copies of the pathogen in the aliquot tested is not sufficient to result reproducibly in a positive signal. In this context, the establishment of a PCR-based assay, not unlike any other assay, requires extensive testing of the volume of the sample to be used. The detection of nearly all HIV-1 infected individuals using seropositivity as a criterion indicates that testing of between 150,000 and 1,500,000 peripheral blood mononuclear cells or the equivalent of approximately 0.05ml of peripheral blood is sufficient. Third, the sequence of the pathogen in the individual being tested is mismatched with the primer such that efficient amplification is not accomplished. As described above, primer design should take into consideration sequence heterogeneity of the pathogen (particularly RNA viruses). A combination of sufficiently low annealing temperatures and primer design to accommodate 3' terminal mismatches and extensive testing should circumvent this heterogeneity.

The exquisite sensitivity of PCR has led to false-positives. Although multiple sources can and have served as the templates for these false-positives, the primary cause is stray PCR products from previously amplified material. Procedural modifications have been recommended that have dramatically decreased their occurrence. Further, the appropriate use of the number and nature of controls allows significant confidence in the interpretation of a positive result.

The extent of sample mixup varies with the laboratory performing the analysis. Results from serological assays that appear to be discordant between testing or relative to the other information available on the nature of the specimen or individual being tested are typically repeated or submitted for the typing of serum proteins. If the HLA region is targeted for amplification as the control for the presence and amount of DNA in a sample prior to retrovirus proviral DNA amplification and given the polymorphism of this region among individuals, convenient sample matching is afforded. Six alleles of the HLA DQ alpha locus can be conveniently detected using sequence-specific oligonucleotide hybridization.

The use of a hybridization step to identify the amplified product is essential. The detection format first used for the detection of amplified products employed

radioactive probes and restriction endonuclease treatment. Radioactive liquid hybridization, without restriction endonuclease treatment, coupled to gel electrophoresis has been shown to have the greatest analytical sensitivity and specificity. More recently, strategies using colorimetric detection have been described. The comparative sensitivity and specificity of these assays relative to the radioactive liquid hybridization assay in preliminary studies shows promise.

Although the detection of antibodies to a specific pathogen are used initially as the assay of choice, direct detection of a pathogenic organism is desired in most cases. The direct detection of human retroviruses is difficult for several reasons. First, only a small number of cells are infected, sometimes as few as one infected cell among as many as 10,000 to 1,000,000 uninfected cells in the peripheral blood. Second, the infected cells may harbor small number of copies of the viral sequences (perhaps as few as 1-10). Third, a latent infection marked by transcriptional dormancy is usually established thereby obviating the use of proteins or RNA to detect the viruses. Fourth, the human genome contains a plethora of endogenous retroviral elements that resulted from infections prior to speciation. The viral genomes present are similar, but distinct from several characterized exogenous animal retroviruses (e.g., murine and baboon) and both intact as well as rearranged and deleted members have come under investigation. Their role in human disease is unclear but they can serve as templates for PCR if the primers for directed amplification are unwisely chosen. Fifth, closely related but distinct members of the oncovirus and lentivirus families may complicate specific detection of a particular virus. For example, at least two members of the human T cell lymphotropic (HTLV) and immunodeficiency (HIV) viruses have been identified. To date, both the HIV type 1 and 2 have been associated with AIDS; however, although HTLV-1 is linked to adult T cell leukemia (ATL) and a chronic progres sive myelopathy [referred to as tropical spastic paraparesis (TSP) or HTLV-I associated myelopathy (HAM)], HTLV-II, even though initially cultured from two patients with T cell variant hairy cell leukemia, has not been implicated in a specific syndrome or disease. An additional hurdle that must be cleared for the detection of these viruses is the often noted viral heterogeneity. HIV variants, for example, have been identified within and among infected individuals. Taken together, these reasons suggested that sensitive and specific detection of the human retroviruses required the specific amplification of viral DNA sequences prior to detection.

A PCR-based assay has demonstrated utility for detecting infection: (1) in neonates, since perhaps only a third of babies born to seropositive mothers are infected with HIV or HTLV, (2) in individuals with ambiguous or indeterminate serological status, (3) by a specific type of virus or multiple viruses, (4) in therapeutic trials to allow the monitoring of infected cell load or the level of viremia, and (5) prior to the generation of antibodies.

Further, it is unlikely that the viruses identified to date represent all of the retroviruses responsible for human disease. Lymphatic disorders, in general, and immunodeficiencies in particular, merit closer scrutiny for a retroviral etiologic agent. Since related viruses share short regions of homology, PCR may be used to detect uncharacterized viruses. For example, PCR targeted to conserved regions of the reverse transcriptase gene of retroviruses allowed the detection of the feline (FIV) and bovine (BIV) immunodeficiency viruses without prior knowledge of their genomic sequence.

In summary, PCR is a powerful but still maturing technology that has played an important role in the detection of characterized human retroviruses. The application of PCR to other viruses, bacteria, fungi, and protozoan pathogens is gathering momentum and promises to make a significant impact on molecular diagnosis.

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