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REVIEW ARTICLE

The Polymerase Chain Reaction and Dermatology

A New Technique with Important Implications for the Study of Skin Inflammation and for Diagnostic Tests of Dermatological Disorders

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The polymerase chain reaction is a powerful and versatile tool for the analysis of nucleic acids. Through a reaction imitating *in vivo* DNA replication, a defined fragment of DNA is repeatedly replicated by a DNA polymerase in an exponential manner. Such selective amplification of a sequence of interest has created new possibilities in molecular biology and related sciences. The basic principle and some of its variations and applications for both qualitative and quantitative analyses of potential interest to dermatologists are described.

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Basically the polymerase chain reaction (PCR) is a technique for selective, exponential *in vitro* amplification of a specific DNA fragment out of a more complex mixture. RNA too can be studied by PCR through reverse transcription into cDNA, which is an excellent template for PCR amplification. The reaction mimicks natural DNA replication, utilizing the properties of one of the naturally occurring DNA polymerases, the enzymes that copy DNA. While the replication machinery *in vivo* copies full length DNA strands, PCR copies a selected, short DNA fragment determined by specially designed primers for the synthesis of the new strands. Due to the rapidity and simplicity of the reaction, its immense power, and numerous ingenious variations, the technique has proved almost revolutionary in many fields of biological and medical science. Where availability of material has been limiting for study or diagnosis, PCR opens quite new possibilities. Thus, from total DNA or RNA preparations from extremely small tissue samples, well-defined fragments can be amplified to amounts amply sufficient for analysis or experiment. This brief review is meant as an introduction to the principles of the technique and its use both for basic studies of dermatological disorders and as a new tool for the diagnosis of diseases. For more comprehensive reviews, readers are referred to Erlich (1) and White et al. (2).

THE BASIC PRINCIPLE OF PCR

The reaction

Like DNA replication *in vivo*, PCR DNA amplification requires a DNA polymerase, a template, and primer together with a pool of deoxyribonucleotide triphosphates for synthesis of a new strand complementary to the template. With two primers, which are usually chemically synthesized oligonucleotides of 18 – 30 bases, complementary to opposite strands of a double-stranded DNA molecule and flanking the sequence of interest, this sequence can be copied into two new double-stranded fragments (Fig. 1A), and the process can be repeated to create four fragments, then eight, etc. (Fig. 1B). For each repetition of the process, the newly synthesized strand must be separated from its template at high temperature. Thermostable polymerases such as the *Thermus aquaticus* (Taq) DNA polymerase survive the high denaturation temperatures and have working optima about 70°C. Therefore, by repeated temperature shifts permitting sequential denaturation of double strands, annealing of primers to templates, and activity of the enzyme, repeated rounds of doubling can be carried out without fresh addition of enzyme and with high specificity (3).

The product

In the first cycle (Fig. 1A), the length of each new strand will vary, but extension time is chosen to permit it to include the sequence complementary to the other primer. After the next round two of the newly synthesized strands have a well-defined length (from primer through primer), and the proportion of amplification product having this exact length increases for each round (Fig. 1B) until at the end of the chain reaction this fragment will constitute the predominant proportion and be the only visible product in gel electrophoresis.

The template

The starting material is not restricted to DNA, but can be RNA as well, usually mRNA after reverse transcription into single-stranded cDNA. In the first cycle one primer binds to the cDNA, and a complementary strand is synthesized containing the binding site for the second primer. Hereafter the process is identical to the one shown in Fig. 1. RNA can also be used directly as the target, since some polymerases used in PCR contain reverse transcriptase activity (4). If mRNA preparations are contaminated with traces of genomic DNA or *vice versa*, amplification products from the two sources can be distinguished by size if primers are designed to flank an intron.

Kinetics

The process is extremely fast. Each cycle takes only a few minutes, and since most experiments require 15 – 45 cycles, they can be completed within a few hours. The theoretical amplification factor is 2^n, where n is the cycle number, predicting a million-fold amplification in 20 cycles (Fig. 2, dotted line). In practice, however, the efficiency is not 100%, and the

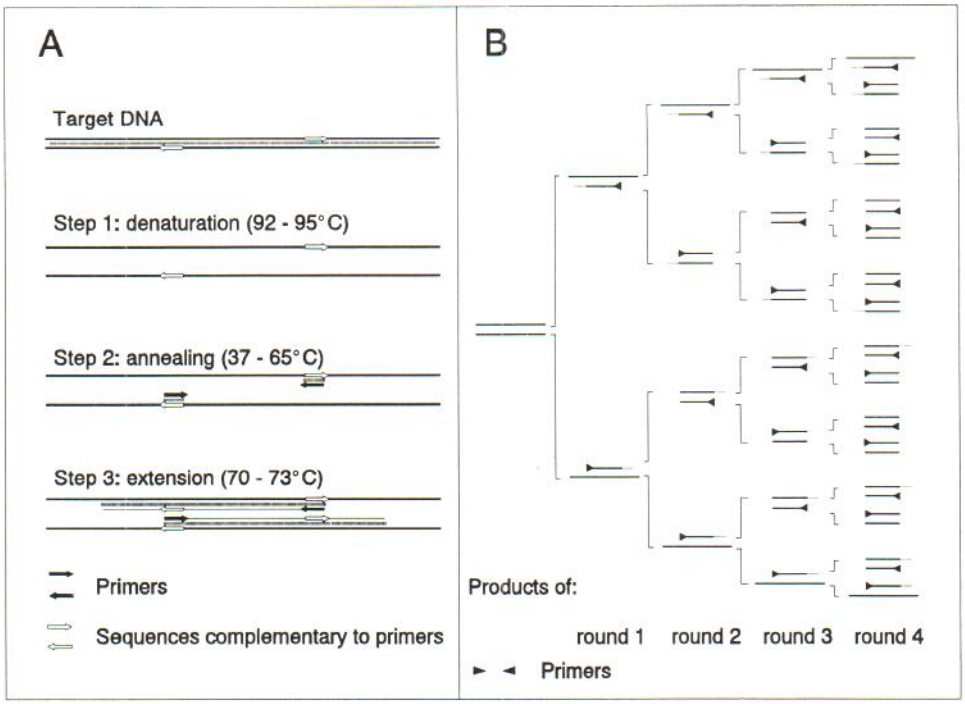

Fig. 1. Principle of the PCR. A: Details of the first cycle. The reaction mixture contains, in an appropriate buffer, template DNA, primers, dNTPs and Taq DNA polymerase. Heating to about 94°C separates doublestranded DNA into single strands. Cooling to an appropriate temperature, typically 50–55°C, permits the oligonucleotide primers to single out and bind to their complementary sequences. After raising of the temperature to about 70°C, polymerization takes place, and a new strand is synthesized complementary to each old one. The direction of arrows is 5' to 3'. B: Accumulation during successive cycles of the fragment defined by the distance between primers.

amplification formula is $Y = I \chi (1 + E)^n$, where Y is the yield after n cycles, I the initial amount of target, and E the efficiency, which is roughly proportional to the length of the product (5). In addition, this formula holds only part of the way, because exponential amplification will at some point be succeeded by a plateau phase (Fig. 2, fully-drawn curve). It is, however, possible to obtain amplification factors of 10^6 to 10^9, which serves most purposes, and re-amplification of the product of one reaction can further increase the yield.

VARIATIONS OF THE TECHNIQUE

Amplifying flanking sequences

The original design described above offers amplification of a sequence *between* two known sequences with primers pointing towards each other. A variant version permits amplification of sequences *flanking* two primer sequences pointing away from each other ("inverse" PCR). By digestion with a restriction enzyme cutting outside the flanking sequences, and circularization of the restriction fragments by self-ligation, the primers are brought to point towards each other through the circularized molecule containing the flanking sequences. These can then be amplified conventionally (6, 7).

Amplifying DNA without two known sequences

If the protein but not the DNA sequence is known, all possible DNA sequences that can encode the particular amino acid sequences of the chosen primer binding sites can be deduced, and mixtures of primers (degenerate primers) designed accordingly. Such degenerate primers will often suffice to amplify a given cDNA sequence. Unknown sequences cloned in e.g. plasmid or bacteriophage vectors can be amplified with primers complementary to vector sequences. Total cDNA may be ligated to vector sequences or specially designed oligonucleotides (8, 9) and amplified by this method, or amplified directly after tailing of first-strand oligo-dT primed cDNA with a dG-sequence, using oligo-dT and oligo-dC primers (10, 11).

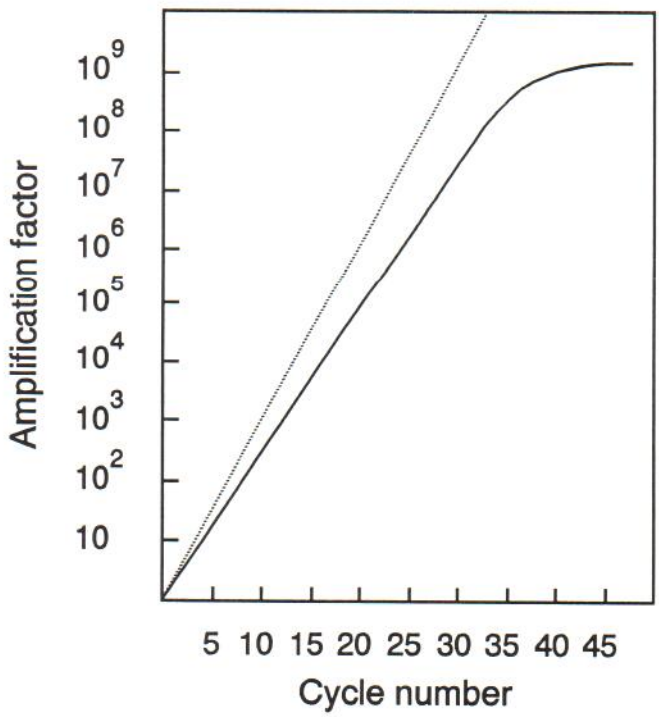

Fig. 2. Theoretical and practical results of 45 PCR cycles. The dotted line represents the theoretical amplification of 2^n, the fully-drawn curve the results of an amplification following the formula $Y = I \chi (1 + E)^n$ with $E = 0.7$ up to 30 cycles, then gradually going into a plateau phase. The range of exponential amplification is both cycle dependent and input dependent.

Altering sequences

A complete match between template and 5'end of primer is not necessary. With primers containing a perfect 3' match and a 5' sequence differing from the template, the PCR can therefore be used to create amplified fragments bordered by altered sequences. This can provide convenient restriction enzyme recognition sequences for cloning of the product. It also provides a more rapid way to site-directed mutagenesis than other methods (12, 13). Building in of the T7 promoter allows *in vitro* transcription of the product, a method which can add to the sensitivity by giving an additional amplification factor of up to 500 (14, 15).

QUANTITATIVE PCR

The formula $Y = I \chi (1 + E)^n$ predicts that the yield of n cycles is proportional to target input. Quantification by PCR should thus be possible. Although E ought to be constant for a given primer set it may, however, vary from one reaction to the next due to variation in any of a number of parameters ("tube effects"). Another complication is the exponential phase being followed by a plateau phase after a number of cycles (Fig. 2), whereupon proportionality is lost (5).

A number of methods have been devised, however, to overcome these problems. These methods can be grouped according to three main principles, which can be varied and combined:

I. Titration with a competitor target

A molecule is designed to be identical with the target except for a single difference in size or sequence, so that the amplification product can be distinguished from that of the natural target by size or restriction enzyme digestion (16–18). Known amounts of the competitor molecule are added to the sample and co-amplified. Where equal amounts of target and competitor products are seen, the initial target amount must have been identical to the amount of competitor added. This approach allows absolute quantification, is independent of tube effects, and is partly cycle independent.

II. Kinetic analysis

This method simply reproduces the picture of the fully-drawn curve in Fig. 2. Aliquots of the sample are amplified at different cycle numbers, and the range of proportionality (the linear part of the curve in a semilog plot) determined. The initial target amount can then by extrapolation be calculated in arbitrary units, or different samples can be compared at a fixed cycle number in the predetermined range of exponential amplification (5, 19, 20).

III. Analysis of dilution series

The range of proportionality is not only cycle dependent, but also input dependent (15, 21–23). Therefore, amplifying a dilution series of a sample at a fixed cycle number exactly reproduces the picture of amplifying aliquots in sequential cycles, and permits determination of a dilution range of proportionality where relative quantification can be performed.

Normalisation

If the concentration of a sample is known, amplification amounts measured can be related to total DNA or RNA content of the original sample. Often the PCR technique is chosen, however, because samples are too small for analysis by other methods, and the concentration will therefore be below the limit of measurement. In such cases, normalisation to a co-amplified internal standard species is necessary. This demands careful analysis of the behaviour of the standard in relation to the parameters analyzed.

Whether it is advisable to co-amplify the target of interest with an external or internal control target in order to correct for tube effects is a matter of discussion. Some authors claim that it is (18), whereas others find the reaction quite reproducible (15, 21, 22). Murphy et al. (21) claim that co-amplification leads to a premature termination of the range of proportionality. The present authors find that if the two targets are present in comparable amounts, amplification of either is seriously disturbed by co-amplification. However, amplification in separate tubes may be performed quite reliably.

DANGERS IN PCR ANALYSIS

False positives

The very advantage of PCR, its extreme sensitivity, is a potential danger. Contamination of samples with as little as a single target molecule can give a false positive signal, a severe complication in e.g. diagnostic applications of the technique, or seriously disturb quantification. Potential sources of contamination are everywhere, especially if the laboratory has been working with the target sequence in the form of plasmid probes, which will often be the case. Also, the product of one PCR amplification is an extremely potent source of contamination of the next. A strict set of hygienic rules must be observed in PCR work and negative control reactions included in every experiment (24, 25). Some persons seem more liable than others to pick up contamination on their skin surfaces simply by entering laboratories containing sources of contamination. These may have to wear hair and face coverings during PCR work (26).

False positives of internal origin can arise from unspecific priming, if sequences more or less related to the primer sequences are present in the genome. It is therefore important to establish annealing conditions that minimize unspecific priming while still giving a reasonable yield of the desired product.

Error rate

The Taq polymerase has no proof-reading capacity, and occasional incorporation of an incorrect nucleotide is therefore not corrected (3). The error rate is low, however, and of no consequence for most purposes, such as the establishment of the presence or absence of a given sequence, quantification, direct sequence analysis of the total product, etc. It must, however, be taken into consideration in sequence analysis of individual amplified molecules in cloned form. Thermostable DNA polymerases with proof-reading activity (Vent, Pfu) have been reported, but their use may require special primer

modifications to equal Taq amplification in yield and specificity (27).

APPLICATIONS

Detection

It will be evident from the above that PCR is an excellent tool for the simple detection of a given RNA or DNA sequence in a small sample. Thus, the presence of bacterial, fungal, or viral pathogens in clinical samples can be demonstrated within a few hours, which is of obvious diagnostic value. It seems likely that the technique will be used extensively in clinical microbiology within a few years, and patents for this application are already a matter of heavy dispute. The expression of a gene can be monitored by amplification of corresponding mRNA sequences out of the pool of total RNA. Thus, the temporal and spatial distribution of the expression of a given gene can be analyzed and abnormal gene expression detected.

Analysis of amplified product

By analysis of amplified products with restriction enzymes, sequencing, or hybridization with allele specific oligonucleotide probes, allele polymorphism and loci correlated with genetic disorders can be studied and acquired rearrangements and point mutations identified. Thus, PCR techniques are powerful for the diagnosis of genetic disorders. The speed and sensitivity of such analyses compared to conventional methods are prominent advantages in e.g. prenatal diagnosis.

Analysis of libraries

It has been mentioned that, with knowledge of a protein sequence, PCR with degenerate primers can be used to amplify a cDNA fragment of unknown nucleotide sequence. This technique is helpful in generating probes to identify cloned genes or cDNAs in existing libraries for further analysis. Generation of representative cDNA libraries may, however, in itself pose a problem when the starting material is limited. In this case the PCR technique can be used to amplify the total cDNA population prior to cloning in bacteria, greatly improving the chance of cloning rare species. The approach, mentioned above, is to bracket, by ligation or tailing, all cDNAs with known sequences to provide binding sites for primers.

Chromosome crawling

Although the fragments that can be successfully amplified by PCR are of limited length, the method can be applied to analysis of larger regions of chromosomes by sequential steps of "inverse" PCR, in which sequence information obtained from one step is used to design primers for the next.

Quantification

Quantitative PCR may be used to estimate gene copy numbers in preparations of genomic DNA (18), thereby identifying deletions or gene amplifications. Quantification of specific mRNAs in different tissues or under different conditions is indispensable in gene expression studies but requires microgram amounts of total RNA with conventional methods, whereas picogram amounts may be sufficient for PCR analysis (21, 23).

PCR AND DERMATOLOGY

Almost all applications of PCR that are useful in medicine in general will also be so in dermatology, both in basic and clinical research as well as in diagnostic work. Detection of pathogens, analysis of loci associated with genetically determined dermatological diseases, identification of somatic mutations in skin affected by disease, cloning and analysis of genes expressed in normal or abnormal skin, quantification of expression of disease-related genes, are all of obvious dermatological relevance.

The sensitivity of PCR in detecting viral and other pathogens associated with skin diseases is an evident advantage both in basic and diagnostic work and has been used in a number of cases already (28). In an interesting recent contribution to the standing discussion about the possible association of cutaneous T-cell lymphoma with a retrovirus, Lisby et al. (29) used PCR under very sensitive annealing conditions to look for sequences related to HTLV-I/II in lesions from 21 CTLC patients without positive results.

To the growing list of dermatological diseases for which the responsible genetic loci have been analyzed by PCR (28) a fresh example has been added with the identification of point mutations in a critical region of the keratin 14 gene in 2 patients with epidermolysis bullosa simplex (30). A number of candidate genes are suspected of being associated with the different forms of epidermolysis bullosa (31, 32). Unambiguous association of these disorders with different genetic lesions will allow for rapid, diagnostic PCR requiring very little material.

It is in principle possible to use PCR for the detection of the mRNA of any given protein/peptide which might be a "marker" for a given disease. Psoriasin is a protein strongly upregulated in psoriatic skin (33). It is at present not known whether this protein is upregulated in other inflammatory skin diseases. If over-expression of psoriasin turns out to be unique for psoriasis, then its mRNA can be used as a diagnostic marker for this disease.

Several cytokines, such as interleukin 1, 6, and 8, have recently attracted considerable interest as being implicated as inflammatory or pro-inflammatory mediators in immunological skin diseases. Since cytokines can be biologically active in extremely low concentrations, the presence of their mRNAs in skin samples can easily escape detection by traditional methods. For the demonstration of such rare mRNAs, the sensitivity of PCR makes this method the obvious choice. Thus, using quantitative PCR conditions, we have recently mapped the distribution of epidermal interleukin 8 expression levels in a large number of dermatological patients (23).

In most of these cases the PCR technique is simpler and more rapid than traditional methods, but its chief appeal to dermatologists lies perhaps in the extremely small amounts of material required for analysis. Since amplification factors up to 10^9 can be obtained, minute epidermal or dermal biopsies

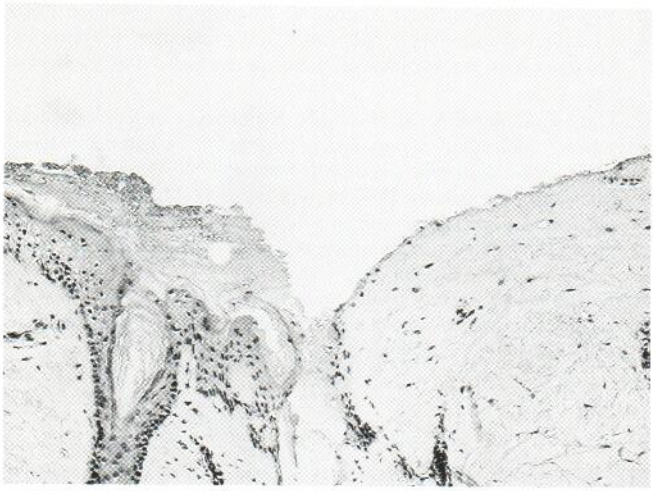

Fig. 3. The figure shows a punch biopsy after epidermal scraping for PCR analysis. The whole of epidermis has been removed on the right side leaving the basal membrane intact. On the left side remnants of epidermis are still left.

yielding only nanogram amounts of DNA or RNA are sufficient for most analyses. In the authors' experience, samples taken by gentle scraping of less than 1 cm^2 of skin, removing the outer cell layers of the epidermis without damaging the basal membrane (Fig. 3), are sufficient for quantitative determination of several cytokine mRNAs which were not detectable in Northern blot analysis of RNA from up to ten suction blisters (34).

Thus, large punch and shave biopsies can in many cases be replaced by gentle curettage or the smallest punch biopsies. With some dexterity in sample taking, it is possible to analyze separately dermal and epidermal skin compartments and a few drops of blood from a biopsy site. With primers for cell-specific mRNAs it may be possible to estimate the cellular composition of various relevant tissues. Thus, it may be possible – and certainly useful – to quantitate the number of various cells (activated T lymphocytes, B lymphocytes, endothelial cells, mast cells, eosinophils, etc.) in a given biopsy. It is possible to follow the time-course of the cytokine cascade upregulation in various tissue compartments during the development of immunological skin reactions. The very modest requirement with respect to sample size is an obvious advantage to patients, making it easier to obtain their consent in participating in experiments, and thus more samples will be available for research.

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