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In Vitro Amplification Techniques for the Detection of Nucleic Acids: New Tools for the Diagnostic Laboratory

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The acceptance of nucleic acid probes as diagnostic tools for the clinical laboratory has been hampered by a number of factors, including laborious techniques and limited sensitivity. The focus of this review is on the recent development of amplification techniques to enhance the signal generated by nucleic acid-based detection systems. Three general areas are discussed: *(1)* amplification of target sequences using the polymerase chain reaction or the transcript amplification system, *(2)* amplification of the probe sequences using Qβ replicase, and *(3)* amplification of probe-generated signals with compound or "Christmas tree" probes. The hope of these new technologies is to simplify yet improve on the sensitivity of nucleic acid-based tests to enable them to attain a more prominent place in the diagnostic repertoire of the clinical laboratory.

INTRODUCTION

The application of the techniques of modern molecular biology to the diagnosis of human disease has recently made significant advances. Some of the most successful applications have been in clinical conditions where classical biochemical markers are either unknown or are inaccessible; under these circumstances, nucleic acid analysis may be the only means of providing diagnostically relevant information [1]. Examples include restriction fragment length polymorphism (RFLP) analysis in the diagnosis of Huntington's chorea trait in affected families [2,3], cystic fibrosis heterozygosity in genetic screening programs [4], and gene rearrangements in lymphomas and leukemias [5]. Other efforts have focused on the diagnosis of infectious agents; the agents chosen for study have typically been those whose identification and characterization are hampered by the lack of a suitable system for *in vitro* cultivation or a paucity of identifying features [7,9–11].

Despite such advances, however, the acceptance of nucleic acid probes as diagnostic tools in the clinical laboratory has been slow to develop. This delay has generally been ascribed to the difficulties in conveying a basic research tool to the clinical laboratory. If maximum sensitivity is sought, radiolabeling of such probes with potentially hazardous radioisotopes such as ^{32}P is a requirement. The relatively short half-life of ^{32}P-labeled probes dictates that the probes be resynthesized on a regular basis, thus increasing radioisotope exposure and intensifying the need for stringent batch-to-batch

Abbreviations: cDNA: complementary DNA CMV: cytomegalovirus HIV-1: human immunodeficiency virus type 1 LTR: long terminal repeat PCR: polymerase chain reaction RFLP: restriction fragment length polymorphism TAS: transcript amplification system $TCID_{50}$: 50 percent tissue-culture infectious doses

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quality control. Furthermore, while many technical improvements have been made, the routine use of radiolabeled probes is still tedious and difficult to automate. The most critical shortcoming, however, is limited sensitivity; this problem is especially apparent in the diagnosis of infectious diseases. The limit of detection for even the most sensitive nucleic acid probes, around 10^3 to 10^4 molecules [20], is still insufficient for direct detection from many clinical samples, in which the number of pathogens varies tremendously and is often small. For this reason, probes for infectious agents have remained largely confined to use in culture confirmation and have not yet obviated the need for primary culture.

The focus of this review is on the recent development of amplification techniques to enhance the signal generated from nucleic acid-based detection systems. The intent of all of these techniques is to provide greatly increased levels of sensitivity, thus raising the possibility that, in certain cases, genetic amplification may replace biological amplification (i.e., growth in culture) as the initial step in the identification scheme. While the use of such techniques is by no means limited to the diagnosis of infectious diseases, it is probably in this area that they offer the greatest promise. In particular, the clinical diagnostic virology laboratory stands to benefit greatly from these techniques because of the difficulty and expense associated with viral culture. Indeed, the first demonstrations of the utility of these approaches have been in the diagnosis of infection with viral pathogens whose detection prior to the introduction of amplification techniques has been difficult or impossible.

AMPLIFICATION METHODS: THE MAINSTAY OF FUTURE MOLECULAR DIAGNOSTIC TECHNIQUES

In this review, we will describe several means of increasing the sensitivity of nucleic acid-based tests using amplification techniques. Because the field is broad and the techniques are varied, we will divide the subject into three general areas: *(1)* amplification of target sequences using the polymerase chain reaction (PCR) or the transcript amplification system (TAS), *(2)* amplification of probe sequences using the Qβ replicase system, and *(3)* amplification of probe-generated signals with compound or "Christmas tree" probes (Table 1). It should be added that coincident with the development of these techniques has been the ability to synthesize DNA oligonucleotides of known sequence easily and inexpensively; all of these methods rely heavily on specific base pairing between oligonucleotides and their target sequences.

Regardless of the detection scheme used, unambiguous identification of an infectious agent depends on the identification of distinguishing nucleic acid sequences. Several groups have focused their efforts on detection of ribosomal RNA. The high copy number of this species lends itself well to direct detection from patient samples; however, the evolutionary pressure to maintain ribosomal RNA structure dictates that interspecies sequence differences be relatively small. The hybridization conditions required for adequate sensitivity and specificity must therefore be stringently controlled; this requirement may not be practical in some settings. The ideal nucleic acid sequence used for detection purposes would be one that is entirely unique to that organism (or that bears substantial sequence divergence from homologous regions of related species), yet is present in sufficiently high copy number to allow maximum sensitivity. Such sequences can be identified by several means, such as differential screening of genomic clones or subtractive hybridization of complementary DNA (cDNA) clones [37,38].

TABLE 1
Amplification Methods

1. Target Amplification
Polymerase chain reaction
Transcript amplification system
2. Probe Amplification
Qβ replicase
3. Signal Amplification
Enzyme-linked probes
Compound ("Christmas tree") probes

Once unique nucleic acid fragments are identified, their nucleotide sequence should be determined and examined for sequence heterogeneity among a number of clinical isolates. The reason for this qualification is that while the gross structure and nucleotide composition of a nucleic acid fragment may be conserved, there may exist regions of microheterogeneity that will preclude efficient base-pairing of the probes generated against the target sequence. The efficiency of base pairing becomes more critical as the probes become shorter, such as in the use of oligonucleotides as probes or primers. If the criteria are met for sequence conservation in the target regions of a variety of clinical isolates, these may be confidently targeted for amplification, using one or a combination of the approaches described below.

TARGET AMPLIFICATION METHODS: PCR AND TAS

The Polymerase Chain Reaction

In 1985, scientists at the Cetus Corporation described an *in vitro* genetic amplification technique known as the polymerase chain reaction (PCR) [15,39–41,46]. This method uses repeated cycles of oligonucleotide-directed DNA synthesis to carry out *in vitro* replication of target nucleic acid sequences (Fig. 1). The oligonucleotides used in a given reaction are synthesized to be complementary to their annealing sites within the two different strands (the sense and nonsense strands) of a target sequence, from 150 to 3,000 nucleotide bases apart.

Each cycle of PCR consists of three steps: *(1)* a denaturing step, in which the target DNA is incubated at high temperature so that the target strands are separated and thus made accessible to the excess primers present in the reaction buffer; *(2)* an annealing step, in which the reaction mixture is cooled to allow the primers to anneal to their complementary sequences on the target sequence; and *(3)* an extension reaction, usually carried out at an intermediate temperature, in which the primers are extended on the DNA template by a DNA polymerase. Each time a cycle is completed, there is a theoretical doubling of the target sequence; while this doubling is probably true in the initial cycles, in later cycles amplification becomes less efficient due to effective dilution of the polymerase by the large number of available DNA templates. Nevertheless, repeating this cycle many times (20- to 60-fold) results in amplification of the target sequence over a millionfold. The amplified sequences can then be detected by gel electrophoresis followed by blotting to nitrocellulose and probing with a ^{32}P-labeled

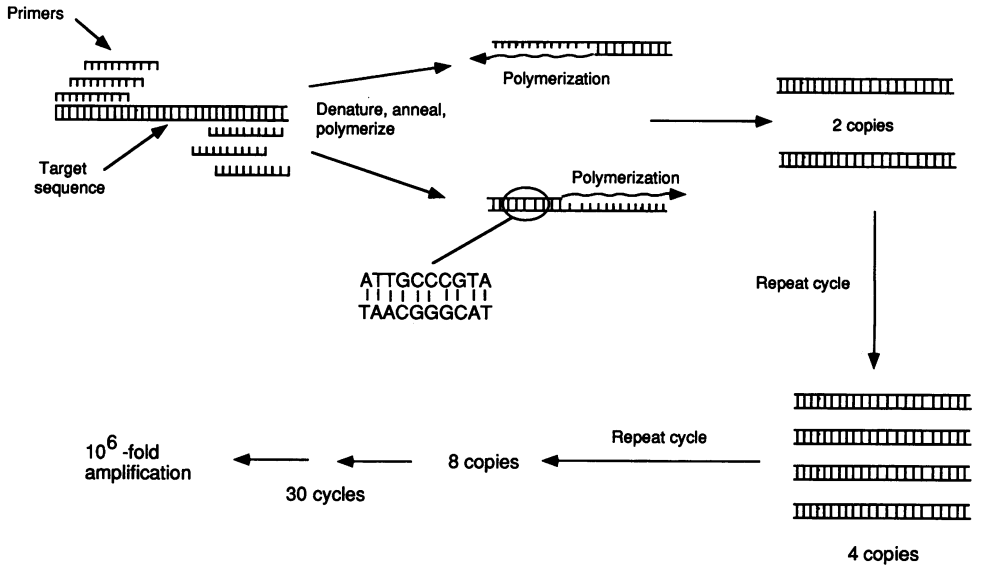

FIG. 1. The polymerase chain reaction. Each cycle of PCR consists of three steps: *(1)* a denaturing step, in which the target DNA is incubated at high temperature so that the target strands are separated ("melted") and thus made accessible to the excess primers present in the reaction buffer; *(2)* an annealing step, in which the reaction mixture is cooled to allow the primers to anneal to their complementary sequences on the target sequences; and *(3)* an extension reaction, usually carried out at an intermediate temperature, in which the primers are extended on the DNA template by a DNA polymerase. The extension of each strand results in the synthesis of a primer binding site for the next round of replication; as a result of the fixed distance between binding sites, the predominant amplification product is of discrete length—that of the distance between the outermost ends of the primers as they are situated on the target DNA. Each time a cycle is completed, there is a theoretical doubling of the target sequence.

probe specific for the amplified region. Alternatively, dot-blot hybridization can be performed with no loss of sensitivity or specificity [19].

The primers are oriented in such a manner that DNA synthesis proceeds toward the annealing site for the primer on the opposite strand; herein lies the process by which PCR gains much of its specificity. While the probability is quite high that a given primer will anneal nonspecifically to multiple sites in the target material, the probability that a second primer will reproducibly (but nonspecifically) anneal to the opposite strand at a precise position and in close proximity to the first is very low. In general, the specifically amplified target sequence is the predominant amplification product and is easily identified by its precisely defined length; nonspecific amplification products tend to be heterogeneous in size, and they do not usually become the predominant product.

Two recent technical innovations have simultaneously simplified and greatly increased the power of PCR. The discovery that the thermostable DNA polymerase (*Taq* polymerase) [47], isolated from the thermophilic bacterium *Thermus aquaticus,* is able to withstand repeated cycles of heating to 95°C has made it possible to carry out PCR without reopening tubes and adding fresh polymerase after each denaturing step. *Taq* polymerase is added once at the beginning of the reaction, thus greatly simplifying tube handling. Furthermore, the ability to perform annealing and extension reactions at a higher temperature significantly reduces nonspecific amplification; the higher temperatures are unfavorable for the formation of imperfectly base-paired complexes between primer and target. A second innovation is the development of the program-

mable thermal cycler. The thermal cycler, essentially a programmable heating block, is capable of carrying out successive heating and cooling cycles unattended and eliminates the tedious task of transferring reaction tubes between water baths or heating blocks.

Recently a method for using mRNA as starting material for PCR has been described [17,42]. Prior to PCR, single-stranded RNA molecules are copied into DNA by reverse transcriptase, using oligonucleotides as primers. The resultant RNA:DNA heteroduplexes are then amplified in the usual manner, using PCR. This technique may have some advantages over that using a DNA substrate; the mRNA represents a larger target because this species is often present in a several hundredfold abundance over the DNA template. Furthermore, because eukaryotic mRNA is post-transcriptionally processed by splicing out non-coding intervening sequences (introns), the relevant coding sequence can be directly examined even though the cognate DNA sequence is scattered across many kilobases. This approach has been taken in the identification of Philadelphia chromosome (9:22) translocations, in which the chromosomal breakpoint may occur over 20 kilobases or more [43].

The true power of PCR is recognized when the technique is employed to amplify rare target sequences in a background of genomic DNA. PCR has been used in the detection of proviral sequences for human immunodeficiency virus type 1 (HIV-1) [12–14,16]. The low prevalence in human mononuclear cells of virus-specific sequences (as few as one copy in 10^4 to 10^5 cells) precludes the use of conventional hybridization techniques. Sequences located in highly conserved regions of the HIV-1 provirus, such as the long terminal repeat (LTR) and the *gag* and *pol* coding regions, were targeted for amplification. The amplification products were then electrophoretically separated and blotted on to nitrocellulose. A labeled probe oligonucleotide, corresponding to sequences flanked by the amplification oligonucleotides, was used to confirm the presence of the specific amplification products. Using this approach, as few as one HIV-1-infected cell could be reliably detected in a population of 10^6 uninfected cells [12]. Subsequent studies using PCR to detect HIV-1 in the peripheral blood of infected individuals have confirmed the sensitivity of this diagnostic procedure [16,17].

In the detection of cytomegalovirus (CMV), PCR has been used to enhance greatly the sensitivity of hybridization [20–22]. Whereas conventional spot hybridization requires at least 10^3 $TCID_{50}$ (50 percent tissue-culture infectious doses) for a positive result [20], the polymerase chain reaction can detect from 1 to 10 $TCID_{50}$ of CMV [21,22]. Other DNA and RNA viruses have recently been detected in similar fashion; some of these are listed in Table 2.

The Transcript Amplification System

An alternative amplification scheme, jointly developed by SISKA Diagnostics and the Salk Institute, has recently been described [36]. This method, termed the transcript amplification system (TAS), can also use DNA or mRNA as starting material. Each step of TAS consists of a cDNA synthesis step, followed by an *in vitro* transcription step, using the cDNA as template. During the cDNA synthesis step, one molecule of DNA is produced for each DNA or RNA target by reverse transcriptase, and a promoter sequence for a DNA-dependent RNA polymerase is incorporated into the newly synthesized strand. This process is made possible because the primers used in TAS are hybrid molecules; one end of the primer consists of a target-specific region,

TABLE 2
Application of Amplification Methods to Viral Diagnosis

Method	Virus	Reference
PCR	Human immunodeficiency virus type 1	[12–14,16–19]
	Human immunodeficiency virus type 2	[19]
	Human T lymphotropic virus type I	[23,24,30]
	Human B lymphotropic virus	[31]
	Hepatitis B virus	[32]
	Human papillomavirus	[33,34]
	Cytomegalovirus	[21,22]
	Rhinovirus	[35]
TAS	Human immunodeficiency virus type 1	[36]

PCR, polymerase chain reaction
TAS, transcript amplification system

while the other end contains a promoter for the phage T7 RNA polymerase (Fig. 2). The result is a DNA copy of the target molecule with a phage RNA polymerase promoter at one end.

A well-established property of T7 RNA polymerase is the ability to produce many molecules of RNA from each molecule of template, often exceeding a 30- to 40-fold ratio of product to template [48]. This feature of T7 polymerase has been exploited in order to provide a potent amplification step; the newly synthesized cDNA serves as template for the synthesis by T7 polymerase of a large molar excess of RNA, which then serves as the substrate for the next cycle. After completion of four TAS cycles, target sequences are amplified 2–5 $\times$ 10^6-fold. This method has been applied to the detection of the *vif* region of the HIV-1 RNA genome, where it was determined that fewer than one HIV-1-infected cell could be detected in a population of 10^6 uninfected cells. The amplified HIV-1-specific RNA was detected after annealing to solid-phase bound oligonucleotides, an approach facilitated by the single-stranded structure of the RNA molecules.

The use of TAS as an amplification technique may have drawbacks. Because the enzymatic reactions of cDNA and subsequent RNA synthesis are carried out at relatively mild temperatures (42° and 37°C, respectively) nonspecific hybridization of the oligonucleotides is more likely to occur. While TAS, like PCR, gains specificity by requiring that two primers anneal to their target sequence in relatively close proximity, experience gained from early experiments with PCR has shown that increasing the annealing temperature of the primers greatly reduces the amount of nonspecific amplification because imperfectly base-paired complexes are less likely to occur. Increased specificity may be possible, however, if a second "nested" TAS primer pair, containing a promoter for a different RNA polymerase (such as the RNA polymerase of either phage SP6 or phage T3) is used after the first round of amplification. Finally, the multiple enzymatic steps of TAS and the lack of thermostable reverse transcriptase or RNA polymerase require replenishment of enzymes at each step, resulting in more sample handling. This last objection is somewhat tempered by the fact that only four cycles of amplification are required for TAS, and that, like PCR, the basic methodology lends itself well to automation.

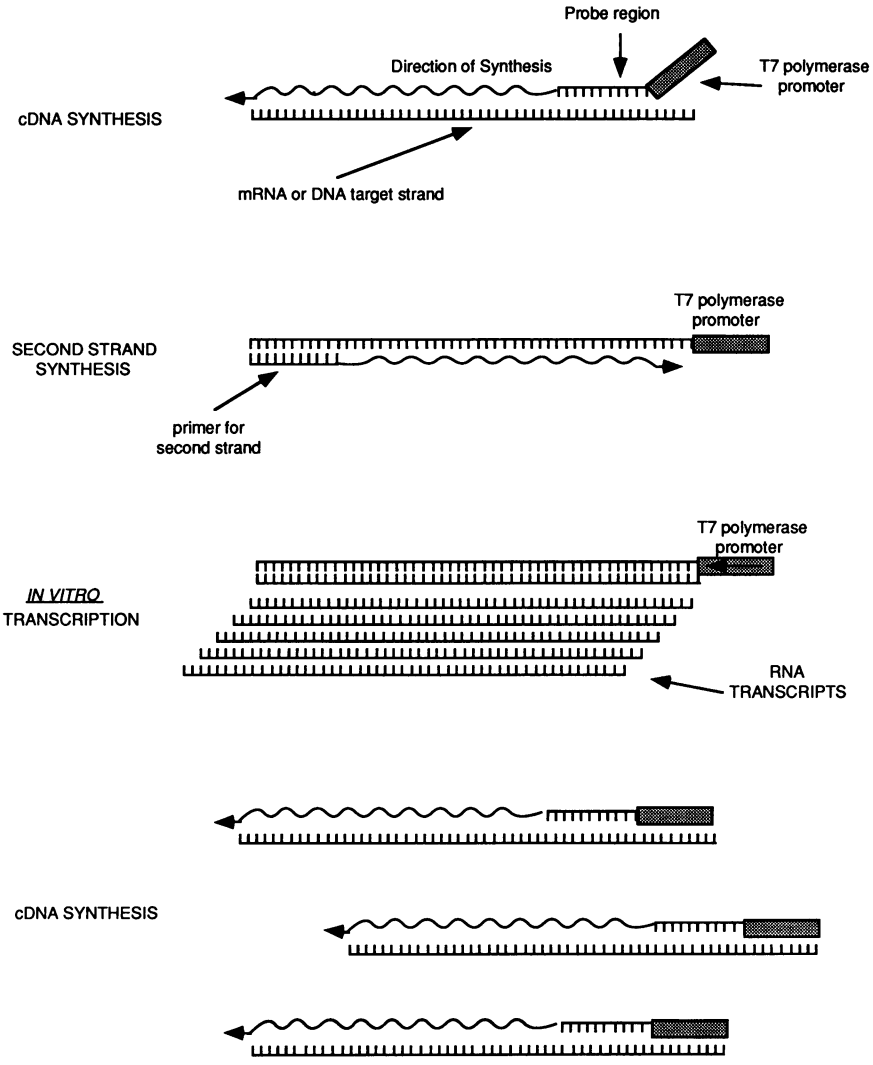

FIG. 2. The transcript amplification system. Each step of TAS consists of synthesis of a complementary DNA (cDNA) from an RNA template by reverse transcription, followed by an *in vitro* transcription step using the cDNA as template. During the cDNA synthesis step, one molecule of DNA is produced for each DNA or RNA target, and a promoter sequence for a DNA-dependent RNA polymerase is incorporated into the newly synthesized strand. This process is made possible because the primers used in TAS are hybrid molecules; one end of the primer consists of a target-specific region, while the other end contains a promoter for the phage T7 RNA polymerase. The result is a DNA copy of the target molecule with a phage RNA polymerase promoter at one end. The newly synthesized cDNA serves as template for the synthesis by T7 polymerase of a large molar excess of RNA, which then serves as the substrate for the next cycle. Four TAS cycles were sufficient to amplify the *vif* region of HIV by 2–4 $\times$ 10^6-fold [36].

POTENTIAL LIABILITIES OF TARGET AMPLIFICATION SYSTEMS

While target amplification techniques represent a new and powerful addition to molecular diagnostics, potential liabilities may exist. One problem may stem from the exquisite sensitivity of these techniques: namely, the issue of how many pathogens constitutes an infection. While, in some cases, small numbers of organisms are tied to the development of disease (such as in infection with HIV), other organisms are ubiquitous, only causing infection when host defenses are attenuated (such as infection with *Pneumocystis carinii*). The clinician must therefore weigh the results of an extremely sensitive test with the clinical picture, knowing that tiny quantities of a pathogen may be present in normal individuals.

Another, potentially serious problem that may arise in the use of these techniques is the amplification of minute quantities of contaminating sequences. The nature of amplified fragments—their short length and the invariant nature of the oligonucleotide binding targets—makes them ideal substrates for further amplification. The transfer of minuscule amounts (and in conventional detection schemes, negligible quantities) of such sequences into a neighboring tube may result in a false positive. When large numbers of clinical samples are processed in a busy clinical laboratory, the problem is compounded; the potential exists for amplified fragments to make their way into buffers, laboratory glassware, and even the air circulation system. For this reason, strict quality control measures must be adopted to insure that patient samples are not contaminated.

AMPLIFICATION OF THE PROBE: THE Qβ REPLICASE SYSTEM

The normal role of Qβ replicase, an RNA-directed RNA polymerase, is to replicate the genomic RNA of the bacteriophage Qβ [44]. The means by which this unique polymerase carries out its task has been the subject of study for several years; it appears that, unlike most polymerases, Qβ replicase needs no oligonucleotide primer to initiate RNA synthesis [45]. Instead, it specifically recognizes a unique folded RNA structure, formed by intramolecular base-pairing of the Qβ RNA genome. Because the replicase is highly specific for this folded structure, its properties have recently been exploited to provide a means of amplifying hybridization signals—by amplifying the probe molecule itself.

Lizardi and co-workers have recently described the first use of Qβ replicase to amplify recombinant molecules containing probes specific for *Plasmodium falciparum* sequences that are linked to the Qβ replication site [27,29]. To do so, sequences from the *P. falciparum*-specific 21 base-pair repeats [28] were inserted into the Qβ replication origin in a DNA vector containing the phage T7 promoter. *In vitro* transcription by T7 polymerase yields RNA molecules whose secondary structure is predicted to be very similar to that of the wild-type origin, except that these molecules harbor extensions in one loop of the folded structure. The recombinant RNA molecules contained necessarily short (22 and 58 nucleotide) inserts due to the presumed size constraints on the placement of unstructured sequences into the otherwise highly structured replication origin. The molecules were then incubated with purified Qβ replicase; despite the presence of the inserts, the replication of the chimeric molecules was as efficient as that of the unmodified parent molecule, approaching 10^9-fold in a single 30-minute incubation. With this degree of amplification, the replicase quickly becomes saturated, and the reaction kinetics go from logarithmic to linear. Thus, reactions containing more of the substrate molecules achieve saturation sooner; the

further accumulation of replicated RNA is strictly time-dependent. This property may make it possible to extend greatly the dynamic range of an assay, allowing a direct linear relationship between the amount of input RNA and the amplified product.

As an approach to increasing the signal generated in a hybridization reaction, the amplification of probe molecules has inherent disadvantages. Chief among these is nonspecific hybridization of the probe molecule to sequences other than the intended target; the result, in the case of the Qβ replicase system, would be amplification of nonspecific signals. If, however, the target DNA (or RNA) is first processed by nucleic acid capture methods, such as hybridization to oligonucleotide-coated beads, background hybridization can be markedly reduced. Another means of reducing background amplification would be to design the Qβ substrates to be conformation-dependent; only molecules specifically base-paired to their targets would be recognized by the replicase. Advantages of the Qβ replicase system—high sensitivity, speed, and the linear relationship between levels of target and amplified product—would, however, seem to warrant considerable investigational effort.

AMPLIFICATION OF THE SIGNAL: COMPOUND OR "CHRISTMAS TREE" PROBES

In the endeavor to increase a hybridization signal, one alternative to *in vitro* amplification is to increase the signal generated from the probe molecule itself. Typically, the probe molecule is a sequence of DNA or RNA, complementary to the target sequence, that has been labeled with a "reporter group." This reporter is most commonly a radioisotope such as ^{35}S or ^{32}P; detection of the signal generated by the probe thus requires exposure to film or quantitation of radioactive decay on a suitable device. While radiolabeled probes generally provide the best sensitivity, their broad acceptance has been hampered by the need to employ hazardous and expensive radioistopes. Alternatively, the probe may comprise a small molecule that serves as ligand for a specific antibody or a binding protein. The best studied of these non-isotopic methods is the biotin-avidin system, in which the probe molecule is synthesized with biotinylated nucleotides; the incorporated biotin molecules are then targeted by an avidin-conjugated reporter group such as horseradish peroxidase or alkaline phosphatase, both of which produce a color reaction in the presence of a suitable substrate [8]. While non-isotopically labeled probes have made significant inroads in some applications, especially in in situ hybridization, their sensitivity is not high enough for many clinical applications.

Increasing the signal generated from nucleic acid probes may be theoretically possible if the reporter group can be concentrated in the probe molecule or if the signal intensity generated by each reporter is increased. One means of accomplishing this task has recently been described by investigators at the Imclone Corporation [25]. This system employs two types of probes simultaneously: a "primary" probe, recognizing the target sequence and containing multiple binding sites for "secondary" probes, and the secondary probes themselves, each of which contains a reporter group (Fig. 3). Incubation of these components with target DNA sequences results in the formation of a "Christmas tree" probe network, in which the primary probe is bound to its target via its target-specific region, leaving the unbound sequence available for binding by the reporter-bearing secondary probes. The probe trees thus generated can assume a variety of shapes, depending on whether the primary and secondary probes are linear or circular, but the net result is the same; the signal-generating capability of the probe

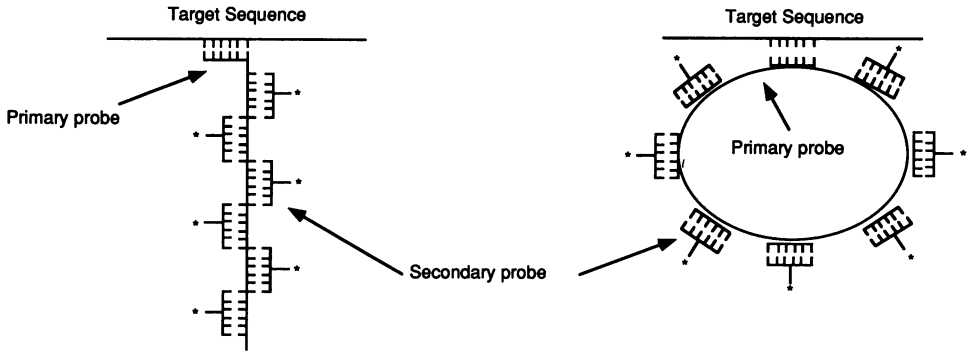

FIG. 3. Compound or "Christmas tree" probes. Compound probe systems employ two types of probes simultaneously: a "primary" probe, recognizing the target sequence and containing multiple binding sites for "secondary" probes, and the secondary probes themselves, each of which contains a reporter group. Incubation of these components with target DNA sequences results in the formation of a probe network ("Christmas tree"), in which the primary probe is bound to its target via its target-specific region, leaving the unbound sequence available for binding by the reporter-bearing secondary probes.

is significantly increased. The outcome, in practical terms, is the ability to detect smaller amounts of a target sequence or to ascertain the presence of a given amount of the target in a shorter period of time.

This approach lends itself well to the use of non-isotopic reporter groups such as calf intestine alkaline phosphatase or horseradish peroxidase. These enzymes are commonly used in immunodiagnostics because they are able to catalyze the production of a relatively large amount of analyzable product, thus increasing sensitivity. Secondary probes, consisting of oligonucleotides coupled to these enzymes, could be used in place of their radioactively labeled counterparts. If high-stringency conditions for hybridization are required, however, enzymatic activity may be lost due to the higher incubation temperatures required. Alternative non-isotopic detection schemes include the use of fluorescent or chemiluminescent secondary probes. The lower signal-generating capacity of probes of this type is offset by their thermal stability; the use of the "Christmas tree" approach may boost the signal produced to practical levels. Fluorescently labeled oligonucleotides have recently been employed as secondary probes in a compound probe system developed by the Chiron Corporation for the detection of *Neisseria gonorrhoeae* and *Chlamydia trachomatis* [26].

CONCLUSIONS

The promise of the technical innovations described here is to increase greatly the sensitivity and speed of nucleic acid hybridization techniques. Although the full ramifications of these developments are difficult to foresee, several predictions can be made regarding their place in the clinical microbiology laboratory of the future. First, these techniques will provide new tools for the identification of pathogens whose identification has previously been limited by the lack of a practical culture system. While an exhaustive list of all such organisms is beyond the scope of this review, Table 2 includes a compilation of viruses that have so far been successfully identified by *in vitro* amplification techniques. (Not surprisingly, the list is dominated by viral pathogens whose detection by these means represents an improvement over existing techniques.) Second, the high sensitivity of these approaches may alter patient

sampling requirements. For example, buccal epithelial cells derived from a mouthwash have been successfully used to identify carrier status for the cystic fibrosis allele [6]. One can envision the use of urinary sediments in the place of urethral swabs in the diagnosis of urethritis, peripheral blood instead of bone marrow or liver biopsy in the diagnosis of atypical myobacterial infections, and peripheral blood mononuclear cells instead of bone marrow biopsy in the detection of recurrent leukemias and lymphomas. Third, the emergence of amplification techniques will decrease reliance on radiolabeled probes; the sensitivity previously attainable only with such probes will probably be achieved by combining amplification methods with non-radioactive reporter systems. Finally, if the potential problems of sample cross-contamination can be addressed and overcome, amplification methods may form the basis of a fully automated nucleic acid detection system.

The clinical implications of rapid and sensitive assays for viral diagnosis are many [15]. Amplification techniques have already demonstrated their utility in the detection of viral pathogens whose *in vitro* cultivation has been difficult (HIV-1, HIV-2, HTLV-1, human B lymphotropic virus), lengthy (CMV), or unavailable (human papillomavirus, hepatitis B virus) (Table 2). Although these techniques have already proven their power in the research setting, the development of sensitive, stable, non-radioactive probes and the simplification of methods will both be required before this technology can make the transition to the clinical laboratory. Significant progress in the development of *in vitro* amplification techniques has been achieved within a relatively short period; the application of these new methodologies to both new and old clinical problems holds great promise for the future.

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