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Gene amplification: present and future in the molecular diagnosis

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Abstract - With the advent of the Polymerase-Chain-Reaction (PCR) new avenues for the diagnosis of various classes of human disorders are accessible to the scrutiny of molecular genetic approaches. The basic principle of this new technique is the log-scale *in-vitro* amplification of specific nucleic acid sequences starting with as little as a single target molecule. As an ultrasensitive tool PCR clearly provides a quantum leap in both sensitivity and speed in the detection of e.g. infectious agents, changes in the genome that are the cause of various human genetic diseases or fingerprinting analyses. In the same sense it will have an impact on the study of the etiology of different malignancies. On the other hand, PCR creates its own, totally new dimension of intrinsic problems: 1) In clinical diagnostic procedures the main problem is that of false positive results. 2) As PCR is an assay strictly at the nucleic acid level, the question has to be raised whether differences from a so called "wild type gene" provide a link to the query disease or merely reflect genetic polymorphisms in an examined population. This article will focus on some examples of different applications in clinical diagnostic procedures that this new technology has been employed for so far.

INTRODUCTION

The Polymerase Chain Reaction (PCR) is a new molecular biology technique and was first introduced at the American Society of Human Genetics Conference in October 1985. Since then it has found wide acceptance throughout the scientific community in basic research and is also increasingly being used in medical diagnostic applications. In order to appreciate the advantages of PCR over the more classical methods of molecular diagnostic procedures in the clinical area, one has to compare their features with respect to specificity and sensitivity, speed and the potential for automation.

PCR AND VARIATIONS OF THE TECHNIQUE

In general, any stretch of nucleic acid sequence can be amplified from any organism, if the sequence of that stretch of DNA is known. To amplify the region of interest, two oligonucleotide primers have to be available that frame the particular sequence by annealing to both strands of the target. A temperature cycling regime will first denature the DNA, thus exposing the specific primer-binding sequences of the target. Following a rapid cooling step that allows for annealing of the oligonucleotides to the target sequence, the heat-stable enzyme Taq-Polymerase will subsequently copy the target in both directions starting from the primer sites. Multiple rounds of temperature cycling lead to an exponentially growing number of copies of the target. One single molecule theoretically is sufficient to be amplified to millions of specific DNA molecules of defined sequence and size within a few hours. A number of variations of the original scheme have been described the most important of which have been compiled recently by M.Innis et al. (ref.1). Some PCR-based modifications that can be especially useful in the design of clinical assays are presented below.

Both sensitivity and specificity of PCR can still be enhanced by the "nested primer PCR" modification (ref.2) which consists of two subsequent PCR assays. An aliquot of the first amplification product is used as the target for the

second assay with a set of oligonucleotide primers that will bind to more internal sequences of the first round's PCR product.

Under certain conditions more than one fragment of DNA can be amplified simultaneously by Multiplex PCR. Using a set of nine primer pairs in a single reaction Chamberlain et al. have reported rapid characterization of around 85% of the deletions in the dystrophin gene (ref.3). This technique may prove exceptionally powerful and rapid for large genes with multiple defects.

The use of RNA as starting material for PCR has first been published by Veres et al. (ref.4) and can be considered as a major contribution. In general this method requires to synthesize a first strand cDNA transcript of the RNA using the enzyme Reverse Transcriptase. Priming of this transcript can be done by either a gene-specific primer or by Oligo-dT or random primers (ref.5). The resulting transcript then serves as the template in the subsequent PCR reaction.

Allele-specific amplification of chromosomal DNA can be achieved with oligonucleotide primers that discriminate allelic mutations at their very 3'end (ref.6). This means that the 3'base of a mutation-specific primer will not anneal to the wild type allele and therefore fail to serve as a start for the Taq-Polymerase.

Gyllensten and Erlich have published a technique, termed "asymmetric PCR" (ref.7) that allows for direct sequencing of a PCR product. In this method, the molar ratio between the primers is between 1:50 to 1:100. This leads to a depletion of one oligonucleotide during the reaction and therefore to a linear increase of single stranded PCR product from the other primer. The single stranded DNA then serves as a template for the sequencing reaction.

For applications that are concerned with amplification of sequences for which only one specific primer can be designed the so called "Rapid Amplification of cDNA Ends" (RACE) (ref.8). In this modification the missing primer site is generated by adding a homopolymer tail towards the unknown end of the target DNA molecule by the enzyme Deoxynucleotidyl-Transferase. A primer that anneals to this tail will amplify, together with the specific oligonucleotide, at a logarhithmic rate while unspecific molecules which only possess the homopolymer tail will only be copied at a linear scale.

CLASSICAL MOLECULAR BIOLOGY TECHNIQUES IN DIAGNOSTIC PROCEDURES

The classical techniques that have been employed include in general either direct assessment of involved genes by nucleotide sequencing or allele-specific probes. In indirect assays, changes like gross chromosomal deletions and translocations, or restriction fragment length polymorphisms (RFLPs) can correlate with the observation of a pathologic phenotype. Both types of assays have certain limiting draw backs which render them inappropriate for the processing of patient samples in a routine laboratory environment:

The prerequisite for direct assays has been the cumbersome cloning of the affected gene and identification of the defect.

In an indirect assay the genetic linkage between the genetic marker and the unknown gene is not always easily assessed, because the polymorphism that is detected by a distant probe, may not in every case be informative.

Finally, all classical procedures rely on sequence-specific hybridization in Blot analyses. For Southern and Northern Blots enough nucleic acids have to be purified from tissues, digested, electrophoretically separated and transferred to nitrocellulose or nylon membranes prior to hybridization to a gene probe. In the case of affections of single copy genes less than 1 pg of a specific sequence has to be detected. Although this can be achieved with sufficient sensitivity by using radioactively labelled DNA probes, assay times are in the range of days to weeks to yield unambiguous hybridization signals. In addition, the usefullness of radioisotopes is strictly limited by half life of the isotope as well as autoradiolysis and therefore hampers routine use either because of the biohazard and waste disposal problem or because of inter-assay variance. In any case, the number of steps involved in the analysis is too high for routine processing or even automation.

The Polymerase Chain Reaction has eliminated a great deal of these complications. I) It has been shown that there is no need for extensive purification of the nucleic acids assayed. On the contrary, whole cells or tissue samples are directly used for PCR. II) The assay can be carried out in 5 hours, depending on the time allowed for the annealing and primer extension steps as well as

for the number of cycles run. Since the temperature shifts (ramps) during the reaction account for a significant time, the assay speed can be increased using longer oligonucleotide primers which possess high annealing temperatures. III) The generation of a vast number of identical copies from as little as a single DNA molecule abolishes sensitivity problems as compared to normal Southern or Northern Blot analyses, since the amplified specific fragment can be visualized in Ethidium bromide-stained agarose gels. IV) The specificity of the PCR product can be assessed by hybridization experiments and signals are usually detected in less than an hour of exposure. This allows the use of stable non-radioactive probes. V) Ultimately, amplification products produced by asymmetric PCR can easily be sequenced.

Unfortunately, the PCR technique has itself generated a totally new dimension of intrinsic problems because of its powerful amplification ability. Among those the most relevant is that vanishing quantities of contaminating DNA molecules will give rise to false positive results. Therefore, stringent measures have to be taken. Only dedicated equipment should be used and PCR work should be restricted to separate areas in the laboratory (ref.9). If sequencing data is to be obtained from PCR products, one has to be aware of base substitutions and additions to the nucleic acid analysed. These artifacts are thought to be due to the fidelity by which the Taq-Polymerase copies the template strand. The rate at which these artifacts occur are 1/7,000 bases for base transitions and 1/40,000 for reading frame errors (ref.10). However, these problems can be overcome by sequencing fragments obtained from individual experiments or by carefully adjusting to assay conditions that increase enzyme fidelity (ref.11).

The following paragraphs will give examples of PCR applications in different areas of medical molecular diagnostics. Specifically, these include the detection of infectious agents, characterization of genetic changes that correlate with malignancies and detection of human genetic disorders.

DETECTION OF INFECTIOUS DISEASES

One area where the PCR technique will undoubtedly become a routine method, is the detection of infectious agents, such as pathogenic bacteria, viruses or protozoa. PCR provides a considerable advantage over other commonly used methods. This is especially true for the identification of non-cultivatable or slow-growing microorganisms such as mycobacteria, anaerobic bacteria etc. or viruses, where tissue culture assays and animal models have to be used or which cannot be cultivated at all. It is immediately obvious that the specific and sensitive results obtained with PCR could indicate microbial contaminations in environmental samples to prevent infections, or in case of manifest infections will more rapidly provide information to guide therapeutic protocols.

The basis for PCR diagnostic applications in microbiology is the discrimination of non-pathogenic from pathogenic strains (e.g. E.coli) by virtue of specific genes. In the case of microorganisms that do not have a normal background flora, all being potentially pathogenic (e.g. S.typhimurium), the detection of any strain has to be goal of the laboratory method. For bacteria and bacteria-like microorganisms an increasing number of appropriate genetic probes has been described. These include *S.typhimurium* (ref.12), strains of mycobacteria including *m.tuberculosis* (ref.13), *L.pneumophilia* (ref.14,15), *enterotoxigenic E.coli* (ref.16), several strains of *Rickettsia* (ref.17), *mycolasma pneumoniae* (ref.18), *B.burgdorferi* (ref.19), *Chlamydia trachomatis* (ref.20) and others. In part, PCR primers as well as genetic probes that specifically detect the amplified fragment are commercially available.

PCR primers have also been reported for intracellular parasites like *T.gondii* (ref.21), *P.falciparum* (ref.22) and for different strains of *Trypanosoma* (ref.23,24).

In virology a large number of PCR assays have been described for the *Human immunodeficiency viruses* (ref.25,26,27), *CMV* (ref.28), *HBV* (ref.29,30), *EBV* (ref.31), *HSV* (ref.32) and others. For example, certain papilloma viruses like types 5 and 8 that are seen in the flat warts, have a 30% chance of turning malignant on sun-exposed areas of the skin, others like types 16 and 18 can be correlated with the appearance of cevical cancers in gynecology. By PCR assays it was shown that the occurrence of a variety of serotypes of Papilloma viruses increased from 25% to 88% in benign and severely dysplastic cells, respectively. In addition, in 90% of cervical carcinoma in-situ and invasive carcinomas only Papilloma Viruses of type 16 and 18 are detected (ref.33). The fact that these assays can be performed directly from cervical smears will also help to further study the correlation of Papilloma Viruses type 16 and 18 with

cervical carcinomas. Clearly, these findings could have a major impact in detecting high-risk populations. For all of the above listed as well as other human viruses, primers and probes are commercially available.

DETECTION OF MALIGNANT DISEASE BY PCR

The detection of leukemias and lymphomas by the PCR method is currently the highest developed in cancer research and is already being used routinely in follow-up studies of patients.

Around 85% of the follicular lymphomas t(14;18) translocations are seen that occur in two well-conserved breakpoints on both chromosomes, the so called "major breakpoint region" and the "minor cluster site". While the sequences involved on chromosome 14 are the Joining genes of the immunoglobulin heavy chain locus, the *mbr*-breakpoints on chromosome 18 are more diverse. However, in around 50% of the cases the *mbr*-sequences are confined to a segment of 150 bp. Therefore, PCR assays can be performed directly on genomic DNA samples to detect translocation-specific malignant cells at a sensitivity which is at least 10,000 fold higher than other methods (ref.34,35).

In around 95% of the cases of chronic myelogenous leukemia as well as some lymphoblastic leukemias, a Philadelphia chromosome is seen. The molecular basis for this translocation is the fusion of the c-abl protooncogene on chromosome 9 to the breakpoint cluster region "bcr" on chromosome 22. While the sequences in the bcr gene that are involved are very confined, the chromosome 9 breakpoints can vary in the range of 100 kb. This is by far outside of the amplification range of PCR assays. To detect the translocation by PCR, the assays therefore have to be performed with RNA as a starting material to amplify a chimeric bcr/abl messages. PCR can then be performed on the first strand cDNA copy of the chimeric message (ref.36). Alternatively, Lee et al. (ref.37) have described a system in which a synthetic oligonucleotide spanning the chimeric junction of the message is protected from S1-nuclease digestion and can serve as template in a subsequent PCR reaction. In case of non-chimeric *abl* and *bcr* messages this oligonucleotide is degraded by S1-nuclease and therefore fails to amplify.

The group of Bartram and coworkers has recently shown a PCR technique for detection of rearranged T-cell receptor δ genes in T-cell lymphomas (ref.38). Between 75% and 87% of these malignancies are said to have rearranged δ genes. Despite a very limited germline repertoire of only 6 known variable genes, 2 diversity genes and 3 Joining-segments, the diversity of the rearranged, functionally active receptor gene is very high, due to imprecise junctions and extensive insertions occuring during the recombination process of the germline components. Following an initial PCR to amplify the whole variable region gene, a second round of PCR is carried out to only amplify the sequences around these diversity genes (nested PCR). The amplified fragment is a specific marker for the receptor gene of that particular patient. There is no cross-hybridization of such clono-specific probes to the T-cell lymphomas of other patients. This approach represents an interesting assay to detect a residual monoclonal disease.

In general, the future work using PCR in prospective studies of this area will help to resolve the following important questions: I) What is the diagnostic and prognostic value of the detection of residual disease ? Residual disease has been detected in patients that have been reported to be in full clinical remission for more than five years. II) What will be the impact of PCR results in terms of therapeutic management of cancer patients ? III) Is there an eventual benefit for the patient who has his residual neoplastic cells detected at an ultrasensitive level and, as a consequence, may undergo a more aggressive therapy ?

Activation of the *ras* oncogenes by single point mutations has been discussed to be involved in the oncogenesis of various human tumours (ref.39,40). Specifically, these mutations occur in codons 12, 13 and 61 and can be detected by PCR (ref.41).

PCR IN HUMAN GENETIC DISORDERS

The number of human diseases which can be tracked down to a genetic cause is rapidly increasing. For most of these data are available that are mainly based on the linkage of a distant genetic marker with a pathologic phenotype and are therefore not amenable to PCR. For less than 100 diseases specific gene sequences are known. Updates on new sequences can be obtained regularly on request (ref.42). In the following paragraph cystic fibrosis serves as an exam-

ple for PCR diagnosis of genetic diseases.

Cystic fibrosis is an autosomal recessive disorder, occurs at a frequency of around 1/2000 in caucasian populations and is regarded the most common fatal genetic disease in humans. From calculation it is estimated that 5% of the population carry a defective allele. Different molecular biology strategies have led to the cloning of the gene for Cystic Fibrosis, called Cystic Fibrosis Transmembrane Conductance Regulator (CFTR) (ref.43). The major defect reported in CFTR is represented by an in-frame deletion of a Phenylalanin residue at the position 508 of the proposed primary structure of the gene product, hence called ΔF_{508}.

Basically, two phenotypes can be distinguished in Cystic Fibrosis. The first is the severely affected group with pancreatic insufficiency and loss of other exocrine functions, the second is more heterogeneous and able to maintain some level of exocrine function. The knowledge of the ΔF_{508} defect has made possible to use PCR to study its distribution in the affected population in more detail. Among 214 affected alleles studied, a total of 68% showed the ΔF_{508} defect. The observation that there are I) severe cases without this defect and II) there are pancreatic sufficient individuals with the defect has lead to the proposal of additional mutations of the CF gene as a cause for Cystic Fibrosis. On the other hand, none of the 198 normal alleles showed the deletion (ref.44). On this basis the following conclusion can be made for the diagnosis of Cystic Fibrosis by PCR. Of the severely affected population 54 % can be accurately diagnosed by PCR using the ΔF_{508} defect as a marker. The results of the PCR assays in this region show that there are other mutant alleles contributing to the severe phenotype, because the ΔF_{508} defect is not found in all patients with pancreatic insufficiency. On the other hand, mildly affected patients can be heterozygous for that ΔF_{508} region, which suggests the existence of mild mutant alleles in these individuals. Since the first report of the CTFR gene more mutations have been described very recently. From 61 communicated mutations, 24 have been published or are "in press" (45). It becomes clear that the number of alleles involved in Cystic Fibrosis may be far bigger than has been originally hoped. Additional data, especially functional studies are necessary to correlate these findings with the CF phenotype. However, with the knowledge of the genomic organisation of the gene, its complete coding sequence and the first mutations identified by PCR and other methods, progress will be more rapid. This may eventually lead to a panel of primers that can be applied for the diagnosis of Cystic Fibrosis by PCR.

SUMMARY

Molecular diagnostic approaches have to meet certain prerequisites to be amenable to routine laboratory work: First, specificity and sensitivity and high reproducibility have to be guaranteed. Furthermore, the speed of the assay may be critical. In addition, the method should be non-radioactive. Finally, the higher automated an assay is, the more easily it is introduced. Under these criteria, Polymerase Chain Reaction clearly has the potential to become the routine laboratory method for diagnosis of a variety of human disorders. Most clearly, the detection of infectious agents surpasses current routine methods in two critical aspects, sensitivity and speed. It also proves valuable for detection of certain malignancies, especially in hematology. Finally, PCR provides an important contribution to the diagnosis of genetic diseases. With the ongoing international effort to sequence the human genome, more nucleic acid sequences will become available. Again, the Polymerase Chain reaction will help to gather this sequence information more quickly than other molecular biology methods.

On the other hand, with the powerful amplification abilities of this method, the contamination of routine laboratory equipment with traces of PCR products has to be the major concern and will require very stringent measures in the laboratory.

There is also another caveat: The recent rapid progress in molecular biology which can in part be attributed to the PCR technology, raises questions in terms of the significance of DNA-sequence differences. The mutations in the CF gene are a good example that molecular biology findings may act in a vacuum of knowledge. The gene for the Cystic Fibrosis has been found by reverse genetics. Originally, there was no protein to fit the data. Postulations with regard to its functional aspects are only based on consensus sequences and homology to similar genes ond their proteins in computer data bases. Within one year more than 60 mutations have been reported (ref.45). While it seems accepted that the ΔF_{508} mutation has a critical impact on the function, the possibility cannot yet be excluded that other mutations which are seen e.g. in

extracellular portions of the protein, are reflecting functional genetic variants. It is also too early to state which combination of polymorphic alleles will cause a mild or even severe phenotype. Therefore, as long as there is no correlation of the sequence to the function, PCR results may largely contribute to a data base of polymorphisms. PCR will get information on all the haplotypes quickly, but since it is not a biological assay, it cannot help to assign that information to the patient's phenotype. As a consequence, so called gene mutations have to be studied in function assays in appropriate animal models or in transfectomas as has recently been done for the ΔF_{508} mutation (ref.46).

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