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Feature Articles

The Polymerase Chain Reaction: A New Tool for the Detection of Minimal Residual Disease In Haematological Malignancies

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The polymerase chain reaction (PCR) is a novel technique for the *in vitro* amplification of specific short DNA fragments, which permits a selective and up to 10^7 fold enrichment of the target sequence. The method is increasingly being used for the molecular genetic analysis of hereditary, infectious and neoplastic disorders. The use of PCR for the detection of minimal residual disease in particular types of leukaemia or lymphoma, such as chronic myelogenous leukaemia expressing specific BCR/ABL-RNA and follicular non-Hodgkin lymphoma with the chromosomal translocation t(14;18) are reviewed. In acute lymphoblastic leukaemia clone-specific sequences from rearranged antigen receptor genes may be molecular markers suitable for amplification. Although PCR holds great promise for "molecular" staging and follow-up, several technical problems have to be kept in mind, and the clinical relevance of PCR-based evidence of minimal residual disease in haematological malignancies requires further investigation.
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THE POLYMERASE chain reaction (PCR) is one of the most fundamental recent advances in molecular genetics, with an exciting potential for use in clinical medicine. It was originally reported in 1985 [1] as a rapid and highly sensitive prenatal diagnostic test for sickle cell anaemia, and has rapidly gained popularity for the molecular genetic analysis of hereditary, neoplastic and infectious diseases [2, 3]. We discuss the application of PCR to the detection of minimal residual disease in haematological malignancies.

PCR is based on an exponential *in vitro* enzymatic amplification of a specific target DNA fragment resulting in a highly specific, 10^5–10^7 fold enrichment of the sequence of interest (Fig. 1) [2, 3]. The use of a heat-stable *Taq* polymerase permits the method to be fully automated and completed within a few hours. PCR-amplified DNA fragments can either be visualised in stained agarose gels, sequenced directly without prior cloning or identified by blot hybridisation with labelled allele-specific oligonucleotide probes and autoradiography [1–4].

MINIMAL RESIDUAL DISEASE

Although for many haematological neoplasms effective treatment resulting in complete remission and sometimes long-term disease-free survival is available, relapses of leukaemia or lymphomas are frequent. In most instances recurrence occurs through proliferation of residual neoplastic cells that had not been eradicated by therapy. The detection of small numbers of such cells at the time of clinical remission is an important and largely unresolved problem. The sensitivity of techniques such as morphology, immunophenotyping, cell culture or detection of molecular tumour markers by Southern filter hybridisation to detect minimal residual tumour cells is 0.001% at best (as reported for immunophenotype analysis with double antibodies and flow cytometry) [5]. However, most of the currently available methods (for example, morphology or Southern blotting) fail to detect malignant cells below the 1–5% level. Amplification of molecular tumour markers by PCR now offers a much more sensitive, rapid and highly specific approach to detecting small numbers of malignant cells in blood or marrow remission samples.

Very small amounts of nucleic acids obtained from fresh blood, bone marrow aspirates or from tumour biopsy specimens are sufficient for PCR analysis. In addition, partly degraded DNA extracted from paraffin-embedded biopsy material may still be suitable for PCR amplification [6]. Serial dilutions of tumour cell DNA with DNA from normal cells have shown that PCR can detect DNA sequences specific for a particular neoplasm at a $1:10^6$–$1:10^7$ level, which is a major improvement in sensitivity over current routine methods [3, 7, 8] (Table 1).

The use of PCR to detect minimal residual leukaemia or lymphoma after treatment requires that a few pre-conditions are met. First, the malignant cells must carry a clonal somatic mutation in their genome that is absent in normal cells. Secondly, the nucleotide sequences flanking the target fragment must be known, to design PCR primers. Thirdly, for practical purposes, the target sequence may not be longer than, say, 5000 to 6000

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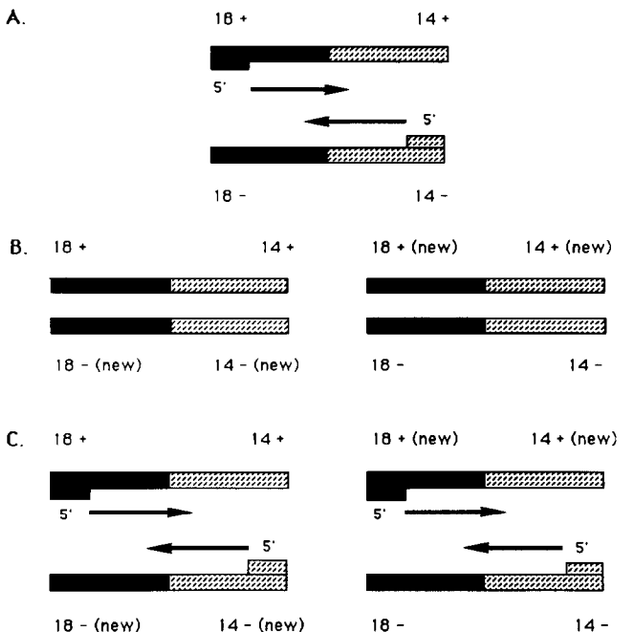

Fig. 1. Principle of PCR amplification of specific DNA sequence, illustrated by example of t(14;18) breakpoint in follicular NHL [7, 13, 14]. Solid bars = DNA sequences from chromosome 18; hatched bars = DNA sequences from chromosome 14. Boxes = primers hybridising to sequences flanking the breakpoints (solid box = primer to mbr on chromosome 18; hatched box = primer to J_H region on chromosome 14).

PCR requires small amount of genomic DNA, including target sequence, heat-stable DNA polymerase (*Taq* polymerase), nucleotides as substrates (ATP, CTP, GTP and TTP, molecular "bricks" to build up DNA strand) and pair of specific synthetic oligonucleotide primers complementary to sequences on opposite strands flanking target sequence. Technique is based on repetitive cycles of three-step *in vitro* reaction: (A) double-stranded hybrid (14;18) DNA (target) is split into two single strands (+ and −) by heating. Cooling of reaction mix permits primers to anneal to their complementary sequences on genomic DNA. These islands of double strands in otherwise single-stranded DNA provide signals to start enzymatic DNA synthesis which proceeds along respective (14;18) single DNA strands (templates) in 5′ to 3′ direction (arrows); (B) after completion of first cycle, two new double-stranded hybrid (14;18) sequences have been synthesised, each containing "old" and new strand; (C) to start new cycle, double-stranded DNA fragments are again heated to become single-stranded; primers anneal to their complementary sequences, and enzymatic synthesis of new strands is resumed (arrows). This second cycle will lead to four-fold amplification of target DNA sequence. Continuing such cycles results in exponential amplification of specific hybrid (14;18) sequences up to about 10^7 fold.

base-pairs [1, 4]. Finally, the "molecular" follow-up of a case requires that the marker mutation remains stable and representative for the malignant clone throughout the course of the disease. In practice, the requirements to ensure successful application are at present only met in a few (nevertheless important) types of haematological malignancies (Table 1).

PCR FOR DETECTION OF MINIMAL RESIDUAL DISEASE IN LEUKAEMIAS AND LYMPHOMAS

Chromosomal translocation t(14;18) in non-Hodgkin lymphoma

Most cases of follicular non-Hodgkin lymphoma (NHL) and 20–30% of high-grade diffuse NHL have a translocation t(14;18) involving the immunoglobulin (Ig) heavy-chain gene locus on chromosome 14 (J_H) and the *bcl-2* gene (*B-cell leukaemia-lymphoma-2*) on chromosome 18 [9]. The breakpoints are clustered within short chromosomal regions, those on chromosome 18 either in a major breakpoint region (*mbr*) at 18q21 [10, 11] or a minor cluster region (*mcr*) further downstream [12], and those on chromosome 14, 5′ to or within J_H [9–11]. The translocation creates a stretch of DNA composed of hybrid t(14;18) sequences unique to the lymphoma and absent in normal cells (Fig. 1). Synthetic primers flanking the crossover sites, one on chromosome 14 (a consensus J_H primer) and one on chromosome 18 flanking the *mbr* or the *mcr*, provide the starting signals for the *in vitro* synthesis of DNA strands crossing from chromosome 14 to 18 and vice versa (Fig. 1). The t(14;18) amplification product can finally by identified through specific hybridisation with "internal" breakpoint DNA probes or direct sequencing. The beauty of this approach is that the vast majority of t(14;18) NHL can be analysed with a limited set of J_H, *mbr* and *mcr* primers to provide a "general" PCR test for the detection of t(14;18) breakpoints [6, 13, 14].

PCR has been used for the "molecular" staging of patients presenting with apparently localised t(14;18) lymphoma or patients with lymphoma thought to be in complete remission. In several such cases, samples from peripheral blood, marrow or lymph nodes that were morphologically normal contained occult lymphoma cells detected by the amplification of lymphoma-specific J_H-*mbr* sequences [13–15]. PCR may also be useful to look for minimal residual lymphoma cells in remission marrow or stem cell harvests taken for programmes with intensive chemotherapy and autologous peripheral stem cell or marrow reinfusion [14, 16, 17]. Recent data suggest that, similar to the t(14;18), the translocation t(8;14) present in Burkitt's lymphoma is suitable for PCR amplification [18].

Table 1. Detection of minimal residual disease in leukaemias and lymphomas by PCR

Neoplasm	PCR target	Sensitivity reported*	Ref.
NHL, follicular or diffuse high grade	t(14;18) hybrid sequences	$1:10^5$ to $1:10^6$	7, 14
CML	BCR/ABL fusion mRNA	$1:10^5$ to $1:10^7$	8, 25
ALL	Ig heavy-chain gene rearrangements		
	V_H-D_H-J_H junction (CD III)	$1:10^4$	35
	V_H251-region	$1:10^4$	38
	TCR gene rearrangements		
	TCR γ Vγ9-Jγ junctions	$1:10^6$	37
	TCR δ Vδ1-D-Jδ1 junctions	$1:10^5$	39

*Sensitivity assessed in mixing experiments with serial dilution of DNA/RNA from malignant cells with DNA/RNA from normal cells (except for ref. 35 where normal and malignant cells rather than nucleic acids were mixed).

Chronic myelogenous leukaemia with the Philadelphia chromosome, translocation t(9;22)

More than 90% of patients with chronic myelogenous leukaemia (CML) have the Philadelphia chromosome (Ph), a non-random reciprocal translocation between chromosomes 9q34 (bearing the c-*abl* oncogene) and 22q11 (carrying the *phl* or BCR gene) creating a novel BCR-ABL-fusion gene [19]. While the breakpoints on chromosome 22 are limited to a 5.8 kb breakpoint cluster region (BCR; also known as "major cluster region"), those on chromosome 9 are scattered over variable positions up to more than 150 kb 5′ to the second exon of the c-*abl* oncogene. Unfortunately, PCR is not readily applicable to amplify translocation sequences with such highly variable breakpoints on one of the chromosomes involved. Despite its genomic variability the BCR-ABL-fusion gene is transcribed into a consistent type of chimaeric BCR-ABL-messenger RNA unique to CML cells [19]. Several elegant studies have shown that sequences from BCR-ABL-fusion RNA can be amplified by initially synthesising a complementary DNA (cDNA) fitting the BCR-ABL-mRNA template with reverse transcriptase. This product can then be amplified by PCR with a BCR-derived and an ABL-derived primer and identified by hybridisation with specific oligonucleotide probes [8, 20–22].

Lee *et al.* (22) studied eight CML patients treated with recombinant α-interferon. At the time of investigation they had been in remission for six months to three years, their marrow was normal morphologically and Ph-negative on cytogenetic analysis, and Southern filter hybridisation failed to detect clonal BCR rearrangements found in samples from initial presentation. However, BCR-ABL-mRNA was amplified from all the samples suggesting that the malignant clone, although suppressed by treatment had not been eradicated. A single case of CML has been reported where recombinant α-interferon apparently resulted in a haematological and molecular remission as assessed by PCR [23]. The fact that the molecular CML marker can still be amplified in remission samples is perhaps not surprising, given that current therapies for CML are not normally curative, except for allogeneic bone marrow transplantation (BMT).

Disease recurrence after BMT still remains a problem. Several studies have used PCR to search for minimal residual CML cells in post-transplant remission samples. Results are contradictory and their clinical relevance must be appraised cautiously. Morgan *et al.* [24] found no evidence of leukaemic RNA in the marrow from CML patients who were in continuing remission up to seven years after BMT. However, several other groups found many of their CML patients in long-term post-BMT remission to be positive for PCR amplification products [25–29]. Serial PCR analyses by Delfau *et al.* [25] suggest that in some cases BCR-ABL-mRNA amplification products may be present only transiently. Cases negative after a first round of PCR cycles can sometimes turn out to be positive if amplified further with a second round of internal ("nested") primers [25, 30]. The improved sensitivity of two-step over single-step PCR for the detection of minimal residual CML is attractive, but systematic comparisons between the various methods are at present not available [31].

In some studies, several CML patients with positive PCR amplification results had received T-cell depleted marrow-transplants [27] while those found to be negative had not [24]. This is intriguing in the light of clinical data that have shown T-cell depletion in the donor marrow to be associated with a high risk of CML relapse [32]. However, if current PCR data are summed up from various reports, there appears to be no firm association between PCR results and T-cell depletion of donor marrow [31].

In conclusion, amplification of t(14;18) DNA or BCR-ABL RNA sequences is possible with standard sets of primers and internal probes applicable to virtually all cases of follicular NHL and CML, respectively. While the simplicity of this approach to detect minimal disease is attractive, its main drawback is the high risk of false-positive results.

Minimal residual disease in acute leukaemias

Virtually all acute lymphoblastic leukaemias (ALL) are characterised by antigen receptor gene rearrangements in the genome of the malignant cells [33, 34] which provide leukaemia-specific markers suitable for PCR amplification. Ig or T-cell receptor (TCR) gene rearrangements are unique to a given B cell or T cell and its progeny, since they are composed of variable (V) segments and diversity (D), joining (J) and constant regions selected from a large germline gene repertoire [33, 35]. Due to the heterogenous nature of these gene rearrangements no PCR method is universally suitable for the detection of minimal residual ALL. Present strategies are based on, firstly, preparing leukaemia-specific probes derived from clonally rearranged Ig heavy-chain or TCR γ and δ gene sequences and, secondly, use of these probes for the specific identification of the respective sequences amplified from remission samples (Table 1). This implies that DNA from the original ALL blasts must be available to characterise individual gene rearrangements before remission samples can be studied.

Leukaemia-clone-specific probes suitable to detect minimal residual ALL can be generated by molecular cloning and sequencing of either amplified VH-DH-JH junctional sequences (including hypervariable complementarity-determining regions) from Ig heavy chain gene rearrangements [35, 36] or V-J-junctional fragments from rearranged TCR γ genes present in ALL blasts [37]. Both methods depend on the *in vitro* synthesis of leukaemia-specific ("custom-tailored") probes to identify residual malignant cells in remission samples, and might, therefore, be too laborious for routine diagnostic application. A somewhat different strategy is based on the amplification of Ig heavy-chain gene rearrangements containing a particular V region (V_H251) preferentially involved in malignant B cells such as ALL blasts [38]. Yet another approach is to amplify a clone-specific DNA fragment from rearranged TCR δ gene junctional regions which can then be used directly as an individual-specific probe to detect residual leukaemic disease in remission samples [39]. Due to the high frequency of J_H, TCR γ and δ gene rearrangements in both B-cell and T-cell ALL, these approaches are likely to be applicable to most ALL cases.

PCR amplification of clone-specific antigen receptor gene rearrangements certainly holds promise for a "molecular" follow-up of patients treated for ALL [36], and possibly NHL. A particular advantage is that apparently the generation of clone-specific probes by PCR is less likely to produce false-positive results caused by contamination than amplification of translocation breakpoints in CML and t(14;18) NHL. However, several other problems must be addressed. Theoretically, clone-specific probes derived from clonally rearranged Ig or TCR genes in leukaemic blasts might either detect residual malignant cells for which the sequence is assumed to be specific, or normal lymphocytes present in a remission marrow which by mere chance happen to harbour the very same type of gene rearrangement. Due to the large variety of antigen receptor gene rearrangements in normal polyconal lymphocytes, the chances of

obtaining such false-positive results are extremely small and control experiments have so far shown that in practice this problem does not occur [37, 38].

Up to 35% of ALL cases have more than one type of Ig gene rearrangement reflecting oligoclonality rather than monoclonality, which may hamper the detection of minimal residual disease by particular clone-specific probes [40, 41]. Ig or TCR gene rearrangement patterns may change over time [34, 42, 43]; for example, up to 40% of relapsed ALL patients show alteration of Ig gene rearrangements due to clonal evolution [42]. Clone-specific probes designed to match rearrangements characterised in presentation samples will fail to detect relapsing ALL subclones with altered Ig or TCR gene rearrangements.

In contrast to ALL, very few molecular markers have been described for acute myeloblastic leukaemia (AML). Molecular analysis of most AML-specific chromosomal translocations has not yet proceeded far enough to permit PCR amplification of leukaemia-specific sequences, with the possible exception of the t(6;9) breakpoints [44]. N-*ras* oncogene point mutations are found in 20–30% of AML cases only [45], and their use is further hampered by the fact that they may be restricted to a few leukaemic cells and that mutations detected at presentation may differ from those seen in relapse [46–48].

CRITICAL APPRAISAL

Application of PCR to the study of minimal residual disease in haematological malignancies has opened new and exciting prospects for a molecular definition of remission, and the technique may soon have a major impact on the management of patients with these disorders. It is therefore mandatory that haematologists and oncologists familiarise themselves with some of the method's inherent problems.

False positives

False-positive PCR results caused by contaminations are a very serious problem. Minute numbers of cells or traces of DNA that contaminate syringes, pipette tips, tubes or gloves may easily be amplified, causing problems in interpreting the results. This can only be avoided by most stringent precautions while collecting samples, during their manipulation in the laboratory and by including appropriate controls (such as amplifying samples containing PCR reagents but no DNA, and DNA samples known to be either negative or positive) [49]. This problem is more likely to affect the amplification of translocation breakpoints with generally applicable sets of probes and primers (for example, in CML) than the detection of minimal residual disease with clone-specific probes, as in ALL.

The sensitivity of PCR to detect minute amounts of a particular nucleic acid sequence depends on the number of amplification cycles performed, as demonstrated by the results of two-step vs. single-step PCR in CML after bone marrow transplantation [25, 30]. However, pushing amplification along for extended numbers of cycles may increase the risk of false-positive results, as perhaps illustrated by a recent report on prenatal sex determination by PCR analysis of maternal blood leucocytes [50]. Two rounds of Y-chromosome-DNA amplification in peripheral blood samples from pregnant women consistently yielded false-positive results (i.e. detection of amplified Y-sequences in blood samples of mothers pregnant with a female fetus) if the second round was extended beyond twenty cycles.

Apart from false-positives, a positive PCR result in a patient with lymphoma or leukaemia in remission means that the particular molecular marker has not been eliminated by treatment, and that, presumably, malignant cells are still around. However, PCR cannot identity their origin, their lineage or their proliferative potential [31]. Also, the method does not precisely tell us how many leukaemic or lymphoma cells are left in a sample, although quantitative estimates of residual malignant cells seem to be possible, for example by combining PCR with a phage-quantitation assay [36].

False negatives

False-negative PCR results could be due to several factors. Experimental evidence suggests that minimal residual leukaemic cells may be distributed non-randomly in the marrow [51], and they could simply be missed at sampling. Similarly, extramedullary relapse could be missed, as illustrated by a case of ALL relapse in the central nervous system with a PCR-negative bone marrow [36]. Despite the method's high sensitivity, cells present at less than $1:10^6$ might still not be detected. Errors could also occur through genetic variation at sites of oligonucleotide primer binding. For example, DNA polymorphism in a sequence expected to be complementary to a primer would prevent its annealing and would lead to failure of PCR to create new DNA from that chromosome [52].

RNA amplification (as in CML) requires that the cells of interest actually express RNA at the time of sampling, which might not necessarily be the case in "resting" malignant cells. It is theoretically possible that some of the negative results of BCR-ABL RNA amplification in CML remission patients [24, 25] are due to the lack of gene expression rather than the absence of malignant cells.

Finally, clone-specific probes designed to match sequences from antigen receptor gene rearrangements found in ALL patients at initial presentation will miss minimal residual disease if Ig or TCR gene rearrangement patterns change due to clonal evolution of ALL.

CONCLUSIONS

Although the clinical relevance of minimal residual disease detection by PCR remains to be determined, this new technology will permit for the first time several important questions to be addressed. What is the natural history of minimal residual leukaemia or lymphoma? Will the detection of minimal residual disease by PCR correctly predict those patients treated for leukaemia or lymphoma likely to relapse and those cured? Is complete eradication of leukaemic cells really necessary to guarantee long-term disease-free survival and cure? Would a positive PCR result in a patient shown to be in complete remission by all other criteria justify additional (in most instances fairly toxic) treatment? There is at present no strong case to be made to treat leukaemia or lymphoma relapse at a very early stage, and for the time being its detection by PCR might well be irrelevant for clinical practice. How many individuals chosen from a healthy normal population would be positive for BCR-ABL-amplification products? How many of those would later develop CML?

It would certainly seem mandatory to incorporate PCR into clinical lymphoma and leukaemia trials to follow up patients prospectively to answer some of these issues. Ideally, PCR conditions would have to be clearly defined within a particular trial or between trials to obtain standardised and, hence comparable, results. Running such studies will require close collaboration by clinicians and their colleagues in the molecular biology laboratory, and success is more likely if both parties are able to understand each other's language.

Note added in proof

In addition to refs 21–30, the further interesting studies on the follow-up of CML patients after allogeneic BMT by BCR-ABL PCR have been reported [53, 54].

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Smoking and Cancer with Emphasis on Europe

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A summary of smoking and cancer in various European countries is presented. Important points are the tobacco/alcohol interaction in the elevated mortality rates from upper digestive and respiratory tract neoplasms in France and other southern European countries, the delay in the lung cancer epidemic in females compared with the situation in North America (with the major exception of the United Kingdom) and the different pattern of lung cancer rates in younger compared with older generations (which suggests that eastern and southern European countries will have the highest lung cancer rates at the beginning of the next century in the absence of urgent intervention). The efficacy of anti-smoking policies in Scandinavian countries which now have the lowest lung cancer rates in Europe and the persisting importance of high-tar dark-tobacco cigarettes in eastern and southern Europe in enhancing the risk not only of cancer of the lung but also of upper digestive and respiratory and bladder neoplasms are also discussed.
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INTRODUCTION

CLEMMESEN [1] comprehensively reviewed the historical development of cancer epidemiology, including associations between tobacco smoking and cancer. Several observations were made in the last century about associations between pipe smoking and the occurrence of cancer of the lip in particular and other oral sites. The general conclusion was that local trauma, including thermal irritation, was an important risk factor. In 1936 Flecksseder [2] reported from Vienna that 51 of 54 patients with bronchial carcinoma were cigarette smokers. Similar findings were reported by Muller [3] from Cologne in 1940, with the advantage of observations in a comparison ("control") series of patients, age-matched to the cases. Thus, associations between smoking and cancer were being noted by European clinicians even before they were quantified in one of the first modern epidemiological studies by Doll and Hill [4] in 1950.

Today it is recognised that cigarette smoking is by far the most important cause of lung cancer, and an important determinant of cancer risk for at least six other anatomical sites—oral cavity and pharynx, oesophagus, larynx, pancreas, kidney and bladder. Estimates of relative risk of cancer from a vast amount of epidemiological data, for current smokers, are of the order of 10 to 20 for the lung, between 5 and 10 for the oral cavity and

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