Introducing a Molecular Test Into the Clinical Microbiology Laboratory

Development, Evaluation, and Validation

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• ***Context.*—In the mid-1980s, the polymerase chain reaction methodology for the amplification of minute amounts of target DNA was successfully developed and then introduced into clinical use; such technology has led to a revolution in diagnostic testing. Despite enormous advances in the detection of infectious agents by amplification methods, there are also limitations that must be addressed.**

***Objective.*—To highlight the pertinent steps and issues associated with the introduction of an amplification assay into a clinical microbiology laboratory as well as the subsequent ongoing activities following its introduction into routine laboratory use.**

***Data Sources.*—Data were obtained from literature searches from 1990 through September 2002 using the subject headings "polymerase chain reaction," "molecular assays," and "amplification" as well as publications of the National Committee for Clinical Laboratory Standards.**

***Data Extraction and Synthesis.*—Using the findings obtained from these studies and publications, the process of introducing a molecular assay into the clinical microbiology laboratory was broken down into 4 major components: (1) initial phase of assay development, (2) polymerase chain reaction assay verification in which analytic sensitivity and specificity is determined, (3) assay validation to determine clinical sensitivity and specificity, and (4) interpretation of results and ongoing, required activities. The approach, as well as the advantages and limitations involved in each step of the process, was highlighted and discussed within the context of the published literature.**

***Conclusions.*—The application of molecular testing methods in the clinical laboratory has dramatically improved our ability to diagnose infectious diseases. However, the clinical usefulness of molecular testing will only be maximized to its fullest benefit by appropriate and careful studies correlating clinical findings with assay results.**

(*Arch Pathol Lab Med.* 2003;127:1106–1111)

In the mid-1980s, the polymerase chain reaction (PCR) methodology for the amplification of minute amounts of target DNA was successfully developed and then introduced into clinical use; such technology has led to a revolution in diagnostic testing. Nucleic acid amplifications such as PCR have become crucial in the diagnosis of selected infectious disease agents. Initially, molecular diagnostic tests were performed only in highly specialized or research laboratories; however, these assays are now more widely performed in all sections of the clinical laboratory. This has been largely a result of commercial assays, such as those advocated by the Food and Drug Administration, having been introduced into the market. Clearly, as the technology advances, additional commercial, Food and Drug Administration–approved methods will become available and enable even the smallest laboratory to employ amplification technologies for the detection of microorganisms. Nevertheless, in the meantime, there is a significant demand for amplification assays to detect the presence of a variety of microorganisms in clinical specimens for which there are no commercially available kits. Thus, clinical laboratories, particularly those associated with academic medical centers, must frequently develop their own "in-house" assays to accommodate the demand for the laboratory diagnosis of infectious diseases by amplification methods.

Despite the significant advantages and strengths that amplification methods offer in terms of the rapid and sensitive detection of infectious agents as well as the quantification of pathogens such as human immunodeficiency virus-1 to monitor therapy or disease outcome, there are limitations and caveats to these assays that must be understood. For example, studies have demonstrated that there is significant variation in the ability of "home-brew" or in-house assays among clinical laboratories to reliably detect infectious agents.[1,2] Moreover, as studies are published that correlate clinical findings with results of amplification tests, it has become exceedingly evident that although molecular diagnostic assays enhance diagnostic capabilities, their results must be clearly interpreted within the clinical context and performance of the laboratory assay. In other words, a thorough understanding of the parameters of the molecular assay, including the respec-

Accepted for publication February 26, 2003.

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Presented at the 11th Annual William Beaumont Hospital Symposium on Molecular Pathology, DNA Technology in the Clinical Laboratory, Royal Oak, Mich, September 12–14, 2002.

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tive procedural limitations and the target organism's microbiology and pathogenesis, is critical for the proper interpretation of results. Clearly, ongoing clinical research to correlate amplification results with clinical findings as well as strict adherence to guidelines for method validation for in-house PCR assays are prerequisite.

However, only guidelines, not universal standards, presently exist for the validation and subsequent quality control and assurance of in-house–developed assays.[3,4] Thus, this discussion will address the specific steps associated with the development, evaluation, and validation of a new in-house amplification assay into the clinical microbiology laboratory. For purposes of discussion, PCR will be the primary amplification format addressed; however, there are numerous other amplification formats as well for which the approach is similar. For purposes of discussion, this process has been broken down into 4 major components: (1) initial phase of assay development, (2) PCR assay validation in which analytic sensitivity and specificity are determined, (3) assay verification to determine clinical sensitivity and specificity, and (4) interpretation of results and ongoing, required activities. Although Food and Drug Administration–approved and/or commercially available assays will not be reviewed, it is clear that many aspects of method validation would be applicable to these assays as well. Considering this approach to the topic at hand, one basic assumption was made—that the laboratory already has the essentials to perform molecular biologic assays. These ''essentials'' include the availability of appropriately designed workspaces to physically control the cross-contamination of samples,[5] the equipment to perform amplification (for example, a thermal cycler), and appropriately trained personnel to successfully perform an in-house–developed assay.

INITIAL PHASE OF ASSAY DEVELOPMENT

As with any new laboratory test, prior discussion with intended users to determine their level of understanding and interest is prerequisite. Once the decision has been made to develop a PCR assay, there are a number of questions that must be addressed after a complete literature review to ensure the clinical utility of the molecular assay. Thus, decisions must be made as to whether a qualitative or quantitative PCR assay is most appropriate, whether the target nucleic acid is to be DNA or RNA, and whether ≥1 target will be detected. For example, the quantitation of hepatitis C virus (HCV) before treatment has been shown to be helpful for predicting and potentially monitoring responses to antiviral therapy in patients with chronic hepatitis,[6] while qualitative HCV RNA tests are used to identify acute HCV infections as well as chronic HCV carriers.[7] Similarly, although highly sensitive, the qualitative detection of cytomegalovirus (CMV) genomic DNA by PCR is not always useful in distinguishing symptomatic from latent or asymptomatic infection. By comparing the clinical outcome with the results obtained with a quantitative, rather than qualitative, CMV DNA PCR assay commercially available from Roche Diagnostics (Indianapolis, Ind), Caliendo et al[8] showed that this assay appeared to have clinical utility in monitoring patients for CMV disease. The choice of target nucleic acid can also affect clinical utility. To illustrate, using CMV messenger RNA as a target for active infection, nucleic acid sequence–based amplification, which is another amplification format, detected the viral transcript prior to the onset of clinical symptoms and determined the clinical improvement due to antiviral therapy in pediatric bone marrow transplant recipients.[9] Similar findings were obtained by Caliendo et al[8] with the NucliSens assay (Organon Teknika, Durham, NC), a qualitative nucleic acid sequence–based amplification assay that detects late CMV messenger RNA in order to better distinguish between active and latent infection. In addition, there may be a need to detect multiple pathogens rather than only one if a molecular assay is to be useful in some clinical settings. This concept is best illustrated by two of the most common sexually transmissible agents, *Chlamydia trachomatis* and *Neisseria gonorrhoeae*. By targeting both of these agents in a PCR-based screening program for genitourinary infections, the potential exists to reduce the prevalence of these diseases in the community and in turn reduce the associated sequelae, thereby avoiding significant health, social, and financial costs to the community.[10] Numerous other niches for either a ''panel'' of individualized or multiplexed amplification assays exist, such as niches for the detection of respiratory tract pathogens.[11,12]

Once the background work has been accomplished, a preliminary evaluation using published protocols or generic default concentrations for the various PCR components is prerequisite. Particular targets must be considered not only from the perspective of specificity and sensitivity, but also, if using fixed, paraffin-embedded tissue, from the perspective of product size, because sample processing leads to significant DNA degradation, allowing only short sequences to be amplified on a consistent basis. As an aside, it is important to check the published primer sequences for accuracy against the published sequence of the target from which they were taken prior to having them synthesized since mistakes in publication can occur. This preliminary evaluation using a default protocol will confirm that the correctly sized product is obtained with newly synthesized primers with nucleic acid extracted from a known target organism. In addition to determining that the correct target is obtained, nucleic acid extracted from closely related organisms must be assayed to confirm the specificity of the reaction. Products obtained in this preliminary evaluation should be hybridized with a target-specific probe to further confirm the assay's specificity that the amplified band actually represents the nucleic acid sequence of interest.

Subsequently, the PCR assay must be optimized or ''fine-tuned.'' Numerous aspects of the amplification mix (eg, pH, concentration of magnesium, primers, deoxyribonucleotide triphosphates) can be manipulated, and numerous generic or proprietary agents can be added to enhance assay performance.[13–15] In addition, there are many different types of polymerases on the market uniquely suited for different circumstances. For example, when amplifying RNA targets, regardless of input RNA level or gene copy number, some reverse transcriptases can provide better sensitivity over others, and there have been *Taq* polymerases developed that are uniquely suited for amplifying large targets (greater than 1000 base pairs). In most assays that we have developed, it has been necessary to go through this exercise of optimization to achieve the greatest sensitivity.[13] This is of paramount importance if more than 1 target sequence is being amplified by virtue of increasing the chance of obtaining spurious amplification products primarily because of the formation of primer

dimers as well as preferential amplification of one target sequence over another.[16] In our experience and that of others,[13,17] the annealing temperature and the concentration of magnesium chloride are key determinants for optimum assay performance. Once the assay has been optimized, the target organism should be serially diluted, and the nucleic acid should be extracted and then amplified using the specified conditions in order to approximate assay sensitivity.

ASSAY VALIDATION—ANALYTIC SENSITIVITY AND SPECIFICITY

Once optimized and its specificity confirmed yet prior to its validation in a clinical setting, an amplification assay must be evaluated for its analytic sensitivity and specificity using a background milieu of clinical material. However, it is essential that key decisions be made prior to further assay development and evaluation employing clinical material. First, the type of specimen (or specimens) acceptable for assaying by PCR must be determined as well as the physical criteria for the specimen's (or specimens') suitability for analysis, including optimum source and volume, appropriate collection method, transport and storage conditions, and specimen longevity. The choice of specimen(s) plays a key role in the performance and interpretation of test results because if any of the criteria are not fulfilled, the sensitivity and specificity of the assay will vary accordingly.[18] This becomes of paramount importance in quantitative amplification assays used to determine viral clearance such as for HCV.[19] In general, plasma or serum is preferred over whole blood or leukocytes when the target organism is predominantly extracellular (eg, HCV RNA, human immunodeficiency virus-1 RNA).

Once the acceptable specimen type (or types) has been delineated, the method for extraction must be selected. There are numerous methods in the literature for extracting either DNA or RNA from clinical specimens; however, they are beyond the scope of this discussion. A host of commercially available kits are available from which to choose as well as a number of automated methods for specimen preparation. These commercially available kits and automated systems are advantageous over manual methods of extraction in that they are more rapid and cost-effective and provide more consistent results. Nevertheless, the most optimal method for extracting nucleic acid must also be determined for each specimen type. The volume of specimen to be used for extraction must be taken under consideration; sample volumes vary considerably depending on the amplification method, but most assays use between 100 and 250 μL. Lastly, the method for detection of the target must be selected. Again, numerous options are available, such as detection by agarose gel electrophoresis or hybridization capture assays employing the capture of a biotinylated amplicon by a target-specific probe and detection by streptavadin-horseradish peroxidase.

Another important feature of any amplification assay is the selection and use of appropriate controls. First, the exquisite sensitivity of the PCR assay is a 2-edged sword. Because of its high sensitivity, there is a correspondingly high potential for contamination of specimens with amplified DNA; even small amounts of amplicon (as little as 2×10^{-8} μL of amplified DNA in an aerosol) can result in false-positive results. Therefore, the inclusion of a sufficient number of negative controls (about 10% of all samples) should be included, which are processed along with patient specimens. This reagent or negative control should include everything but the target sequence in order to monitor for cross-contamination. In addition to reagent controls, the inclusion of a positive control containing a low copy number of target is requisite on each run. In addition, measures including the design of the workspaces used for amplification (physically separate areas for pre- and postamplification manipulations), direction of workflow, and other contamination control measures—including inactivation of amplicons, meticulous laboratory technique and precautions, aerosol-resistant pipette tips, positive displacement pipettes, and dedicated space, equipment, supplies, laboratory coats, masks, and gloves—should be employed.

Although false-positive PCR results are a limitation, false-negative PCR results may also be a problem. The chief causes of false-negative PCR results are inhibitors of the DNA polymerases used in amplification of target sequences (for example, hemoglobin, heparin, and bilirubin) and a low copy number of target sequences in specimens submitted for PCR testing; inhibitors can also be introduced during specimen processing. Therefore, it is crucial that inhibition be monitored for each sample. This can be accomplished by either spiking each patient specimen with target nucleic acid and assaying along with the ''native'' specimen in a separate reaction or by using an internal control. An internal control can be constructed that contains nucleic acid of a different size and sequence compared to the target with the upstream and downstream primer recognition sequences on either end (see Eisenach et al[20] for an example of such a construct); once constructed, this internal control should be added to each PCR reaction to monitor for inhibition. A housekeeping gene such as β-globin can be used to assess inhibition as well as sample adequacy for some specimens. For some assays, particularly those for which nucleic acid is recovered from fixed, paraffin-embedded tissues or other tissue types, an extraction control should be included as well.

Manufacturers have recognized the critical need for stable, well-characterized amplification controls; some are available or under development at this time. Recently, Ambion (Austin, Tex) introduced a product, Armored RNA, in which a bacteriophage coat is assembled around an RNA target resulting in pseudoviral particles.[21] A number of ready-to-use RNA targets such as those derived from human immunodeficiency virus, HCV, hepatitis A virus, enterovirus, Norwalk virus, and West Nile virus are available in this construct. The company is also able to design and manufacture Armored RNA for specific needs. These preparations, which are stable for at least 11 months and compatible with a number of amplification platforms, can serve as positive controls and standards for quantitative PCR assays.

Once these important decisions have been made, it is essential to determine the analytic sensitivity and specificity of the assay. The analytic sensitivity, or the lower limit of detection, refers to the lowest number of organisms that can be reliably and reproducibly detected by the assay. This process is accomplished by serially diluting the infectious agent using pooled, infectious target-negative, clinical material as the diluent. Nucleic acid is extracted from each dilution and then assayed using the optimized reaction protocol; all appropriate controls should be run as well. In our experience, extraction protocols or other

aspects of the PCR reaction might need to be modified from published methods or generic defaults in order to achieve optimum sensitivity with clinical material. The assay should continue to be optimized using spiked clinical specimens until the appropriate sensitivity and specificity are achieved. For quantitative molecular biologic diagnostic testing, additional parameters including precision, accuracy, and tolerance limits must be determined prior to clinical validation. The reader is referred to an excellent review regarding these aspects for validating quantitative molecular assays by Wolk et al.[22]

ASSAY VALIDATION—CLINICAL SENSITIVITY AND SPECIFICITY

Once the analytic sensitivity and specificity have been determined, the amplification assay is then evaluated for its clinical sensitivity (the assay's ability to identify a patient with a particular infection) and specificity (the assay's ability to identify a patient without a particular infection). Several issues are associated with validating an amplification assay on clinical specimens. First is the selection of the ''gold standard,'' whose results will be compared to those obtained with the amplification assay. Traditionally, laboratorians have been accustomed to accepting culture as a final arbiter as to whether a clinical specimen is truly positive or not for a given pathogen. However, numerous incidences during the past decade have arisen that have demonstrated that culture may in fact be an imperfect standard. Two examples in which PCR has been shown to be frequently more sensitive than culture is the detection of *Bordetella pertussis*[23] and herpes simplex virus[24] in respiratory and cerebral spinal fluids, respectively. Recognizing that the gold standard may be imperfect, the second issue pertains to the manner in which discrepancies are resolved. This is a significant challenge when validating the clinical sensitivity and specificity of an amplification assay. One approach to resolving this relatively new problem and thereby allowing for validation of a new amplification assay without total dependence on culture has been through the use of discrepant analysis.[25–27] However, an objection to this approach for discrepancy resolution was raised by Hadgu,[28] who reasoned that this approach was inherently biased in favor of the PCR test being evaluated; others have felt that this objection was of little consequence. In some instances, discrepancies between culture-negative, PCR-positive specimens are resolved on the basis of not only performing repeat testing, ruling out possible cross-contamination, and using primers in a different region of the organism's genome, but also on clinical grounds—an approach used by Lakeman et al.[29] Thus, while of potentially considerable significance, additional data are required to determine the magnitude of the bias for discrepancy analysis. In addition to the limitations of culture as a gold standard for evaluating an amplification assay, some assays must be evaluated and the results correlated strictly on clinical grounds alone and possibly other laboratory data such as serology if the target organism is nonculturable.

Another challenge facing laboratorians attempting to validate the clinical sensitivity and specificity of an amplification assay is the paucity of positive clinical samples in some circumstances in which only one or a few patients present to their institution with a particular disease such as herpes simplex encephalitis. The procurement of known positive clinical specimens from colleagues is often necessary to validate the sensitivity of the assay; these specimens need to be blinded prior to performing and interpreting the amplification test.

INTERPRETATION OF RESULTS AND ONGOING ACTIVITIES

Laboratory results obtained by amplification assays need to be carefully interpreted. For these results to be used appropriately, it is imperative that clinicians as well as laboratorians have a thorough understanding of the advantages and limitations of the PCR assay as well as the target organism's basic microbiology and pathogenesis. As previously discussed, inherent problems and limitations associated with PCR in terms of false-negative results due to inhibition or inadequate sample collection, transport, and processing as well as false-positive results due to cross-contamination must be taken into consideration when interpreting test results. In addition to these caveats, sampling error due to analysis of an inadequate sample volume can also lead to a false-negative test result. Thus, results should be interpreted in the context of the patient's history, physical examination, and clinical course.

Clearly, as more studies are published regarding the use of PCR assays in a clinical setting, the spectrum of disease attributable to a particular agent has frequently broadened. For example, PCR has been used to detect herpes simplex virus DNA in the cerebrospinal fluid of patients who present with ''atypical'' clinical syndromes. Herpes simplex virus DNA has been detected in cerebrospinal fluid specimens obtained from patients with radiculomyelitis,[30] Bell palsy,[31] cluster headaches,[32] migraines,[33] and even common recurrent headaches.[34] It has been suggested that herpes simplex virus infection or reactivation may be a cause of such illnesses. A similar situation is unfolding with respect to enterovirus infections in adults.[35] Thus, interpretation of a PCR test result may change with time as greater understanding is gathered regarding new relationships that may be revealed between a clinical syndrome and an infectious agent as a result of molecular testing. One last factor that contributes to difficulties with interpretation of test results is the lack of standardization of amplification assays among published studies, particularly those developed in-house. An additional requisite for multicenter studies evaluating the utility of an amplification assay is the availability of reference materials.

In some situations in which a PCR test is positive but the culture is negative and/or disease is not supported on clinical grounds, a ''positive'' PCR test may need to be defined as to what constitutes a significant level of microbial DNA for a given clinical situation. The key to evaluating the clinical utility or ''niche'' of molecular assays is clinical correlation with laboratory results. Two examples illustrate this point. First, studies have been published in which PCR was used to rapidly identify *Streptococcus pneumoniae* in blood. But in one study, blood samples from 17% of healthy children assayed by PCR were found to have pneumococcal DNA.[36] In another study, cerebrospinal fluid was assayed by PCR for the presence of bloodborne herpesviruses in 662 patients because of clinical suspicion of viral etiologies for neurologic symptoms.[37] On the basis of their data, the authors concluded that the finding of herpesvirus DNA in cerebrospinal fluid may represent different degrees of the clinical evidence ranging from a causal relationship in well-defined conditions, such as herpes simplex encephalitis, to a possible viral reacti-

vation of little clinical significance. Therefore, levels of microbial DNA need to be defined for a given organism and then clinically correlated with the site and situation. Also, it may well be that another diagnostic test would be more appropriate in a given situation despite the exquisite sensitivity and specificity of PCR. This has already been demonstrated for diagnosing human parvovirus B19 infection, with subsequent guidelines published for appropriate test selection depending on the patient[38]; serologic testing is considered most appropriate in immunocompetent individuals, whereas PCR should be chosen to detect parvovirus DNA in individuals lacking an adequate antibody response.

It is clear that many questions remain unanswered, all of which may influence the manner in which PCR results are interpreted. Fredericks and Relman[39] raised a number of pertinent questions about the application of PCR for the detection of infectious agents in clinical samples. Although there are presently no answers, such questions as to how long microbial DNA persists in tissues after disease resolution or antibiotic treatment and how PCR can distinguish between colonization, latent infection, active infection, and relapsing infection must be ultimately answered if PCR is to become a useful and powerful tool for diagnosis.

Once the PCR assay has undergone validation, it is imperative that the laboratory participate in some proficiency testing program to ensure the reliability of the test and to confirm the expertise of the laboratory performing the test. Because of the lack of PCR assay standardization, particularly with respect to in-house–developed assays, a number of published studies have underscored the need for proficiency panels for monitoring the quality of diagnostic laboratories performing PCR testing.[40,41] Although there are programs offered through the College of American Pathologists as well as the Centers for Disease Control and Prevention for certain agents, laboratories are frequently faced with the dilemma of no sources of proficiency testing materials. In such instances, the laboratory must implement their own procedures to test for the internal consistency of their results. In addition, the quality of PCR test components must be ensured on a regular basis. A functional validation to determine the efficacy of new primers/reaction mix run in parallel with old primers/reaction mix with some samples must also be performed.

One other topic not previously mentioned is the issue of cost for amplification assays. In general, PCR and other amplification tests are perceived as expensive. Not only are there costs for reagents and equipment, but there are also training costs associated with teaching microbiologists to perform these molecular diagnostic assays.[42] Through rapid diagnosis, more expensive and/or invasive procedures may be prevented, hospital stays shortened, or the unnecessary administration of antibiotics prevented. Presently, in light of the expense of these assays coupled with an ever-increasing demand for testing as the list of clinical syndromes increases for some infectious agents, the appropriate use of these assays is imperative. Studies centering on the use of molecular assays in terms of cost and other benefits are now being published.[43–46] More of these types of studies need to be performed so that the appropriate use of molecular assays is accomplished.

Thus, the application of molecular testing methods in the clinical laboratory has drastically improved our ability to diagnose infectious diseases. However, the clinical usefulness of molecular testing will only be maximized to its fullest benefit by appropriate studies correlating clinical findings with assay results. As methods become more refined, automated, and standardized, the use of amplification methods to detect infectious agents will become more valuable. Because amplification methods and the interpretation of their results are continuously evolving and becoming more refined, it is imperative for clinical microbiologists as well as clinicians to remain current and knowledgeable in all aspects, including the chemistry and microbiology of the infectious organism as well as in general medicine. Questions regarding basic laboratory issues including the clinical need, the cost benefit for testing that pertains to reimbursement, the patent, and other issues as well as direct and indirect costs must be addressed and answered. Of great import, the specific clinical niche for the amplification test (ie, the clinical question that a PCR result will answer) must also be delineated. And finally, whether an amplification assay is and remains the best approach must be addressed during as well as after assay development.

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