Polymerase Chain Reaction Test Interpretation

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ABSTRACT: The polymerase chain reaction (PCR) technique is a powerful biologic tool that can simplify the diagnosis of many infectious diseases. However, PCR is not without limitations and is an inappropriate tool in diagnosing some diseases. This article discusses the basic concept of PCR and a variety of advanced PCR techniques used in commercial diagnostic tests. It analyzes the advantages and limitations of PCR techniques and discusses in detail the interpretation of results from PCR-based diagnostic tests.

The invention and refinement of the polymerase chain reaction (PCR) technique have revolutionized the detection of specific genetic material. In equine medicine, as in many other areas of medicine, this powerful technique has transformed pathogen identification. PCR can amplify the genetic material from any pathogen, but the clinician cannot assume that every PCR test will be more valid than other conventional diagnostic methods. If the test is correctly designed, PCR can be extremely sensitive and specific and can often identify a specific pathogen at much smaller concentrations than can other conventional diagnostic methods. However, false-negative and false-positive test results can occur secondary to inhibition of the reaction, sample handling errors, and the limitations of laboratory techniques.

The correct interpretation of test results is as important as understanding the technique and laboratory procedures involved in each diagnostic test. PCR pathogen tests rely on the detection of genetic material (DNA or RNA). A positive or negative PCR test result may correctly reflect the presence or absence of genetic material in the submitted sample but may not correctly reflect the infection status of the animal from which the sample was derived. A negative PCR test result can be generated in an infected and clinically affected individual if no genetic material is present in the sample. An example is the apparent lack of *Sarcocystis neurona* DNA in the cerebrospinal fluid (CSF) of horses with equine protozoal myeloencephalitis. Conversely, the presence of genetic material does not guarantee the presence of live organisms; a horse that has a positive PCR test result for *Salmonella* spp may not be actively shedding viable bacteria.

**DNA REP LICATION AND THE PCR TECHNIQUE**

The PCR technique uses a DNA polymerase to exponentially replicate (amplify) a specific target DNA sequence, generating a large amount of this genetic material so that it can be visualized. A key element of PCR is the use of thermostable DNA polymerases from thermophilic bacteria that can survive the high temperatures required during the PCR process. PCR also uses short DNA primers that bind to the specific target DNA sequence, initiating
strands of the double-stranded DNA template are copied during every cycle (two strands become four). At the end of the cycle, each of the four DNA strands can now act as a template for further replication, resulting in exponential amplification of the original double-stranded target sequence that is encompassed by the two primers (Figure 1). The resultant PCR product produced from amplification of specific target DNA is known as an amplicon.

Although primers are designed to be sequence specific, amplification of unwanted sequences can occur through nonspecific primer binding. Nontarget amplicons are usually distinguished from target amplicons based on size and are usually present at a lower level. However, increasing the PCR cycle number increases the level of nontarget amplicons and can affect detection of the target amplicon. As a result, the number of PCR cycles that can be run is usually limited to 30 to 40. Excessive PCR cycling can produce false-positive results through the amplification of either contaminating DNA or DNA with similar sequences, and minimization of PCR cycling should be part of any good clinical diagnostic laboratory quality-control program. If the pathogen is present in the clinical sample at a very low level, 30 to 40 cycles may still not be sufficient to amplify the target DNA. In this situation, more complex variations of the PCR technique can be used to achieve optimal specificity and increase detection of the target DNA. Some of these variations are discussed below.

MODIFICATIONS OF THE PCR TECHNIQUE

Reverse-Transcriptase PCR

PCR uses a DNA polymerase, which, in turn, demands the presence of DNA. Reverse-transcriptase PCR (RT-PCR) is used to detect RNA viruses, such as Eastern equine encephalitis virus. This technique involves the use of a reverse-transcriptase enzyme to

Figure 1. Schematic depicting one PCR cycle, which consists of three temperature steps (denaturation, annealing, extension) in which different parts of the reaction occur. At the end of the cycle, each of the four DNA strands can act as a template for further replication, resulting in exponential replication of the original target sequence.
create a complementary DNA strand from an RNA template, resulting in PCR amplification.

**Nested PCR**

Nested PCR is used to increase the sensitivity of detecting pathogens, such as *Neorickettsia risticii*, that may not be detectable after 30 to 40 cycles of regular PCR. Unlike regular PCR, nested PCR uses two pairs of primers and two sequential series of PCR amplification. The first amplicon is created using the first (outer) pair of primers. This initial amplicon is then used as a template for the second PCR, which uses the second (inner) primer pair (Figure 2). The second, smaller DNA amplicon is the product of these two “nested” PCRs. Only a small amount of the original sample is passed through to the second series of amplification, thereby diluting possible PCR inhibitors while simultaneously increasing the specificity and sensitivity of the target amplicon. Nested PCR is also valuable in speciation applications because the outer primer pair can be designed for specificity toward a DNA sequence common to a larger class of microorganisms, whereas the inner primers can be designed for specificity for individual subtypes.3

**Internal Control PCR**

A variety of internal controls are available to monitor aspects of a PCR-based test. Internal controls are generally included for one of the following reasons:

- **To ensure DNA was appropriately extracted from the sample.** These controls monitor sample handling and DNA extraction techniques by including a second set of primers (see Multiplex PCR on page 191) that amplify a gene present in all samples. For example, for a blood sample, primers specific for mammalian ribosomal DNA might be included. Alternatively, the test sample can be spiked with an unrelated source of DNA before DNA extraction. Simultaneous PCR amplification of this second DNA source can act as a control for DNA extraction, reagent integrity, specimen processing, and PCR inhibition.9 This type of control does not ensure that primers against the specific pathogen DNA are working appropriately.

- **To detect false-negative results (no DNA are amplified, but pathogen DNA are present) due to sample handling errors, poor primer performance, or the presence of contaminating inhibitory substances in the sample.**10,11 In this technique, which monitors PCR success, an artificial target DNA (mimic) is manufactured and can be amplified using the same primer pair as the pathogen target DNA but is distinguishable in post-PCR analysis. The mimic is included along with the target DNA in each PCR test. If neither the mimic nor the clinical target DNA is amplified, the PCR was unsuccessful and the protocol should be carefully examined for handling errors, primer performance, and possible PCR inhibition. If the mimic is amplified but the target DNA is
Key Points

- The strength of PCR diagnostic tests is based on the ability to design pathogen-specific PCR primers that amplify pathogen, but not host, genetic material.
- A positive PCR test result indicates the presence of pathogen genetic material but does not necessarily confirm active infection.
- A negative PCR test result indicates the absence of pathogen genetic material but does not necessarily rule out active infection.

not, a true-negative result is more likely, although sample handling errors, such as ineffective pathogen DNA extraction or pathogen DNA degradation, are still possible.

Multiplex PCR

Multiplex PCR uses multiple primer pairs within the same amplification series. This allows detection of multiple DNA sequences within the same sample as well as the inclusion of internal controls to assess the reliability of the PCR itself. Because multiple primer pairs are designed for use in the same reaction, care must be taken to identify primer pairs that function at the same annealing temperatures. Furthermore, for ease of post-PCR analysis, designing primer pairs that result in sufficiently different-sized PCR products for the multiple target DNA sequences is essential. The use of multiplex PCR is also increasing in popularity in running panels of PCR tests. For example, a panel of respiratory virus PCR tests run in a multiplex reaction might include primers specific for equine
herpesviruses 1, 2, 4, and 5; equine adenovirus 1 and 2; equine arteritis virus; and equine rhinitis A virus.\textsuperscript{12}

**Real-Time PCR**

Real-time PCR combines the amplification process and the subsequent amplicon detection process into a single step. In addition to the regular primer pair used for target DNA amplification, a third, fluorescent DNA sequence (called a fluorogenic probe) is included that specifically binds to the amplicon, resulting in increasing fluorescence as more amplicons are produced.\textsuperscript{13} Because amplification and detection occur in a single tube, the advantages of real-time PCR include increased ease in handling large sample numbers, more consistent results, decreased turnover time (no post-PCR processing), and fewer false-positive results.\textsuperscript{14} The disadvantages include the expense of the instruments and possibly increased sensitivity of the reagents to inhibition. In equine PCR diagnostics, real-time PCR has been used in an attempt to increase the specificity of the test because the fluorogenic probe must specifically bind the target amplicon DNA and should not bind to possible extraneous amplicons.\textsuperscript{14,15} Real-time PCR is sometimes confusingly referred to as RT-PCR, which should be reserved for referring to reverse-transcriptase PCR.

**INTERPRETATION OF PCR-BASED DATA**

Accurate identification of infectious pathogens can be critical to the development of an appropriate treatment protocol. Therefore, it is important to consider how both positive and negative PCR test results should be interpreted. The gold standard for infectious pathogen identification has traditionally involved isolation of the pathogen by bacterial culture, viral isolation, or histopathologic identification.\textsuperscript{16–19} While isolation of a pathogen decisively demonstrates the presence of that organism, failure to isolate the pathogen using the “standard” method does not always rule out infection. Studies designed to compare the clinical accuracy of PCR testing to other “standard” diagnostic methods usually consider four outcomes\textsuperscript{6,18} (Table 1). When the results of PCR and “standard” isolation methods agree (both results are either positive or negative), the result is unequivocal. Questions generally arise when the results of PCR testing do not support standard isolation results.

**Table 1. Comparison of PCR and Gold-Standard Pathogen Identification Results**

<table>
<thead>
<tr>
<th>PCR Result</th>
<th>Standard Result</th>
<th>General Interpretation</th>
<th>Alternative Interpretation</th>
</tr>
</thead>
<tbody>
<tr>
<td>Positive</td>
<td>Positive</td>
<td>Pathogen present</td>
<td>—</td>
</tr>
<tr>
<td>Positive</td>
<td>Negative</td>
<td>Pathogen DNA present (no indication of live pathogen)</td>
<td>False-positive PCR result (due to contaminating DNA) or False-negative standard result (due to pathogen being present but dead or too rare to isolate, errors in handling or processing the sample, or prior antibiotic use)</td>
</tr>
<tr>
<td>Negative</td>
<td>Positive</td>
<td>Pathogen present</td>
<td>Lack of pathogen DNA in PCR sample or False-negative PCR result (due to the presence of inhibitory substances, poor DNA extraction, or poor reaction performance)</td>
</tr>
<tr>
<td>Negative</td>
<td>Negative</td>
<td>No pathogen present</td>
<td>—</td>
</tr>
</tbody>
</table>
PCR results can occur through the undetected inclusion of contaminating DNA within the test reaction, excessive PCR cycling resulting in amplification of DNA similar to the target DNA, low specificity of the PCR primers (primers that amplify similar nonpathogen DNA), or carryover contamination in post-PCR analysis using conventional PCR techniques. Contamination can be monitored through inclusion of negative control reactions in which the test protocol is identical, but target DNA is not included.6

The development of a new PCR test should include primer testing using target DNA with similar sequences (e.g., primers for equine herpesvirus should be tested against those for equine herpesvirus 4)21 to ensure that the primers have good specificity to the target DNA. Careful sampling protocols, PCR design, and quality-control protocols should also be a routine part of good clinical pathology laboratory practices.

**Interpretation of a Negative PCR Result**

Negative PCR results from infected animals can occur in two ways. First, negative PCR results can occur when samples from infected animals do not contain target DNA (or RNA). Negative PCR results can be obtained from CSF samples that lack *S. neurona* schizonts22 or from the serum of infected animals that have passed the short-lived viremic phase of infection with encephalitis virus.7 Second, false-negative PCR results can occur when samples contain appropriate target DNA but undergo handling or laboratory errors (poor DNA extraction from the sample, DNA degradation, poor primer performance, poor reaction optimization, transcriptional errors) or have been exposed to inhibitory substances (urea and heme3,4) or antimicrobials. The inclusion of positive controls and internal standards as well as the use of techniques such as nested PCR can minimize these problems.8,10

**CONCLUSION**

Rapid and accurate pathogen detection is critical in developing appropriate treatment protocols and limiting the spread of contagious diseases. Although isolation of a pathogen confirms the presence of viable isolates of that organism, failure to isolate the pathogen using the standard method does not always rule out infection. For example, prior antimicrobial use or a low number of organisms can inhibit growth in culture. The exponential amplification of pathogen genetic material using PCR generally leads to an increase in positive results compared with most other conventional methods. In addition to the basic PCR technique, other advances in PCR technology (nested PCR, real-time PCR) have been used to increase the specificity and sensitivity of PCR-based diagnostic testing.

Several factors may limit the validity of specific PCR-based pathogen detection tests. False-positive PCR test results can occur if the sample was contaminated with pathogen genetic material (during either sample collection or sample handling and DNA extraction). In addition, the PCR process itself can introduce false-positive results through excessive cycling, poor primer performance, or post-PCR analysis contamination. False-negative PCR test results can result from low numbers of amplicons, poor primer performance, or the presence of inhibitory substances (urea and heme3,4) in the sample. The following have been used to monitor PCR success and reduce potential problems: better DNA extraction techniques, detailed PCR protocols with carefully tested primers, the inclusion of appropriate controls, and the use of techniques such as nested PCR and real-time PCR.2,8,14 Understanding the potential causes of false test results and how they can be avoided, or at least monitored, is crucial to correctly using PCR-based diagnostic tests.

In addition to false test results, it is possible to obtain correct PCR test results (based on the presence or absence of pathogen genetic material in the sample) that, nevertheless, do not accurately reflect the infection status of the tested animal. Positive PCR test results from animals that are no longer infected can be obtained through amplification of genetic material from nonviable organisms (e.g., DNA from nonviable *Salmonella* spp in fecal and environmental samples20). Negative PCR test results from infected animals can occur when the tested sample does not contain pathogen genetic material (e.g., lack of *S. neurona* schizonts in CSF samples,21 lack of encephalitis virus particles in the serum of infected animals that have passed the short-lived viremic phase,23,24 lack of the targeted virulence genes in some pathogenic strains of *Rhodococcus equi*18). Therefore, in addition to understanding the possible causes of false test results, practitioners must carefully evaluate the usefulness of each pathogen PCR test based on the likelihood that the presence or absence of genetic material in the submitted sample will correlate with the disease status of the tested animal.

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REFERENCES


1. PCR can be used to identify pathogens because it
   a. measures the host antibody response to pathogen infection.
   b. amplifies genetic material from the pathogen.
   c. uses improved techniques to culture fastidious bacteria.
   d. is a new microscopic technique to directly identify microorganisms.

2. PCR can identify pathogen genetic material through the
   a. use of enzymes that specifically recognize the pathogen genetic material.
   b. creation of primers that specifically bind pathogen DNA.
   c. use of different stains to identify different pathogen genetic material.
   d. none of the above

3. PCR works through
   a. exponential amplification of target pathogen DNA.
   b. the use of a thermostable DNA polymerase.
   c. repetitive temperature cycles.
   d. all of the above

4. Reverse-transcriptase PCR refers to the use of a reverse-transcriptase enzyme to create
   a. complementary DNA from pathogen RNA.
   b. complementary RNA from pathogen DNA.
   c. an internal control for monitoring inhibition within the PCR process.
   d. none of the above

5. PCRs may be monitored for possible inhibition by using _______ PCR.
   a. reverse-transcriptase
   b. internal control
   c. multiplex
   d. none of the above

6. Multiplex PCR
   a. can be used to increase the number of pathogens that can be detected in one PCR test.
   b. uses two sets of primers in sequential PCR tests.
   c. is limited by decreased sensitivity compared with one-step PCR tests.
   d. none of the above

7. Real-time PCR
   a. is inefficient for handling large sample numbers.
   b. involves the use of a fluorogenic probe.
   c. involves considerable post-PCR processing.
   d. does not use PCR primers.

8. Pathogen PCR tests can be complicated by
   a. inhibition by fecal compounds.
   b. the presence of nonviable pathogens.
   c. DNA extraction and handling errors.
   d. all of the above

9. A positive PCR test result indicates
   a. vaccination.
   b. the presence of pathogen genetic material.
   c. inhibition.
   d. all of the above

10. A negative PCR test result may indicate
    a. lack of genetic material, with no infection.
    b. lack of genetic material, with possible infection.
    c. inhibition of PCR, with possible infection.
    d. all of the above