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Review article

Application of the polymerase chain reaction to the diagnosis of human genetic disease

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Summary. In vitro DNA amplification by means of the polymerase chain reaction is currently revolutionizing human molecular genetics. Since its inception in 1985, a wide variety of different methods and their applications in the diagnosis of disease have been described. This review is intended to serve as a brief guide to current and emerging possibilities in this rapidly expanding field.

Introduction

Following the first report of the cloning of a DNA fragment into a plasmid vector (Cohen et al. 1973), modern molecular genetics became virtually synonymous with the in vitro recombination of DNA. The subsequent development of monoclonal antibodies (Köhler and Milstein 1975) provided molecular biologists with a powerful new tool of tremendous analytical potential, but did not alter the initial requirement for amplified DNA sequences to study nucleic acid structure and function.

In 1985, Saiki et al. described an elegant new method that, by circumventing the need to clone specific DNA fragments, has revolutionized the practice of molecular biology. The original principle had, however, already been described by Kleppe et al. (1971). This technique involves the primer-mediated enzymatic amplification of specific target sequences in genomic DNA by repeated cycles of (1) heat denaturation of the double-stranded template, (2) primer annealing and (3) extension of the annealed primers with DNA polymerase. Target specificity is determined by the choice of short oligonucleotide primers that are designed to hybridize to opposite DNA strands flanking the sequence to be amplified, with their 3′ ends facing inwards. Successive cycles of amplification result in a continuous doubling of, and exponential increase in, the sequence copy number as newly synthesized copies become availabe for primer binding. This versatile technique is now well known as the polymerase chain reaction (PCR) and is typically capable of amplifying a single copy DNA sequence from the human genome approximately 10^9 fold (corresponding to 30 cycles of complete PCR). The method, however, only became widely established once Kogan et al. (1987) had introduced the use of the thermostable *Taq* DNA polymerase (Chien et al. 1976), thus avoiding the necessity of adding thermolabile Klenow enzyme after each denaturation step. This has allowed automation of the procedure with its important advantages of sensitivity, speed and convenience. The remaining question of whether the known high error rate of *Taq* DNA polymerase (one misincorporation per 10000 bases; Tindall and Kunkel 1988) would affect the reliability of the technique in a diagnostic context has been firmly excluded by Krawczak et al. (1989), providing a sufficiently high copy number is employed.

The theory and practice of PCR have previously been reviewed (Mullis et al. 1986; Saiki et al. 1988b; White et al. 1989). More recently, Vosberg (1989) has comprehensively reviewed the PCR technique from the technical standpoint and has briefly described its many applications over the whole spectrum of biomedical research. The dramatic effects of the introduction of a novel technique are nowhere more apparent than in the application of PCR to disease diagnosis. Indeed, the number of reports of the direct sequencing of PCR-amplified disease gene alleles has multiplied exponentially. In this short review, we have attempted to summarize the current applications of PCR to the analysis and diagnosis of human genetic disease, to describe some of the relevant variations of the basic technique reported to date, and to examine the prospects for future advances in the field of diagnostic medicine.

Disease analysis

The genetic diseases that have been analysed to date by PCR methods are summarized in Table 1. Since for any

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Table 1. PCR-based DNA diagnosis of human inherited disease. Unless indicated otherwise, entries refer to direct diagnosis, i.e. detection of the mutation itself (point mutation, deletion, etc.). Closing date for the survey was October 20, 1989

Disease	Reference	Disease	Reference
Acute intermittent porphyria (AIP)	Grandchamp et al. 1989	Haemophilia B (F9 deficiency)	Reitsma et al. 1988 Reiss et al. 1990[a]
Adenosine deaminase (ADA) deficiency	Akeson et al. 1988		
Alpha-1-antitrypsin (AAT) deficiency	Newton et al. 1988 Abbott et al. 1988[a]	Heparin cofactor II deficiency	Blinder et al. 1989
		Hereditary fructose intolerance	Cross et al. 1988
Amyloidotic polyneuropathy	Nichols et al. 1989	Hereditary persistence of fetal haemoglobin (HPFH)	Gilman et al. 1988
Antithrombin III (ATIII) deficiency	Perry et al. 1989		
ApoB deficiency	Collins et al. 1988	Hypoxanthine phosphoribosyl-transferase (HPRT) deficiency	Cariello et al. 1988
ApoC deficiency	Fojo et al. 1988		
ApoE deficiency	Emi et al. 1988	Hypophosphatasia	Weiss et al. 1988
Becker muscular dystrophy (BMD)	Chamberlain et al. 1988 Roberts et al. 1989[a]	Insulin resistance type A	Moller et al. 1988
		Laron dwarfism	Amselem et al. 1989
C1 inhibitor deficiency	Ariga et al. 1989	Lesch-Nyhan syndrome	Gibbs et al. 1989b
Cystic fibrosis (CF)	Kerem et al. 1989 Feldman et al. 1988[a] Williams et al. 1988b[a]	Leukocyte adhesion deficiency	Kishimoto et al. 1989
		Maple syrup urine disease	Zhang et al. 1989
Duchenne muscular dystrophy (DMD)	Chamberlain et al. 1988 Roberts et al. 1989[a]	Non-insulin-dependent diabetes mellitus (NIDDM, type II diabetes)	Kadowaki et al. 1988
Dysfibrinogenaemia	Koopman et al. 1989	Osteogenesis imperfecta	Cohn et al. 1988
Ehlers-Danlos syndrome IV	Tromp et al. 1988	Ornithine transcarbamylase (OTC) deficiency	Hata et al. 1989
Ehlers-Danlos syndrome VII	Weil et al. 1989		
Elliptocytosis	Roux et al. 1989	Phenylketonuria (PKU)	Lyonnet et al. 1989
Encephalomyopathy, mitochondrial	Johns and Hurko 1989	Protolipid protein (PLP) deficiency	Jengnic et al. 1989
Fabry disease	Bernstein et al. 1989	Retinoblastoma	Horowitz et al. 1989
Familial hypercholesterolemia	Leitersdorf et al. 1988	Sandhoff disease	Nakano and Suzuki 1989
Glucose-6-phosphate dehydrogenase (G6PD) deficiency	Vulliamy et al. 1989	Sickle cell anaemia	Saiki et al. 1985
		Spondyloepiphyseal dysplasia	Lee et al. 1989
Gaucher's disease	Zimran et al. 1989	Tay-Sachs disease	Arpaia et al. 1988
Gerstmann-Sträussler syndrome	Hsiao et al. 1989	Thalassaemia beta (HBB)	Wong et al. 1987
Gyrate atrophy	Mitchell et al. 1988	Thalassaemia delta (HBD)	Losekoot et al. 1989
Haemophilia A (F8C deficiency)	Levinson et al. 1987 Kogan et al. 1987[a]	von Willebrand disease (VWF) type IIA	Ginsburg et al. 1989

[a] Indirect RFLP analysis (segregation or linkage disequilibrium assessment)

one condition, the number of reports has usually increased rapidly after the first application has been described, only the first published report in each category is included for the sake of brevity. For the purposes of tabulation, the identification of the disease-causing mutation is regarded as equivalent to the possibility of carrier detection or prenatal (direct) diagnosis in that family. Inclusion of a disease in Table 1 should not however be taken as meaning that prenatal diagnosis has necessarily already been carried out. In most cases, the diagnosis at the DNA level was retrospective in that direct sequencing of the PCR-amplified disease allele was employed to analyse the gene sequence of an individual already suffering from the disease. Determination of the gene lesion has also been accomplished by subjecting PCR-amplified DNA to restriction enzyme cleavage, oligonucleotide discrimination hybridization and mismatch analysis.

To illustrate the rapid development and recruitment of individual applications in disease diagnosis, we shall describe the development of the utilisation of PCR in the analysis of six different genetic diseases.

Sickle cell anaemia

The first report of the use of in vitro-amplified DNA in disease diagnosis described the discrimination of β(A)- and β(S)-alleles for the direct diagnosis of sickle cell anaemia (Saiki et al. 1985). Since the precise base-pair change responsible for the sickle cell disease phenotype had been known for some time, direct analysis of the amplified material could be carried out by oligonucleotide discrimination hybridization, and no further development was required. Whereas this analysis was performed solely with cell line DNAs, Embury et al. (1987) reported its use in prenatal diagnosis using both aspirated and cultured amniocytes as sources of fetal DNA. Although the analysis of PCR-amplified DNA only confirmed the results of a simultaneous Southern blotting analysis, it did serve to illustrate that diagnosis was feasible within one day of obtaining fetal DNA. Radioactivity was still used as a label because of the small fragment size of the respective alleles. Saiki et al. (1988a) further simplified the procedure by employing an automated thermocycler and a non-radioactive enzymatic detection

system. In all these examples, sickle cell disease was used essentially as a test system, diagnosis being both retrospective and confirmatory.

Alpha-1-antitrypsin deficiency

The first report of PCR-based alpha-1-antitrypsin (AAT) deficiency diagnosis (Abbott et al. 1988) described the prenatal determination of intragenic *Mae*III restriction fragment length polymorphism (RFLP) alleles closely linked to, and in linkage disequilibrium with, the PI ZZ genotype. This indirect RFLP analysis was again tentatively performed using previously established phenotypes as retrospective controls. Detection was achieved without further signal intensification by simple ethidium bromide staining of the amplification product. The diagnostic result was only acted upon after confirmation by Southern blotting.

Direct analysis of the AAT-gene Z-allele (G to A transition in exon V) by oligonucleotide probing of PCR-amplified material has also been described (Petersen et al. 1988), but seems difficult to perform in practice. This mutation is the cause of the vast majority of cases of AAT deficiency, and hence diagnosis of this condition is straightforward. Since no restriction fragment length change is introduced by the Z-allele mutation, a viable diagnostic alternative, both convenient and time-saving, is provided by the direct sequencing of amplified DNA. This approach has been described by Newton et al. (1988) and has been used in prenatal diagnosis in two cases. The results (both unaffected) were confirmed postnatally by isoelectric focussing of blood samples.

Haemophilia A

Kogan et al. (1987) first described the use of thermostable *Taq* DNA polymerase and presented the analysis of two intragenic RFLPs (*Bcl*I and *Xba*I) in the factor VIII gene in families with haemophilia A. At the same time, they described rapid DNA isolation procedures that brought a complete diagnosis, together with prenatal sex determination by PCR amplification of Y-specific sequences, within the scope of a day's work. This article was the first to describe an optimized approach to the use of PCR analysis for routine laboratories. The alleles can readily be distinguished by ethidium bromide staining after digestion and electrophoresis of the amplification products. Carrier detection and prenatal diagnostic results were confirmed by oligonucleotide probing of the PCR-amplified samples, Southern blot analysis of genomic DNA and cytogenetic sex determination.

Levinson et al. (1987) demonstrated that direct detection of two previously known *Taq*I site (TCGA) mutations in the factor VIII gene was possible by mismatch analysis of PCR-amplified material using RNase A cleavage of unpaired bases. Although no novel information was gleaned by mismatch cleavage analysis in this particular case, the study illustrated the potential of this method in screening for mutations in genetic diseases exhibiting allelic heterogeneity and where the large size of the defective gene has previously made analysis difficult.

Haemophilia B

The first report of the analysis of the factor IX gene using PCR-amplified material was reported by Reitsma et al. (1988) who sequenced the promoter region of the gene from a patient with a putative regulatory defect, haemophilia B Leyden. However, since multiple sequence deviations occur in the region, functional studies will be required to confirm the involvement of the single base pair change tentatively implicated in the altered developmental expression of factor IX in this family. Siguret et al. (1988) described the unambiguous identification of a CGA to TGA point mutation corresponding to amino acid residue 252 within the factor IX gene causing haemophilia B. The site of mutation had been previously pinpointed by Southern blotting experiments, but PCR-amplified material was used to perform both restriction site analysis and oligonucleotide dot-blot hybridization. This complex approach permitted both carrier detection and prenatal diagnosis without the need for confirmation by Southern blotting. Further reports of point mutations in the factor IX gene (mutations characterized after PCR amplification of specific exons) have rapidly accumulated (e.g. Attree et al. 1989; Green et al. 1989).

Reiss et al. (1990) used the completely known factor IX gene sequence, including introns, to design oligonucleotide primers for indirect PCR diagnosis by RFLP segregation analysis. At the same time, these authors described the amplification of all functional parts of the gene (eight exons covering 34 kb of X-chromosomal DNA). The absence of specific amplification products, noted in the presence of adequate controls, identified a gene deletion that was subsequently confirmed by Southern blot analysis.

Montandon et al. (1989) described the use of chemical mismatch analysis by base-specific modification followed by piperidine cleavage for rapid mutation detection within amplified factor IX gene fragments. Diagnostic applications were presented and amply demonstrated the potential of this ingenious method for future gene analysis.

Cystic fibrosis

The diagnosis of cystic fibrosis (CF) differs from that of other genetic diseases in several ways. The underlying gene has only recently been cloned (Riordan et al. 1989). Since cystic fibrosis is the most common and severe inherited disease in Caucasians, a sufficient number of large and informative families have presented worldwide enabling extremely precise linkage studies to be performed thereby allowing very accurate genetic mapping. The RFLP markers, hitherto used in diagnosis, are not only very closely linked (< 0.1 cM) to, but also exhibit very significant linkage disequilibrium with, the disease allele. For obvious reasons, the anonymous DNA probes used to detect the linked

RFLPs have not been studied as thoroughly as functional genes would have been. Sequence data, required for oligonucleotide primer design, have therefore had to be specially obtained before PCR diagnosis was possible. Feldman et al. (1988) and Williams et al. (1988b) have both described the application of PCR to RFLP analysis for two of these loci (KM19 and CS.7). These authors performed a "same-day" antenatal diagnosis and described a non-invasive and rapid method for obtaining patient material from buccal cells for DNA isolation. Diagnostic results were checked by Southern blot analysis.

Northrup et al. (1989) have reported PCR analysis of the D7S8 locus. Use of this marker serves to increase both the precision and the accuracy of diagnostic results, since it is located on the other side of the CF locus. The widely used CF-markers XV2c (Rosenbloom et al. 1989) and pMP6d-9 (Huth et al. 1989) completed the PCR analysis of RFLPs with linkage disequilibrium to the CF locus (Rommens et al. 1989). The identification of the predominant CF-mutation, present on 70% of the identified CF "chromosomes", will certainly lead to the direct diagnosis of CF by PCR (Kerem et al. 1989).

Duchenne and Becker muscular dystrophy

Because of the mammoth size of the DMD/BMD (Duchenne and Becker muscular dystrophy) gene, even the use of RFLPs located within the gene for segregation analysis is probabilistic as a result of the high frequency of intragenic recomination events. For this particular disease, direct DNA diagnosis is clearly to be preferred. By carefully choosing known deletion "hot spots" for deletion screening, Chamberlain et al. (1988) performed PCR analysis on DMD patients. Approximately 50%–70% of DMD or BMD patients exhibit deletions as shown by Southern blot analysis using the complete cDNA sequence (Koenig et al. 1987) as a probe; the analysis of six chosen deletion 'hot-spots' by DNA amplification of the surrounding sequence permitted the detection of the disease-causing deletion in nearly 40% of patients.

It is important to be aware that deletion screening is qualitatively different from RFLP tracking; the extreme sensitivity of the PCR method makes it extremely vulnerable to contamination. This might easily arise in prenatal diagnosis by maternal cell contamination during biopsy. Both Chamberlain et al. (1988) and Hentemann et al. (1990) have examined the effects of simulated maternal contamination; studies such as these have highlighted the need for very thorough multiple parallel controls to be employed in all diagnostic tests. Deletion screening by PCR has recently been further augmented by the amplification of another deletion hot-spot in the DMD/BMD gene (Speer et al. 1989); this reveals deletions in approximately 10% of DMD patients.

However, there still remains a large proportion of patients with no recognizable gene deletion, and in these cases, only indirect RFLP analysis can presently be offered to at-risk family members. The PCR analysis of three intragenic RFLP markers (pERT87–15/*Xmn*I; pERT87–15/*Bam*HI; pERT87–8/*Taq*I) has been described by Roberts et al. (1989). Since 70% of women should be heterozygous for one or more of these RFLPs, a rapid method of diagnostic screening is now available for the majority of families.

The study of Feener et al. (1989) demonstrated very elegantly that PCR is suitable not only for repetitive routine diagnoses, but is also applicable to very sophisticated analyses that have hitherto not been possible. These authors demonstrated differential splicing of the dystrophin mRNA transcript in muscle and brain by PCR amplification of reverse-transcribed mRNA derived from the two tissues. Such methodology may usefully be applied in a diagnostic context since the huge number of exons within the DMD gene (> 75 at the latest count) implies that splice site mutations may not be an infrequent cause of Duchenne muscular dystrophy.

Other medical applications

For some X-linked diseases, DNA diagnosis is either not yet available or of uncertain reliability (e.g. fragile-X mental retardation syndrome). For many parents, prenatal sex determination thus provides an alternative option. The application of PCR to the detection of Y-specific repetititve sequences has been described by Kogan et al. (1987). The amplification of these sequences is subject to the same difficulties as encountered in deletion screening. However, the chromosome specifity of the alphoid satellite family has successfully been used to distinguish between the X- and the Y-chromosome (Witt and Ericksen 1989) and appears to be more reliable than alternative methods.

The extreme sensitivity of the PCR technique and its suitability for use on partially degraded DNA may also be exploited to determine the genotypes of deceased individuals in cases where conserved material is still available. Such data may often help to complete RFLP segregation analyses; appropriate techniques for DNA extraction have been described for formalin-fixed, paraffin-embedded tissues (Impraim et al. 1987) and Guthrie-spots (Williams et al. 1988a).

Technical variations

The in vitro-synthesis of DNA from a genomic template gives rise to an amplification product that does not contain 5-methylcytosine and cannot therefore reflect any methylation pattern present in the original template. This has led to the identification of novel RFLPs, which had previously gone unnoticed because of the inhibition of restriction enzyme cleavage by cytosine methylation, but which can be exploited by PCR amplification (e.g. the *Hha*I RFLP in the factor IX gene; Winship et al. 1989).

In the vast majority of the RFLP analyses described above, the actual discrimination of the two alternative alleles is effected by restriction enzyme digestion of the amplified product. The only exception is the *Dde*I RFLP in the factor IX gene. This polymorphism is of the unusual insertion/deletion type and can thus be directly

recognized by the size of the amplification product (Reiss et al. 1989). In true restriction site polymorphisms, the digestion step may be replaced by allele-specific oligonucleotide hybridization, which can also be used to distinguish binary alleles. Alternatively, allele-specific oligonucleotides (ASOs; Gibbs et al. 1989a) can be used to discriminate between alleles during the PCR amplification itself. With appropriately chosen stringency conditions for annealing and adequate controls, the presence or absence of PCR products can be used to determine the alleles. This has so far been achieved for both sickle cell anaemia (Wu et al. 1989) and AAT-deficiency (Newton et al. 1989), being termed ASPCR (allele-specific PCR) or ARMS (amplification refractory mutation system), respectively. An interesting variation of this system is the allele-specific template-dependent ligation described by Wu and Wallace (1989) and named ligation amplification reaction (LAR).

Gel electrophoresis is usually employed to check for the presence or absence of a specific PCR amplification product. This would probably not be suitable for mass screening projects, and more routine laboratories might find a simpler test more appropriate. An amplified DNA assay (ADA) as described by Kemp et al. (1989) might be able to substitute for the electrophoresis step by a simple non-radioactive colorimetric detection system. For repeated investigations, a "green or red dot"-system seems feasible: this could in principle be readily adopted for commercial test kits.

Sequence data are clearly a prerequisite for the design of target-specific oligonucleotides to direct the PCR reaction. Counter-intuitively, PCR may also be used to amplify genomic DNA outside the sequence defined by a pair of oligonucleotide primers. This requires appropriate restriction enzyme digestion of the template followed by religation of the digestion products. The PCR primers are located within a known core region of the digested molecules with their 3′ ends facing outwards in contrast to their normal orientation. Religation then joins restriction fragment ends originally located left and right of the core region. During annealing in PCR amplification, the primers that now face each other with their 3′ ends pointing inward, direct the in vitro synthesis of the sequence originally located 5′ and 3′ to the core region. This elegant technique has been named inverse PCR (IPCR) by Ochman et al. (1988) and Triglia et al. (1988) and makes sequence data available immediately adjacent to cloned DNA fragments, e.g. regulatory regions upstream of cloned cDNAs. In a diagnostic context, this technique might be helpful in the identification of polymorphic sites recognized by, but not located within, anonymous DNA probes, thus enabling direct PCR analysis of the polymorphism. Similarly, mutations might be sought in promoter regions of genes (e.g. haemophilia B Leyden) without the necessity of cloning the gene from the DNA of the patient to be studied.

Genes can be mapped to specific human chromosomes by another variation on the basic PCR theme (Abbott et al. 1989) with a precision dependent upon the resolution provided by established lines of somatic cell hybrids. After localization, virtually unlimited numbers of chromosome- or region-specific DNA probes can be created by microdissection of banded chromosomes and enzymatic amplification (Lüdecke et al. 1989).

Future developments

When a new experimental method that promises time-saving and other advantages becomes available, its future application in diagnostics is dependent upon both its accuracy and reliability compared with alternative procedures. It is important to stress that the findings of Krawczak et al. (1989) have not in any way reduced the absolute necessity for contamination-free work (Lo et al. 1988; Kwok and Higuchi 1989). Premixing of reactions, parallel running of "blanks" and restriction-positive controls in RFLP analysis (Reiss et al. 1990) should at least allow early detection of the various possible sources of error.

Improvements have been suggested ever since the development of the PCR procedure, e.g. for contamination-free non-invasive sampling by using epithelial cells in urine rather than buccal cells as described originally (Gasparini et al. 1989). The handling of prenatal samples, where it may never be possible to completely exclude maternal cell contamination, has been dealt with (Chamberlain et al. 1988; Hentemann et al. 1990). Finally, it is unclear whether direct comparisons between PCR- and Southern blotting-derived results provide a suitable means of estimating the reliability of PCR, since Southern blotting cannot itself be regarded as 100% reliable in the context of mutation detection.

PCR amplification of cDNA sequences promises to be an effective tool in disease diagnostics. Mutation detection in large and complex genes exhibiting allelic heterogeneity (e.g. factor VIII gene/haemophilia A) should be greatly facilitated by the use of PCR-amplified cDNA sequences as a substrate either for direct sequencing or for mismatch pairing analysis. Harbarth and Vosberg (1988) first described a method to prime specifically the reverse transcription of a myosin cDNA. Gene-specific oligonucleotide primers have also been used to amplify cDNAs derived from rare mRNA transcripts (Frohman et al. 1988) such as those originating from the basal level of illegitimately transcribed tissue-specific mRNAs (Chelly et al. 1989; Sarkar and Sommer 1989). The vast expansion of applications of PCR in diagnostic medicine, even with its relatively short history, indicate that this powerful technique will eventually be widely used.

It is certainly worthy of note that the medical exploitation of the PCR technique is not limited to diagnosis, but may also be applied to the treatment of human genetic disease. The technical foundation has already been laid for the correction of single bases in long DNA sequences (Dulau et al. 1989); the precise reconstructive engineering of complete genes and their control elements may even be possible (Yon and Fried 1989).

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