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MINIREVIEW

Polymerase Chain Reaction: Trenches to Benches

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The application of molecular diagnostic techniques to the diagnosis of infectious disease is based on the identification of unique "signature sequences" in the DNA or RNA of a pathogen; the detection of characteristic nucleic acid traces in a clinical specimen is presumed, in most cases, to be evidence of present (or recent past) infection. However, despite the auspicious introduction of nucleic acid probes for the detection of infectious agents, relatively few laboratories employ them on a regular basis, largely because they are still technically demanding and difficult to automate and often lack the sensitivity required for microbiological specimens (5, 20). Thus, with only a few exceptions, probes for infectious agents are still confined to culture confirmation and have not eliminated the need for primary culture.

Enter the polymerase chain reaction (PCR) (23, 24). Of the basic techniques in molecular biology that have been developed in the last decade, none has had a greater impact. First described in 1985, this ingenious method uses repeated cycles of oligonucleotide-directed DNA synthesis to carry out in vitro replication of target nucleic acid sequences, forming the basis of an extremely sensitive system for the amplification and detection of specific nucleic acid sequences (Fig. 1). In addition to numerous published applications in human genetics and clinical microbiology (reviewed recently in references 5 and 20), PCR has provided the means to accomplish in the laboratory what only a decade ago was impossible, such as the recovery of DNA from mummified tissues (18) and the identification of human pathogens in archived material (21). Indeed, published reports of new PCR applications for the diagnosis of infectious disease, in the spirit of the amplification technology they exploit, are accumulating at a seemingly exponential rate.

However, despite the extraordinary enthusiasm surrounding this technique and the considerable investment of human and financial resources in its applications, PCR is routinely performed as a clinical service in only a few centers. Why has this important technology not emerged from the developmental "trenches" in the 8 years since its moonlit conception (16)? Despite complaints from the diagnostic community that restrictions on licensing agreements have hindered the widespread use of PCR, it is in fact an array of technical problems, some created by the technique itself, that have prevented it from becoming a clinical laboratory bench procedure. The purpose of this minireview is to briefly summarize these problems and describe the measures that are being taken to address them.

FALSE POSITIVES DUE TO AMPLICON CARRYOVER

The greatest problem facing the diagnostic application of PCR (and other nucleic acid amplification methods) is false positivity due to contaminating nucleic acids (13). Here, the exquisite sensitivity of PCR proves to be its undoing; the transfer of minuscule quantities of such sequences into a neighboring tube may result in a false-positive result. Nucleic acid contamination may result from three sources. One source consists of clinical specimens containing large numbers of target molecules, which results in cross-contamination between specimens (this type of contamination is already well known to clinical microbiologists). Another source is contamination of reagents used in PCR by previously cloned plasmid DNA, a particularly aggravating problem for laboratories that have been studying a particular organism for many years. Plasmid clones derived from the organism that have been previously analyzed and sequenced to obtain the signature sequence may be present in large numbers in laboratory equipment and reagents. The third source is accumulation of PCR products (amplicons) in the laboratory by repeated amplification of the same target sequence.

Amplicon contamination is the most serious kind of contamination and unfortunately the most likely to occur because of the large numbers of molecules that are generated in a standard reaction. Each PCR vessel may contain as many as 10^{12} copies of an amplicon (13); thus, even the tiniest aerosol droplet (10^{-6} μl) may contain up to 10^5 potential targets. Amplicons are by definition proven PCR substrates and are thus ideal targets for further amplification. When one considers the fact that hundreds to thousands of amplification reactions may be performed in the optimization and testing of a new set of reagents, it is not surprising that "amplicon buildup" can manifest itself in the contamination of reagents, buffers, laboratory glassware, autoclaves, and ventilation systems. This problem is especially acute in the diagnosis of infectious diseases, in which assays are generally tuned for maximum sensitivity (1 to 10 template molecules). With a number of reports claiming that the sensitivity of PCR exceeds that of the prevailing gold standard, the burden of proof now lies with investigators who make such claims; formal retractions directly attributable to amplicon contamination have recently appeared in the literature (6).

To avoid amplicon carryover, PCR applications laboratories must take specific precautions (reviewed in reference 13), including the use of disposable laboratory materials, prealiquoted reagents in quality-controlled lots, and positive displacement pipets and analysis of amplification products in an area that is physically separated from the area where reagents and samples are prepared. If a laboratory employs these measures from the start, it is possible to have no problems with contamination. When it does occur in these settings, contamination is most often observed at the 1- to 100-molecule level (in those PCR tests that are optimized for maximal sensitivity). Second target testing is also recommended, especially in the early stages of development.

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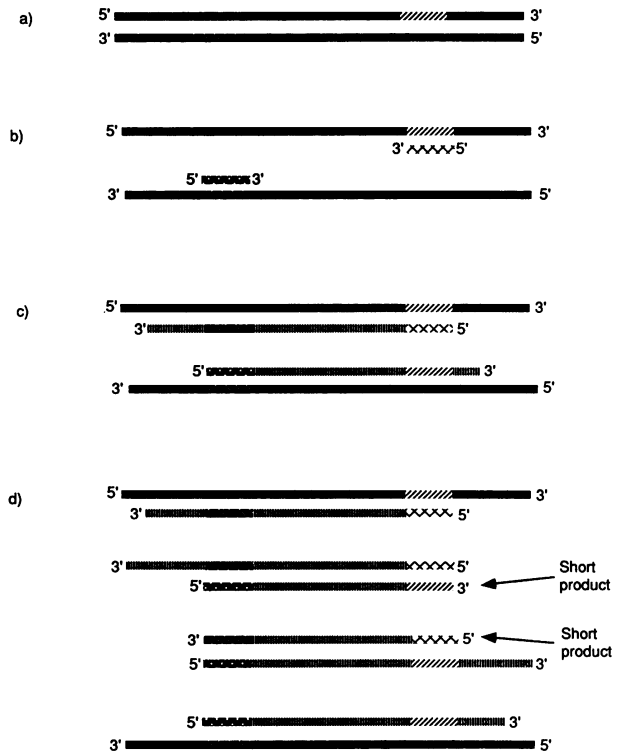

FIG. 1. The PCR. In the first cycle, A double-stranded DNA target sequence is used, with the primer-binding sites indicated by diagonally hatched lines (a). These two strands are separated by heat denaturation, and two synthetic oligonucleotide primers (cross-hatched lines) anneal to their respective recognition sequences in the 5′-to-3′ orientation indicated (b). Note that the 3′ ends of each primer are facing each other; *Taq* DNA polymerase initiates synthesis at the 3′ ends of each primer (c). Extension of the primer via DNA synthesis (broken line) results in new primer-binding sites. The net result after one round of synthesis is two copies of the original target DNA molecule. In the second cycle, each of the four DNA strands shown in panel c anneals to primers (present in excess) to initiate a new round of DNA synthesis (d). Of the eight single-stranded products, two are of a length defined by the primer-annealing sites; this short product accumulates exponentially in subsequent cycles.

However, while these precautions described here can be adopted by research laboratories, they will represent severe limitations to service laboratories until prepackaged quality-controlled diagnostic kits become available. Present-day clinical microbiology laboratories have neither the space to devote exclusively to PCR nor the inclination to troubleshoot false positives frequently.

Fortunately, for the problem of amplicon buildup, relief appears to be in sight. Two amplicon sterilization methods, one enzymatic and one photochemical, have recently been described. In the enzymatic method (15, 26), dUTP is substituted for TTP in all amplification reaction mixtures, resulting in incorporation of U in place of T in the amplicon. Thus, amplicons can be distinguished from authentic target DNA by the presence of an "unnatural" nucleotide base. The bacterial enzyme uracil-*N*-glycosylase (UNG) is then added to the reaction mixes (the physiologic role of this enzyme is to cleave uracil residues created by spontaneous deamination of cytosines from the phosphate backbone). During a brief incubation step prior to amplification, uracil-containing DNA strands that are carried over from previous amplifications are enzymatically degraded and thus rendered ineligible to serve as substrates for further amplification. The UNG itself is then inactivated by heating to 94°C. Because naturally occurring target DNA does not contain large numbers of uracil residues, this method distinguishes between U-containing amplicons carried over from previous reactions and the T-containing DNA from an organism in a clinical specimen. Thus, the UNG protocol allows "live" amplicons to accumulate in the laboratory, but a pre-PCR sterilization step selectively eliminates them prior to amplification (Fig. 2).

An alternative, post-PCR method that exploits the photochemical properties of the psoralen derivative 4′-aminoethyl-4,5′-dimethylisopsoralen (4′-AMDMIP) has recently been described (3, 9). This compound is added to the PCR mixture prior to amplification; it does not substantially interfere with primer annealing or *Taq* polymerase activity and is thermally stable. After amplification (but before the polypropylene reaction tubes are opened), the tubes are exposed to long-wave UV light, which penetrates them and photochemically activates the isopsoralen but does not otherwise damage the DNA. The activated psoralen then forms cyclobutane adducts with pyrimidine residues on the amplified DNA that prevent *Taq* polymerase from traversing the molecule in a subsequent amplification. The efficiency of this process is dictated in part by probability and can be extremely high, depending on the length and nucleotide base composition of the amplicon. In general, for amplicons greater than 300 bp in length with roughly 50% G+C content, virtually complete sterilization can be achieved. Moreover, in contrast to the enzymatic methods, the original input DNA is also sterilized, resulting in a reduced risk of target DNA accumulation from the clinical samples themselves.

Both sterilization methods have left room for improvement. In the photochemical procedure, inhibition of PCR has been observed at high isopsoralen concentrations (concentrations that might be necessary to inactivate very short or highly GC-rich amplicons). In addition, when internal hybridization probes are used for detection of the amplicons, lower hybridization stringencies may be required to compensate for the presence of isopsoralen cross-links in the amplified DNA (3, 9). Potential problems with the UNG protocol include incomplete ablation of UNG activity at the elevated temperatures used for denaturation and annealing in the PCR procedure. (Residual UNG activity may affect the sensitivity of the system because in the early cycles of PCR, the uracil-containing strands may be inactivated as soon as they are made.) In addition, the substitution of dUTP for TTP in many PCR protocols results in lower amplification efficiency, requiring adjustment of the deoxynucleoside triphosphate pools to regain sensitivity (19). Future improvements in these techniques will likely include new isopsoralen compounds with higher affinities for amplified DNA and the introduction of more thermolabile forms of UNG.

Neither method described here can be used as a quick solution for an existing (T-containing) amplicon contamination problem. These protocols will only serve to help avoid future problems with amplicon buildup. Furthermore, as no sterilization protocol is likely to be either 100% efficient or completely foolproof, good laboratory practice, including physical separation of pre- and postamplification procedures and observance of previously proposed guidelines, is still highly recommended (13). Nonetheless, sterilization methods are likely to have a major impact on the automation of

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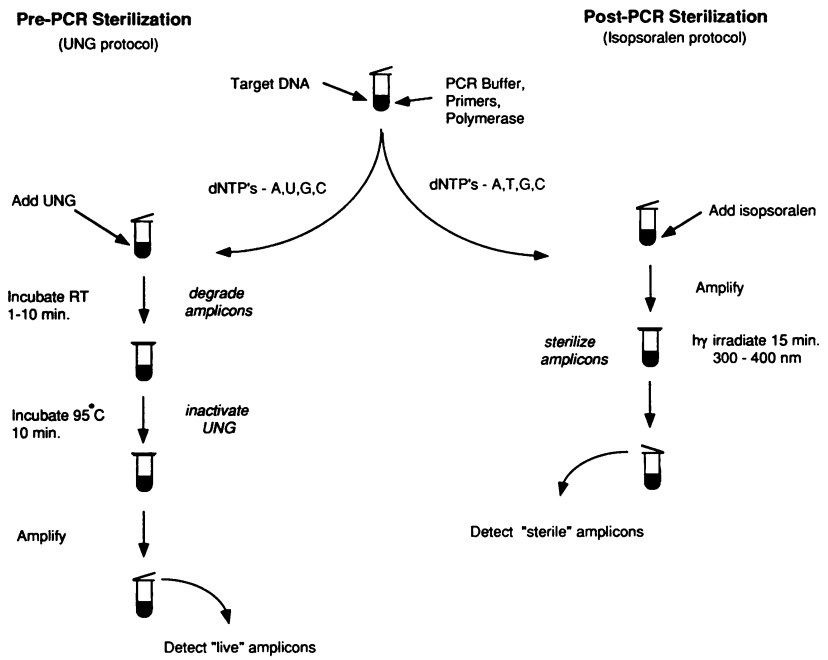

FIG. 2. Pre-PCR and post-PCR sterilization methods. In the pre-PCR (enzymatic) method, previously amplified DNA (containing U in place of T) is selectively degraded prior to amplification. In the post-PCR (photochemical) protocol, an isopsoralen compound is included prior to PCR. After amplification, but before the products are removed for analysis, the tubes are exposed to long-wave UV light, resulting in cross-linking of amplified DNA. RT, (hγ), room temperature; dNTP, deoxynucleoside triphosphate.

the technique and on the importation of nucleic acid amplification methods into clinical laboratories.

POSTAMPLIFICATION DETECTION FORMATS

Another obstacle to the widespread introduction of nucleic acid amplification techniques into clinical laboratories has been the means of detecting the amplicons after amplification by PCR. To provide maximum sensitivity and specificity, most PCR applications have used gel electrophoresis along with liquid or membrane hybridization with radiolabeled probes to demonstrate the presence of the amplified DNA. While they provide excellent sensitivity, these methods are generally time-consuming and labor intensive and require special training of laboratory personnel. Furthermore, the use of radioisotopes in the production of the probes makes such methods impractical for routine laboratory use.

The greatest improvements in detection technology will occur when amplification and nonisotopic detection formats combine forces with automation (1, 2, 4, 8, 10–12, 17, 25, 26). One method combining PCR amplification with nonisotopic detection is the reverse dot blot described by Saiki et al. (25). In this system, several oligonucleotide probes are affixed to nylon membranes via a homopolymeric tail, leaving the target-specific portion of the probe free to hybridize. Target DNA is amplified with biotinylated primers and then hybridized to the membrane; hybridized DNA is then detected with a streptavidin-horseradish peroxidase conjugate, which in turn catalyzes a color change on the membrane surface. While this detection method can be both sensitive and specific, it is not easily adapted to laboratory automation because membrane strips must be individually processed and the results must be visually recorded.

Other techniques that employ 96-well microtiter plates to provide sensitivity, while taking advantage of existing laboratory technology for plate handling and quantitation of results, have been described. In one approach (27), target DNA is amplified with modified oligonucleotides so that the amplicons contain two functional groups; one end is biotinylated, and the other contains a recognition sequence for a high-affinity DNA-binding protein. The latter moiety is attached to a microtiter dish and is used to adsorb amplified DNA (via the recognition sequence) from the reaction mixture. An avidin-horseradish peroxidase conjugate is then used to detect the adsorbed amplicons. This strategy produces excellent sensitivity, but it does not discriminate between specific and nonspecific amplification products. Specificity, therefore, must be conferred by the amplification step itself; this can be achieved with a nested PCR protocol, but such protocols carry a greatly increased risk of amplicon contamination (28).

Detection of amplified sequences internal to the primers also improves specificity. To this end, sandwich hybridization formats that trap the amplified target DNA via hybridization to DNA sequences flanked by the primers have been developed. Similar in concept to the reverse dot blot, these methods theoretically provide excellent sensitivity and specificity but have a format more amenable to laboratory automation than the former method (Fig. 3). Keller et al. described a method for detection of PCR-amplified hepatitis B virus DNA using microtiter plates to which "capture DNA" was covalently attached (11). PCR amplification of hepatitis B virus target DNA resulted in a molecule with homology to both the capture probe and a biotinylated detection probe. Incubation of the detection probe and amplicon together in the capture probe-coated well results in the formation of a molecular bridge that forms the basis of a

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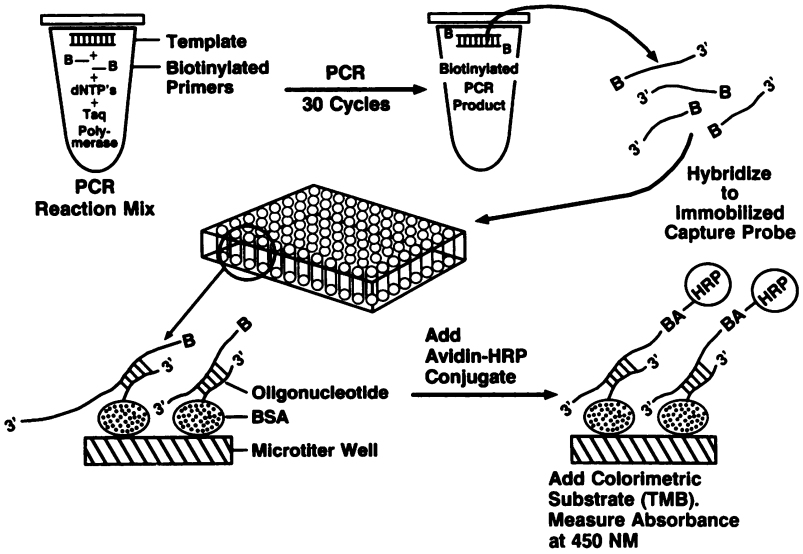

FIG. 3. Microtiter plate-based colorimetric detection of PCR amplification products. An oligonucleotide probe specific for the amplified target sequence is bound to wells of the plate. Biotinylated amplified target DNA is hybridized to the wells and then washed and detected with avidin-horseradish peroxidase (HRP) and a chromogenic substrate. B, biotin; BSA, bovine serum albumin; BA, biotin-avidin complex; TMB, tetramethylbenzidine; dNTP, deoxynucleoside triphosphate. (Reprinted by permission of Roche Diagnostic Systems.)

sensitive and specific detection system. A similar system was described for detection of human immunodeficiency virus (10).

Permutations of this basic strategy are in various stages of development. Using PCR and a microtiter-bound antibody specific for target-probe complexes, Bobo et al. detected chlamydial DNA in cervical specimens (2). Nickerson et al. used template-dependent ligation to detect PCR-amplified alleles of various human genetic loci in 96-well dishes with an automated workstation (17). In the latter method, oligonucleotide primers are designed so that in the presence of an amplified target molecule they lie head-to-tail on the target; their point of ligation exactly straddles the nucleotide position of a known mutation or polymorphism. Efficient ligation and the eventual detection of a ligation product occur only when the probes are perfectly base-paired to the target sequence; a single base change at the point of ligation prevents the reaction from occurring. The ligation products are detected by adsorption onto wells of a 96-well dish and subsequent colorimetric detection. While this approach was first used to examine human genetic alleles, applications for infectious disease are numerous and could include detection of mutations associated with drug resistance and discrimination of nucleotide differences in regions of small subunit RNA (16S) genes, where single nucleotide changes may serve to distinguish one species from another (21).

PROSPECTS

Several predictions regarding the impact of PCR and other amplification techniques on clinical microbiology laboratories can be made. First, these techniques will have their greatest impact on the detection of pathogens for which in vitro cultivation systems are lengthy, inconvenient, dangerous, prohibitively expensive, or simply unavailable and will thus greatly extend the diagnostic repertoire (and the accompanying responsibilities) of clinical laboratories. Furthermore, previously unrecognized or unidentified pathogens (some initially identified through the use of the technique itself) will be added to the laboratory litany (22). Second, the implementation of amplification techniques will create a demand for laboratory professionals with training in these techniques. Currently, very few medical technologist training programs, pathology residency programs, or clinical microbiology fellowship programs offer formal instruction in molecular techniques. Consideration must be given to the fact that those entering programs now will be directly confronted with this technology when they finish their training. Third, continuing-education programs will have to be developed to provide laboratory professionals with an understanding of the principles of molecular diagnostics along with a realistic picture of the power and limitations of the new technology. Finally, there will arise a need to provide national standards for test methods and to effect laboratory quality assurance and proficiency-testing programs for molecular diagnostics. Though many amplification-based tests will initially be offered on an experimental basis, it would be prudent to begin developing molecular-diagnostic versions of proficiency examinations such as those currently offered through the College of American Pathologists. The National Committee on Clinical Laboratory Standards has already anticipated the need for laboratory standardization in this area; subcommittees for standardization of molecular diagnostic tests have recently been assembled.

CONCLUSIONS

Although the tone of this minireview is meant to be optimistic, it may be a considerable length of time before clinical microbiology laboratories become the oft-predicted "PCR playgrounds." Many details remain to be worked out, especially in the areas of patient sample requirements, sensitivity cutoffs, rapid sample preparation techniques, and elimination of inhibitors of PCR that are present in blood and other biological samples (7, 14). Furthermore, while amplification methods are rapidly becoming the standard methods

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for some genetic and infectious disease tests, conventional culture for many pathogens is rapid, inexpensive, and as sensitive as PCR and allows detection of multiple organisms from a single procedure. Culture also allows assessment of traits such as antibiotic resistance, virulence factors, disease-associated antigens, and strain differences that are currently difficult or impossible to determine by amplification alone.

As with many other techniques available to clinical laboratories, the decision to use nucleic acid amplification rather than conventional methods will likely be dictated by cost along with other factors. The sensitivity and specificity of the amplification method must be weighed against the low-cost, bench-proven conventional method, with consideration given to turnaround time and clinical needs. The cost per test will eventually be driven down by automation and increased sophistication (not to mention market forces), leading to increased application in areas for which the cost/benefit ratio was previously limiting. Ultimately, it is expected that the application of this technology will lead to vast improvements in diagnostic capabilities and to a better understanding of clinical infectious disease.

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