LEADING ARTICLE

THE POLYMERASE CHAIN REACTION - A NEW TOOL IN THE DIAGNOSTIC LABORATORY

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The concept of the polymerase chain reaction (PCR) first came to Kari Mullis in April 1983 as he was driving down a moonlit mountain road in Anderson Valley, Northern California. He had been considering a variation in the technique of DNA sequencing when the idea for multiplying limitless copies of DNA struck him; as he was later to remark "Sometimes a good idea comes to you when you are not looking for it"[1].

The difficulty in obtaining sufficient amounts of pure DNA for easy analysis had previously hampered the application of molecular biological techniques in the work of most clinical laboratories. Using the PCR technique, however, a single molecule of DNA can be copied by more than a million times within a space of one afternoon. And, because the starting material can be obtained from even trace amounts of blood or other tissues or cell samples, the problems of insufficient or non-viable samples are eliminated. DNA has even been amplified from mouthwashes, hair, urine or paraffin-embedded tissues. The hallmarks of PCR - selectivity, sensitivity, speed - have enabled its diagnostic application in infectious diseases, genetics and cancer. It has improved on existing techniques in paternity testing, identification of individuals in forensic cases and in donor-recipient matching in organ transplants.

The PCR technique involves a three-step cycling process: denaturation, hybridisation and polymerase extension[2,3]. A typical reaction requires two synthetic oligonucleotide sequences, known as primers, that flank the sequence of DNA to be copied, the DNA polymerase enzyme and the four nucleotide bases (adenine, cytosine, guanine, thymidine). In the first step, the two strands in the double stranded DNA that is to be copied are separated by heating to a high temperature. This is followed by the hybridisation step, where the reaction is cooled down to allow the primers to anneal to the target molecule. The third and final step involves holding the reaction at an optimum temperature for DNA synthesis using the DNA polymerase. DNA synthesis will then proceed across the space between the primers, doubling the amount of that segment of DNA. If the extension product of the primer includes sequences of the other flanking primer, the extension product made in one cycle will serve as the template for extension in the next cycle; the result is an exponential increase in the product as a function of the cycle number. The average cycle takes less than 3 minutes; commonly, 20 to 30 cycles are performed.

The 'nested PCR' technique[4] is a modification of this basic technique, where the initial reaction is followed by a second reaction using the first PCR product as the template for a second pair of primers; this helps to increase the sensitivity and specificity of the reaction. PCR can also be used to amplify ribonucleic acid (RNA) if the RNA transcripts are first converted to complementary DNA (cDNA) using reverse transcriptase.

Subsequent analysis of the amplified DNA can be obtained either by gel electrophoresis or by hybridisation with allele-specific oligonucleotide probes. Qualitative changes are identified in the diagnosis of genetic and malignant diseases, while quantitative analysis is usually required in the diagnosis of infectious diseases.

In the field of diagnostic microbiology, the speed and sensitivity of the technique makes it particularly useful in rapid diagnosis of slow-growing or non-cultivable pathogens, a classical example being the mycobacteria[5]. In tuberculous meningitis, laboratory diagnosis is further hampered by the difficulty in obtaining sufficient organisms from cerebrospinal fluid samples for analysis; treatment has thus been based empirically on clinical diagnosis. As described in the article by Lee et al[6] in this issue of the journal, the technique of amplification by PCR followed by detection with hybridised probes is sufficiently specific and sensitive to be used for the rapid diagnosis of this disease[6]. The only limitation in the use of PCR has been the inability to estimate the susceptibility of the organism to various drugs.

This exquisite sensitivity has also been applied to the diagnosis of viral infections, where the majority of tests have so far relied on the use of techniques that detect antibody formed in response to viral antigens. Viral antigen detection tests have been limited by the relatively minute amounts of viral particles available for diagnosis by conventional techniques. With PCR techniques, the small amounts of viral genome present can now be expanded to sufficient levels for detection. This has enabled the detection of infection in the 'window period' prior to seroconversion, and in overcoming problems with diagnosis due to 'passive' antibody. It is also able to differentiate between true immunity and persistent infection in the presence of antibody, and in the differentiation of strain differences such as in human immunodeficiency virus types 1 and 2 (HIV 1/2), human T-lymphotropic virus types I and II (HTLV I/II), hepatitis C virus (HCV) and hepatitis B virus (HBV) variants[7]. Even in HBV detection, current techniques for antigen detection may eventually be replaced by more sensitive tests using genome amplification and molecular analysis.

The specificity and sensitivity of PCR has been applied to the early diagnosis of genetic diseases, including the detection of carriers and antenatal diagnosis. The identification of abnormal and altered gene sequences has also helped the histopathologist in the diagnosis of different malignancies, in the determination of clonality and in the detection of residual

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tumour cells[8]. Because it can be performed using paraffin-fixed tissues, retrospective studies of DNA in pathological samples stored in formalin-fixed paraffin-embedded tissues and analysis of DNA in tissues of extinct plants and animals are now possible[9].

HLA Class II (DP, DQ, and DR) typing can now be determined at the level of the gene by hybridisation with allele-specific (ASO) probes to detect specific variations in DNA[10]. A more recent development has been to selectively amplify a specific allele using sequence-specific primers (SSP)[11]. These methods have overcome the problems associated with current methods, which include the scarcity of monospecific antisera, and the difficulty in obtaining sufficient cells for analysis in leucopenic patients and patients with immune dysfunction affecting HLA expression. It is also an extremely powerful method for detecting and comparing polymorphic target sequences and 'DNA fingerprinting' using PCR technique[12] is now the method of choice in paternity testing and forensic science.

Despite the obvious advantages of the PCR technique, however, inherent disadvantages exist that still limit its use in routine diagnostic laboratories. A major problem is the occurrence of false positive reactions due to contamination from the product of previous amplifications. Because of the extreme sensitivity of the technique, even the most minute contamination of sample before amplification can result in significant quantities in the end product. This will be a major problem in large clinical laboratories where automated sampling systems with washable probes are used to handle a high sample throughput. Measures to minimise product carryover have included performing the pre-PCR and post-PCR technique in different laboratories, using sterile reagents and equipment, and adding positive and negative quality control samples in every procedure[13].

False positives can also occur if the primers are not carefully chosen to avoid similarity with endogenous human sequences. Choice of sample is important and cell-associated organisms may not be detected if serum samples are collected. Where nucleic acid hybridisation is used to enhance the sensitivity of detection, the use of radio-isotopic markers such as ^{32}P are required; this makes it unpopular for routine clinical laboratory use. Product detection by gel electrophoresis is also relatively laborious and interpretation subjective.

Recent advances have, fortunately, concentrated on overcoming these problems and will be briefly described. Three new strategies have been developed to reduce the incidence of false positive reactions due to product carryover. One method utilises a photochemical reagent to cross-link amplified DNA before the reaction[14]. Another method uses deoxyuridinetriphosphate (dUTP) to substitute for thymidinetriphosphate (TTP) in all the reactions. The enzyme uracil-N-glycosylase (UNG) is then added to the sample before the PCR procedure, and this will destroy the carry-over PCR product but not the desired DNA template. The enzyme is heat inactivated prior to amplification[15]. In the third method, short-wavelength ultra-violet irradiation of the reaction tube contents is used to damage any contaminating sequences before the DNA template and primers are added[16].

The isolation of a heat-resistant DNA polymerase from an organism living in hot springs called Thermus aquaticus (Taq) has enabled primer annealing and extension to be carried out at higher temperatures[17]. This has reduced the non-specific primer annealing and hybridisation that occurred with earlier DNA polymerases with low optimum temperature, and eliminated the need to add fresh enzyme after each cycle. Automation was thus possible using machines with controlled cooling and heating capacity called thermal cyclers. The technique of 'hot-start' PCR was also developed to increase specificity by further reducing non-specific annealing[18].

Newer enzymes recently isolated include the DNA polymerase from Thermus thermophilus, which has both reverse transcription and DNA polymerisation activities, allowing PCR amplification from RNA and DNA sequences with secondary structures[19]. New primers have also been designed to amplify heterogeneous targets and to improve sensitivity of detection. Co-amplification of sequences from different organisms may allow for the detection of several agents in one assay. To eliminate the need for radioactive probes, non-isotopic systems have been developed[20] and simplified and rapid microplate colorimetric systems are now available.

These recent developments will help to bring the PCR technique out of the research setting and make it more accessible to routine clinical laboratories. Commercial test systems using PCR technology are already available for several diagnostic applications. Laboratories wishing to make the transition to PCR methods should, however, ensure that they are familiar with the technical pitfalls involved and implement stringent standards of quality control. With this transition, the clinical diagnostic laboratory is poised to enter a new era of diagnostic technology.

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