Molecular Testing for Infectious Disease

Donald Jungkind

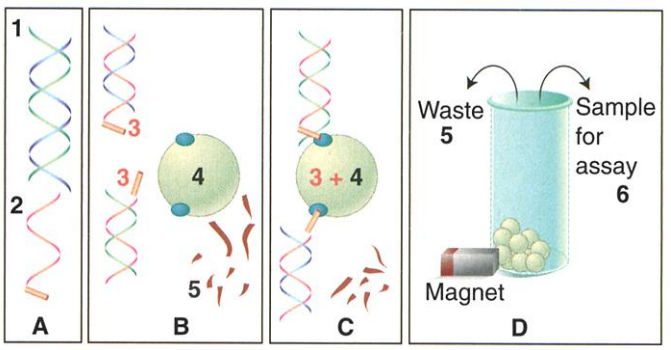
Today, the clinical microbiology laboratory does much of its testing with variations of classical procedures that have been used for half a century or more. Classical culture methods are labor-intensive (*1*, *2*), and they require labile natural products such as sheep red blood cells in agars or extracts of plant and animal products in broths or agar media. Delicate, living tissue cultures are required for growth of viruses. Immunological assays require the production of antibodies by animals or cell cultures. The high-volume automated instrumentation used in clinical chemistry and hematology laboratories does not exist for microbiology. The need for less labor-intensive clinical microbiology tests increases as advances in treatments for viral infections are made, the need for a safer blood supply becomes more urgent, and the importance of identifying food pathogens becomes clear. Pressure to rein in health care costs via rapid diagnosis, treatment, and discharge of patients has also encouraged the development of clinical procedures that can be executed more quickly than classical methods (*2*, *3*).

Among the most promising of the new technologies are nucleic acid amplification (NAA) methods, such as the polymerase chain reaction (PCR). These methods have improved sensitivity and specificity of tests in a format compatible with automation. Several companies offer NAA diagnostic tests, each of which uses an enzymatic replication of key regions of an organism's nucleic acid targets, such as the genome, rRNA, mRNA, or plasmid. Several examples of these methods are summarized here.

PCR as developed by Roche Molecular Systems (Pleasanton, CA) was the first NAA method and has been the most successfully implemented technology to date (*2*). By now, the principle of PCR is well known (*4*). A DNA target is required, but RNA pathogens can also be amplified using reverse transcriptase. The key components are two primers specific for the target region and a thermostable DNA polymerase. With alternating temperatures, the PCR technique denatures target strands, anneals primers, and amplifies the strands by replicating the coordinate strand from the primed complexes, creating double-stranded nucleic acid product called amplicons. The cycle is repeated several dozen times until large quantities of amplified product are produced. PCR has had widespread use in basic research and clinical application. The menu of pathogen PCR tests available from Roche is currently the most extensive, including qualitative tests for *Chlamydia trachomatis, Neisseria gonorrhoeae, Mycobacterium tuberculosis, Mycobacterium avium, Mycobacterium intracellulare*; qualitative tests for HIV-1 viral components (proviral DNA), hepatitis C virus (HCV), human T lymphocyte virus 1 and -2, cytomegalovirus (CMV); internal controls; and quantitative assays for HIV, hepatitis B and C, and CMV (*5*–*12*).

After PCR amplicons are made, they must be detected. The COBAS AMPLICOR was the first system to automate both the amplification and detection steps of the PCR testing process (*7*). The instrument, which sits on a bench top, enables routine PCR testing as an aid to diagnosis. COBAS AMPLICOR can deliver up to 50 detection results per hour after the initial amplification process. The new COBAS TaqMan instrument automates PCR amplification and detection by providing real-time quantitative test results within minutes using fluorogenic reporter molecules.

Specimen preparation is the first step of the PCR process, but it was the last step to be automated. The COBAS AmpliPrep system automated specimen preparation by releasing target nucleic acid from the microbe and capturing it with specific oligonucleotide probes. These target-probe complexes then become attached to magnetic beads via a biotin-streptavidin binding reaction (*2*). Once attached to the beads, the target is purified and concentrated automatically by the instrument (see figure below). This instrument reduced hands-on time for specimen preparation of HCV and HIV by 76% compared with the manual specimen preparation method.



Automated specimen preparation for PCR using the AmpliPrep instrument. (**A**) Lyse solution is used to hybridize target nucleic acid (1) to biotinylated nucleic acid probes (2) to form complex (3). (**B**) Magnetic beads (yellow sphere, 4) coated with streptavidin (green ovals) bind to the probe and form a complex with the captured target (**C**). (**D**) Magnetic washing removes unwanted material (5), and purified sample (6) is released and transferred to a storage tube.

The Gen-Probe transcription-mediated amplification (TMA) system (San Diego, CA) is an amplification system that uses an isothermal process. With TMA, up to 10 billion RNA amplicons can be produced from a single target molecule in 30 min. This process can be used with rRNA, mRNA, or DNA. It uses two primers and two enzymes, an RNA polymerase, and a reverse transcriptase. After an initial thermal denaturation step during specimen processing, the remaining steps are performed at a single temperature. The DNA template is produced as an intermediary in the reaction. The RNA polymerase binds to a promoter sequence in the DNA template. Acridinium ester-labeled DNA probes, which specifically bind to the target amplicon, are added to detect the amplicons produced in the reaction. The hybridized probe, which gives off a chemiluminescent signal, can then be measured. TMA is currently used for *C. trachomatis, N. gonorrhoeae*, and *M. tuberculosis* (*13*, *14*) and can also perform automated tests for detection of HIV-1, HIV-2, HCV, and HBV in blood products. A high-volume multiple-assay instrument developed by Gen-Probe, the TIGRIS system, will replace the manual and semiautomated tasks required for the first-generation TMA system (*15*) (see photo, next page).

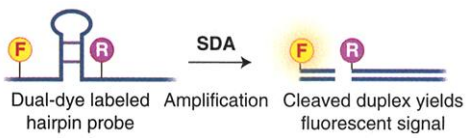
Another NAA test is a thermophilic strand displacement amplification (tSDA) assay (Becton Dickinson, Sparks, MD) (*16*)

The author is at the Clinical Microbiology Laboratories and the Departments of Pathology and Microbiology, Thomas Jefferson University Medical School, Philadelphia, PA 19107, USA. E-mail: Donald. Jungkind@mail.tju.edu

that is well suited to isothermal amplification of DNA targets. In tSDA, an organism-specific primer containing an overhanging restriction recognition site for Hinc II is hybridized to a single-stranded target DNA sequence. The DNA is then replicated in a way that modifies the restriction site such that it can only be nicked on one side and not cleaved all the way through. Hinc II then nicks the recognition site and a DNA polymerase extends the 3′ end from the nick site, displacing the downstream fragment. A second primer with a recognition site on the complementary strand of the target DNA fragment can be added to the reaction to generate exponential amplification with each new product, providing a template for the opposite primer. Detection of amplified product uses fluorogenic reporter probes that can be extended and displaced by the DNA polymerase, generating a real-time increase in signal within an hour (see figure below). tSDA has been used for detection of *C. trachomatis*, *N. gonorrhoeae*, and *M. tuberculosis*. The full automation of sample preparation and all reagent pipetting is in development.

Gen-Probe TIGRIS. This machine is an example of instruments for automating nucleic acid purification, amplification, and detection. Such devices use a computer that is hooked up to the housing in which the automated activity is performed.

Nucleic acid sequence-based amplification (NASBA) is another isothermal NAA method well suited for amplification of RNA targets and mRNA expression targets (bioMérieux, Durham, NC). NASBA, marketed as the NucliSens assay, relies on simultaneous activity of three enzymes: a reverse transcriptase, RNase H, and T7 RNA polymerase. Two oligonucleotide primers specific for each analyte are required. The coordinated interaction of the enzymes and primers creates a self-sustaining target sequence replication process that allows amplification (to as many as 10^9 copies) of a single target nucleic acid sequence in 90 min or less. Commercial diagnostic assays based on this method are available for CMV mRNA and quantitative HIV-1 (*6*, *17*, *18*). The system also has a versatile nucleic acid extraction system, referred to as the Boom method, which is compatible with a large variety of patient specimens. It uses silica particles for a solid phase extraction process to capture all nucleic acids after lysis of the microbes in a sample.



Detection of amplified product using fluorogenic reporter probes. DNA polymerase can extend and displace the reporter probes, generating a real-time increase in signal with every cycle of successful amplification. Fluorescence is generated when a quencher molecule and a fluorescent reporter are separated during amplification.

Another NAA variation is the nucleic acid probe amplification assay. The ligase chain reaction (LCR; trade name, LCx) from Abbott Laboratories (Abbott Park, IL) uses two pairs of complementary oligonucleotide probes to bind a DNA target sequence. The probe pairs are designed to bind complementary sites adjacent to each other on the target molecule. If base pairing of the oligonucleotide probes occurs in a perfectly compatible manner, a DNA ligase will link the two probes into one molecule that mimics one strand of the original target sequence. After strand separation, the newly ligated oligonucleotide probe serves as a target template for a repetition of the probe amplification process. Thus, amplification occurs as a result of ligase action and, like polymerase-based methods, can yield millions of copies of the probe. Using labeled probes during the entire amplification process allows ligation product to be detected. This test has been used for chlamydia, *N. gonorrhoeae*, *M. tuberculosis*, and HIV-1 (*14*, *18*–*20*).

Current NAA tests are most useful for detection of organisms that are either impossible or difficult to grow or those that grow well but at too slow a rate to be clinically useful. Another useful application of NAA tests is to provide therapeutic monitoring for treatment of HIV infection and chronic hepatitis. Viral load tests are now standard for monitoring these patients. PCR in combination with sequence analysis is useful for identification of fungi and bacteria that are hard to identify by other means and for determining viral genotype.

NAA tests have one bottleneck. In all NAA testing, the clinician must name a suspect organism. However, the most common test in clinical microbiology is a culture and susceptibility test, which asks two open-ended questions: (i) what pathogen can be found in the specimen collected from the site of infection and (ii) what antimicrobial agents seem to be effective in the treatment of the pathogen? To replicate this common test with NAA techniques would require testing a large number of relevant targets. Multiplex PCR amplifications and the use of microarrays to detect all pertinent oligonucleotide targets may eventually provide a way to replace the culture and susceptibility test with an automated chemistry-based assay

The use of commercial Food and Drug Administration–approved NAA test kits and instrumentation would bring great advantages because results would be standardized, greater numbers of clinical laboratories could use them, and technologists could benefit from training and support. All of this translates to hands-on time savings because of automation. These newly developed assays represent the beginning of an evolutionary process that will bring incremental improvements over time. Clinical microbiology is at the stage where clinical chemistry laboratories were in the 1960s, when classical manual chemistry methods were just beginning to be replaced with automated instrumentation. Managing the direction and pace of this development will be an exciting challenge for microbiologists and clinicians in years to come.

References and Notes

1. P. D. Klapper *et al.*, *Clin. Chem.* **44**, 1737 (1998).
2. D. Jungkind, *J. Clin. Virol.* **20**, 1 (2001).
3. V. Foubister, *CAP Today*, September, 84 (2000).
4. T. J. White, R. Madej, D. H. Persing, *Adv. Clin. Chem.* **29**, 161 (1992).
5. C. A. Bass, D. L. Jungkind, N. S. Silverman, J. M. Bondi, *J. Clin. Microbiol.* **31**, 2648 (1993).
6. K. G. Beavis, M. B. Lichty, D. L. Jungkind, O. Giger, *J. Clin. Microbiol.* **33**, 2582 (1995).
7. D. G. Murphy, L. Cote, M. Fauvel, P. Rene, J. Vinclette, *J. Clin. Microbiol.* **38**, 4034 (2000).
8. D. L. Jungkind, S. Direnzo, K. G. Beavis, N. S. Silverman, *J. Clin. Microbiol.* **34**, 2778 (1996).
9. J. M. Pawlotsky *et al.*, *J. Virol. Methods* **85**, 11 (2000).
10. P. Wattanamano *et al.*, *J. Clin. Microbiol.* **38**, 727 (2000).
11. H. L. Zaaijer *et al.*, *Vox Sang.* **66**, 78 (1994).
12. D. J. Brambilla, S. Granger, C. Jennings, J. W. Bremer, *J. Clin. Microbiol.* **39**, 1121 (2001).
13. J. S. Bermann, G. Yuoh, G. Fish, G. L. Woods, *J. Clin. Microbiol.* **37**, 1419 (1999).
14. W. H. Goessens *et al.*, *J. Clin. Microbiol.* **35**, 2628 (1997).
15. W. K. Roth, E. Seifried, *Transfus. Clin. Biol.* **8**, 282 (2001).
16. M. C. Little *et al.*, *Clin. Chem.* **45**, 777 (1999).
17. A. Kulkarni *et al.*, *Clin. Microbiol. Infect.* **7**, 179 (2001).
18. N. Zanchetta *et al.*, *J. Clin. Microbiol.* **38**, 3882 (2000).
19. R. Lumb *et al.*, *J. Clin. Microbiol.* **37**, 3102 (1999).
20. K. C. Carroll *et al.*, *J. Clin. Microbiol.* **36**, 1630 (1998).
21. The author was a major clinical investigator of the AMPLICOR/AmpliPrep methods described herein.