Nucleic Acid Amplification Tests for the Diagnosis of Pneumonia

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Molecular diagnostic techniques, such as polymerase chain reaction (PCR), are promising tools for the rapid etiological diagnosis of pneumonia. PCR offers potential advantages over conventional tests for the detection of *Mycoplasma pneumoniae*, *Legionella* species, and *Chlamydia pneumoniae*. For pneumococcal pneumonia in adults, PCR adds little to existing diagnostic tests and is unable to distinguish pneumococcal colonization from infection when testing respiratory samples. Although PCR is probably more sensitive than are conventional microscopy-based methods for diagnosing *Pneumocystis carinii* pneumonia, the specificity is uncertain, because *P. carinii* can occasionally be detected in the absence of clinical symptoms. PCR is useful for the diagnosis of viral pneumonia in immunocompromised patients. Further work is required to better characterize the role of PCR versus the role of other tests for diagnosing pneumonia and to develop standard PCR assays that can be readily adopted by routine diagnostic laboratories.

Pneumonia is one of the most common infectious diseases among adults and children. Despite recent advances in diagnostic testing, the etiological diagnosis of pneumonia is infrequently achieved with confidence. Even in the most rigorous studies, it is difficult to establish an etiological diagnosis in 50% of cases of community-acquired pneumonia [1]. In the real world, the diagnostic rate is much lower. This has led some investigators to question the usefulness of routinely performing microbiologic tests for patients with pneumonia [2–4].

Although there may be some justification for not routinely ordering microbiologic tests for every patient with pneumonia, it is important to recognize recent advances in diagnostic testing. The most promising advances have been with antigen and nucleic acid detection assays. New immunochromatographic urinary antigen detection assays for *Legionella pneumophila* and *Streptococcus pneumoniae* are easy and quick to perform and have relatively high sensitivities and specificities [5, 6]. Moreover, with further refinement and development, it is expected that many other antigen detection tests for pneumonia pathogens will soon be commercially available. Similarly, during the past decade, there has been considerable evaluation of nucleic acid amplification techniques as diagnostic tools for pneumonia. Although none of these assays are readily available or in routine use, it is expected that the situation will change during the next few years.

This review focuses on the current role of molecular diagnostic techniques for determining the etiology of pneumonia. PCR will be the only technique discussed, because this has been the nucleic acid amplification method almost exclusively evaluated in published studies. The detection of pulmonary mycobacteria will not be discussed because this has been the subject of recent reviews [7, 8].

MOLECULAR DIAGNOSTIC TECHNIQUES FOR PNEUMONIA

PCR is an attractive tool for diagnosing the cause of pneumonia, because it can detect minute amounts of nucleic acid from potentially all pneumonia pathogens, does not depend on the viability of the target microbe, is probably less affected by previous antimicrobial therapy than are culture-based methods, and provides results quickly. Moreover, increased mechanization means that PCR is becoming increasingly available to laboratories outside of specialist tertiary referral centers. The development of real-time PCR technology enables testing to be...
performed in <1 h in a single reaction vessel, thereby reducing the chance of contamination. These advantages may make PCR the front-runner for the ideal diagnostic test for pneumonia [9], although there are some disadvantages of relying on molecular approaches rather than culture. Such disadvantages include the limited ability to perform antimicrobial susceptibility testing and the lack of an isolate archive if future testing is required.

As yet, no PCR assay has been approved by the US Food and Drug Administration for diagnosis of pneumonia, although commercial assays are becoming available. For this to happen, optimal protocols need to be established. Several key parameters need to be determined before PCR becomes part of the routine diagnostic workup for a particular pneumonia organism, notably sensitivity and specificity, reproducibility, and optimal sample types.

**SENSITIVITY AND SPECIFICITY**

The accurate calculation of clinical sensitivity for a PCR assay is hindered by the lack of a suitable diagnostic “gold standard” for most infections. This is an inherent problem with PCR, which is likely to be a more sensitive technique than are most culture-based diagnostic tests. Sensitivity may be affected by the presence in samples of PCR inhibitors, usually those of an unknown nature, which cause false-negative results [10]. These inhibitors can be detected by the inclusion of specific positive internal controls, which should be part of every PCR assay. Given the small volumes of PCR reaction mixtures, sampling errors may also reduce sensitivity, which may be improved by increasing the sample volume in the reaction mixture or through concentration of the sample.

The risk of false-positive results is a major problem facing all PCR assays. This is largely a consequence of the extreme sensitivity of PCR and may result from contamination by exogenous material or from detection of low levels of colonizing organisms in the sample. False-positive results may also be the result of amplification of microorganisms that have similar genome sequences to the target organism. False-positive findings may be avoided by use of appropriate controls, good laboratory practice to prevent contamination, and confirmation of all positive results by an independent method. The targeted nucleic acid sequence of all newly developed PCR assays should be checked for specificity by means of a tool such as the Basic Local Alignment Sequence Tool (available at: http://www.ncbi.nlm.nih.gov/BLAST/). This is a useful exercise even for previously published sequences for PCR assays, because errors in publication of primer sequences have resulted in transfer of the error to assays used by other investigators [11]. This search may also identify similar sequences from other microorganisms that have been published since the assay was initially developed.

Sensitivity and specificity may also be influenced by the type of PCR assay. Nested PCR is generally more sensitive than single-step PCR, but there is an increased risk of contamination for nested PCR, because the reaction tubes need to be opened after the first stage to add reagents for the second round of amplification. Multiplex PCR assays, which amplify >1 target sequence in the same reaction tube, are generally less sensitive than are single-step PCR assays. The specificity of multiplex PCR may also be reduced if the annealing temperatures for the individual primer pairs are not identical. The use of hybridization with specific probes, fluorescence, or EIA to detect amplified PCR products ensures the specificity of the PCR product and may increase sensitivity, compared with standard visualization of PCR products after agarose gel electrophoresis.

**REPRODUCIBILITY**

It is essential that PCR protocols are reliable and robust enough to be used outside of research laboratories. Unfortunately, it is often difficult to compare the findings from different investigations, even when the same PCR target is used. Clinical samples are often from disparate patient populations, and protocols may differ with respect to sample preparation, nature of controls, and PCR conditions. Few assays have been independently evaluated by outside laboratories. There is a real need for more-standardized approaches to diagnostic testing, and it is hoped that the recently published recommendations for *Chlamydia pneumoniae* testing [12] will prompt similar action for other pneumonia pathogens.

**OPTIMAL SAMPLE TYPES**

The sensitivity of PCR will vary with the sample type tested. For pneumonia, both respiratory and nonrespiratory samples have been used. Most studies have evaluated lower respiratory tract samples (especially sputum and bronchoalveolar lavage samples), which are likely to have the highest diagnostic yield. Samples obtained by transthoracic needle aspiration may have an even higher microbiological yield and have a lower risk for contamination, compared with other lower respiratory tract samples [13–17], but this method is not widely practiced. PCR is particularly useful for detection of pathogens that are not known to colonize the human respiratory tract, such as *Legionella* species, for which a positive result provides strong supporting evidence of infection. A major limitation of lower respiratory samples is that they may be difficult to obtain. For example, fewer than one-half of patients with legionnaires disease produce sputum [18], and invasive procedures, such as bronchoscopy, are only performed for a few selected patients. For some pathogens that do not colonize the oropharynx,
thor swab or oral wash samples may be useful and are usually easy to obtain.

Pathogens may also be detected in samples of blood (whole blood, serum, plasma, and peripheral leukocytes) and urine, but the usefulness of each sample type will vary with the specific pathogen tested and the stage of illness. These non–lower respiratory tract samples have the advantage of usually being readily obtainable from all patients.

**APPLICATION OF MOLECULAR DIAGNOSTIC TECHNIQUES FOR THE DIAGNOSIS OF SPECIFIC PNEUMONIA PATHOGENS**

**Streptococcus pneumoniae.** *S. pneumoniae* is the most common cause of community-acquired pneumonia in both adults and children. However, a definitive diagnosis of pneumococcal pneumonia is difficult to establish by means of conventional diagnostic tests. Blood culture lacks sensitivity, and isolation of *S. pneumoniae* from sputum samples may represent oropharyngeal colonization. Newer pneumococcal urinary antigen tests are promising [6, 19] but may be less reliable for children [20].

The use of PCR as a tool for diagnosis of pneumococcal pneumonia has been fairly extensively evaluated in both adults and children (table 1). In contrast to evaluations of PCR assays for other pneumonia pathogens, which mainly test respiratory samples, most studies of *S. pneumoniae* PCR have focused on testing blood samples (whole blood, serum, or plasma) for the purpose of detecting occult pneumococcal bacteremia. Only one study has tested multiple respiratory and nonrespiratory samples obtained from the same group of patients [11]. When a positive blood culture result is used as the diagnostic “gold standard,” the reported sensitivities for detection of *S. pneumoniae* in blood samples have been 29%–100% in adults [11, 16, 21–25] and 57%–100% in children [26–29]. When the definition of pneumococcal pneumonia also includes patients with positive sputum culture results, the reported sensitivities have been 26%–88% [16, 21, 22]. The reason for this variation is unclear, because similar PCR protocols were used in most studies. Positive PCR results were also recorded for control subjects in many of these studies, and these findings are not readily explained.

For the few studies that have evaluated PCR for detection of *S. pneumoniae* in respiratory samples, rates of PCR positivity for sputum samples have been high (83%–100%) [11, 30–32]. One of these studies tested both sputum and throat swab samples obtained from a large number of patients with pneumonia, as well as throat swab samples obtained from a control group of patients without pneumonia [11]. Although PCR was more sensitive when testing sputum samples than when testing other sample types, the authors concluded that PCR testing of respiratory samples is an unreliable tool for diagnosis of pneumococcal pneumonia and that this approach cannot distinguish colonization from infection. These conclusions were based on the finding that the PCR positivity rate for throat swabs was

### Table 1. PCR assays evaluated in studies of pneumonia.

<table>
<thead>
<tr>
<th>Pathogen</th>
<th>Target genes</th>
<th>Sample types tested</th>
<th>Comments</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Streptococcus pneumoniae</strong></td>
<td>Pneumolysin, autolysin</td>
<td>Sputum, throat swab, serum, plasma, WBC, urine, TNA</td>
<td>Problems distinguishing colonization from infection when testing respiratory samples; variable reported sensitivities for blood samples</td>
</tr>
<tr>
<td><strong>Mycoplasma pneumoniae</strong></td>
<td>P1 adhesion, 16S rRNA, ATPase operon</td>
<td>Sputum, nasopharyngeal, throat swab, BAL, TNA</td>
<td>More sensitive than culture; throat swabs may be the preferred sample type</td>
</tr>
<tr>
<td><strong>Legionella species</strong></td>
<td>5S rRNA, 16S rRNA, mip</td>
<td>Sputum, BAL, ETA, throat swab, serum, WBCs, urine</td>
<td>At least as sensitive as culture when testing lower respiratory tract samples; needs further evaluation for non–respiratory samples</td>
</tr>
<tr>
<td><strong>Chlamydia pneumoniae</strong></td>
<td>Cloned PstI fragment, 16S rRNA, MOMP, 53 kDA protein, 60 kDA protein, 16S-23S spacer rRNA</td>
<td>Sputum, nasopharyngeal, throat swab, BAL, oral wash</td>
<td>More sensitive than culture</td>
</tr>
<tr>
<td><strong>Chlamydia psittaci</strong></td>
<td>MOMP 16S rRNA, gseA</td>
<td>Sputum, throat swab, blood, lung tissue</td>
<td>Not extensively evaluated</td>
</tr>
<tr>
<td><strong>Pneumocystis carinii</strong></td>
<td>Mitochondrial rRNA, 18S rRNA, 5S rRNA, thymidylate synthase, dihyrofolate reductase, MSG</td>
<td>Sputum, throat swab, BAL, oral wash, ETA, blood, lung tissue, serum</td>
<td>May be most useful when clinical suspicion of <em>P. carinii</em> pneumonia is high but cytological test results are negative</td>
</tr>
<tr>
<td><strong>Viruses</strong></td>
<td>Various</td>
<td>Nasopharyngeal, throat swab, sputum, BAL</td>
<td>Not extensively evaluated for pneumonia; most useful for immunocompromised patients</td>
</tr>
</tbody>
</table>

**NOTE.** BAL, bronchoalveolar lavage; ETA, endotracheal aspirate; MOMP, major outer membrane protein; MSG, major surface glycoprotein; TNA, transthoracic needle aspiration.
the same for both patients with pneumonia (55%) and control patients (58%), suggesting that the positive results are more likely to represent colonization than infection. Furthermore, in patients who produced sputum, almost all of those with positive throat swab samples also had positive sputum samples. By extrapolation, these findings imply that many of the positive sputum PCR results indicate detection of colonizing organisms rather than pathogens. It is possible that quantitative PCR may be useful in this setting and that infection can be distinguished from colonization by a higher bacterial burden in the former [33], but this is yet to be systematically evaluated.

It is also possible that the PCR assay used in this study was detecting oropharyngeal colonizing bacteria other than S. pneumoniae and that this was partly responsible for the relatively high rate of throat swab PCR positivity in both case patients and control subjects. The PCR assay used in this and in most other studies targets the pneumolysin gene, which is considered specific to S. pneumoniae. Recent evidence has questioned this specificity: genes encoding pneumolysin and autolysin have been found in other streptococci that closely resemble Strep-tococcus mitis [34]. These findings highlight the necessity of periodic reevaluation of the specificity of PCR assays and the need for more-specific targets for S. pneumoniae.

For the diagnosis of pneumococcal pneumonia, PCR remains a research tool. No current PCR assay has been clearly demonstrated to offer significant advantages over existing diagnostic tests.

Mycoplasma pneumoniae. M. pneumoniae is a fastidious organism, and culture on artificial media is laborious and time-consuming. Consequently, laboratory diagnosis of M. pneumoniae infection has largely relied on serological testing. Although there have been recent improvements, especially with the development of IgM antibody assays, the sensitivity and specificity of serological testing is suboptimal [35].

The limitations of conventional diagnostic tests have prompted numerous studies of the use of PCR to provide a rapid and sensitive method for detection of M. pneumoniae [36–51].

Although these studies have used a variety of PCR assays, patient populations, and sample types, the findings have been generally consistent. PCR is more sensitive and considerably more rapid than culture. In general, there is a good correlation between PCR results and the results of serological testing, although most studies have reported that a significant number of samples test positive by only 1 of these 2 methods.

Both upper and lower respiratory tract samples are suitable for PCR testing. Of interest, upper respiratory tract samples (throat swabs and nasopharyngeal samples) appear to be the preferred sample types because of both high sensitivity and convenience, and throat swabs may have a further advantage over nasopharyngeal aspirates as a result of the higher rate of PCR inhibitors in the latter [43]. During the investigation of a large outbreak of M. pneumoniae infection in a closed community, 15% of patients continued to have M. pneumoniae DNA detected in their throats 2–6 weeks after the initiation of antibiotic therapy [51]. PCR testing of samples obtained by transthoracic needle aspiration appears to be specific, but it is less sensitive than testing upper respiratory samples [45]. PCR of throat swab samples may be the best existing diagnostic test for M. pneumoniae, but standardized protocols are needed before this is introduced into widespread use.

Legionella species. Legionella species do not colonize the respiratory tract and, consequently, detection of Legionella bacteria in clinical samples indicates infection. For this reason, and given the major shortcomings of other diagnostic tests for Legionella infection [18], there has been considerable interest in investigating the role of PCR for diagnosing legionellosis.

PCR was first used to detect Legionella species in environmental water samples but has now been successfully used to test a variety of clinical samples. When testing lower respiratory tract samples, PCR has repeatedly been shown to have a sensitivity equal to or greater than culture [52–58]. A consistent finding in most studies has been the identification of samples that are PCR positive but culture negative. Whether this represents contamination or the increased sensitivity of PCR is unclear, although the latter is likely to be a major contributor to the increased yield.

The main disadvantage with testing sputum samples is that fewer than one-half of patients with legionnaires disease produce sputum [18]. This has prompted investigation of the role of PCR for testing other sample types. Legionella DNA can be detected in urine samples obtained from guinea pigs and humans with legionellosis [59–64]. By use of a 5S rRNA PCR assay, Helbig et al. [61] detected Legionella DNA in urine samples obtained from 42 of 58 patients with confirmed legionellosis and concluded that urinary PCR complements other rapid diagnostic tests, especially urinary antigen detection. Other researchers who used similar PCR assays have recorded sensitivities of 46% and 86% when testing urine samples [60, 64], whereas others noted a sensitivity of only 30% when a 16S rRNA PCR assay was used [63]. Although Lindsay et al. [65] detected Legionella DNA in both acute- and convalescent-phase serum samples obtained from 5 patients with legionellosis, others have recorded sensitivities for serum PCR of only 30% and 43% [60, 66]. In a guinea pig model, peripheral leukocytes had a greater PCR positivity rate than did plasma or urine samples [62], but evaluation of this sample type in humans is limited to a single case report in which leukocyte samples remained PCR positive for up to 56 days after the onset of symptoms [67]. Throat swabs may also be a suitable sample for PCR testing, but this application has only been evaluated in one study, which found Legionella-positive samples for 5 of 6 patients with legi-onellosis [68]. Further investigation of nonrespiratory sample
types is needed to establish their role in the diagnosis of legionellosis. Regardless, the sensitivity of PCR is likely to increase when testing samples obtained early in the course of illness and when testing ≥1 sample type from each patient [60].

The development of an optimal PCR assay has been complicated by the intermittent contamination of some commercial DNA extraction kits with Legionella DNA [69]. Use of these kits to process samples may result in false-positive results, and it highlights the importance of including appropriate controls in each assay run. Of all the common pneumonia pathogens, Legionella species probably presents the greatest risk for PCR contamination, given the organism’s environmental habitat. PCR could be considered the test of choice for legionellosis in patients who produce sputum, but, like PCR assays for other pneumonia pathogens, standardized protocols need to be developed.

**Chlamydia species.** Use of cell culture for detection of *C. pneumoniae* is technically demanding and time-consuming, and cell cultures generally have a low yield. As a consequence, the diagnosis of *C. pneumoniae* infection largely relies on serological testing that uses microimmunofluorescence [12]. A serological diagnosis of *C. pneumoniae* infection requires the testing of both acute- and convalescent-phase serum samples; therefore, diagnosis can only be made retrospectively.

These major limitations have encouraged many investigators to explore the use of PCR for diagnosis of *C. pneumoniae* infection. Unfortunately, test results from different studies are often contradictory, and the findings from some laboratories are unconfirmed by others, despite the use of identical protocols. Although vascular tissue specimens, serum samples, and PBMCs have been tested by PCR to investigate the association between *C. pneumoniae* and cardiovascular disease [70], respiratory samples have been almost exclusively tested when studying pneumonia [71]. In general, PCR appears to be at least as sensitive as culture, but specificity is difficult to assess because of the lack of an appropriate “gold standard” [71]. *C. pneumoniae* DNA can be detected in both upper- and lower respiratory tract samples, but it is unclear which respiratory sample is preferred for PCR testing.

Some investigators have found a higher PCR positivity rate for upper respiratory tract samples than for lower respiratory tract samples [72], but this may reflect colonization rather than invasive disease. Indeed, the use of highly sensitive PCR techniques may increase the ability to detect *C. pneumoniae* carriage, the clinical relevance of which is unclear. A recent interlaboratory study that used a standardized protocol questioned the reliability of a *C. pneumoniae* nested PCR assay because of the high risk of contamination [73]. PCR results must clearly be interpreted in the context of the patient’s clinical presentation and together with the results of other investigations. A standardized approach to *C. pneumoniae* diagnostic testing has been recently recommended by the US Centers for Disease Control and Prevention (Atlanta, GA) and the Canadian Laboratory Centre for Disease Control (Ottawa, Ontario, Canada) [12].

PCR assays have been developed to detect *Chlamydia psittaci*, although there is limited experience of their use for diagnosis of human psittacosis [74–76]. Wider use of these assays will undoubtedly identify many otherwise unrecognized cases.

**Pneumocystis carinii (Pneumocystis jiroveci).** The standard method for diagnosis of *P. carinii* pneumonia (PCP) is cytological staining of bronchoalveolar lavage or induced sputum samples via immunofluorescent or tissue stains, such as methenamine silver, Giemsa, or toluidine blue O. Several studies have confirmed that PCR has greater sensitivity for detection of *P. carinii* than do cytological methods. Although the specificity of PCR is also usually high, many of these studies have identified samples that test positive by PCR but negative by standard tests and/or were obtained from patients without clinical features of PCP [77–86]. These findings are difficult to interpret and may represent *P. carinii* colonization of uncertain clinical relevance. The risk of false-positive results is greater for nested PCR than for single-step PCR assays, especially when testing samples obtained from non–HIV-infected patients [84]. Preliminary evidence suggests that quantitative PCR may help distinguish between *P. carinii* colonization and infection [87], but this needs further evaluation.

The preferred sample type for PCR testing is similar to that used for cytological methods for detecting *P. carinii*. Bronchoalveolar lavage specimens probably have the highest yield, although induced sputum samples are satisfactory for HIV-infected patients. In one series, the sensitivity and specificity of PCR were 100% and 98%, respectively, for bronchoalveolar lavage specimens and 95% and 94%, respectively, for induced sputum specimens, prompting the authors to suggest that PCR testing of induced sputum specimens may obviate the need for bronchoscopy in some patients [83]. Oral washes may be an alternative noninvasive sample for HIV-infected patients with a high fungus load, although the sensitivity of PCR of these samples is less than that for lower respiratory tract samples [82, 86, 88, 89]. *P. carinii* DNA can also be detected in blood samples, although the clinical utility is uncertain [88, 90, 91].

The role of PCR for diagnosis of PCP has yet to be established. Unlike standard tests for other pneumonia pathogens, cytological methods for detecting *P. carinii* can be performed in the same time or faster than PCR, and, therefore, turnaround time is not a consideration when evaluating the role of PCR for diagnosis of PCP. At present, PCR may be best used to test samples obtained from patients with a high clinical suspicion of PCP but for whom the results obtained by cytological screening methods are negative.
RESPIRATORY VIRUSES

Pneumonia in a patient with a viral infection may occur either as a primary viral pneumonia or secondary to bacterial infection. When systematically evaluated, viruses account for ~30% of pathogens identified in adults and in ~70% of children hospitalized with community-acquired pneumonia [92, 93], with respiratory syncytial virus, influenza A and B viruses, parainfluenza viruses, and adenoviruses being numerically the most important. At present, immunofluorescence, viral culture, and antigen detection methods are most widely used for the laboratory diagnosis of viral respiratory infections.

PCR assays have been developed for all of the important respiratory viruses. A commercial multiplex PCR assay for respiratory viruses has been developed [94], but it has yet to receive approval by the US Food and Drug Administration. At this stage, the role of PCR for the diagnosis of pneumonia remains uncertain, because most studies have focussed on ill-defined or nonpneumonic respiratory tract infection, particularly in children. PCR is likely to improve the ability to diagnose viral pneumonia, but studies of patients with well-defined pneumonia are needed.

At present, the main clinical applications of PCR are for the diagnosis of specific viral pneumonias for which PCR is a preferred diagnostic tool (e.g., hantavirus pulmonary syndrome) and for the diagnosis of pneumonia in immunocompromised patients. Respiratory viruses have been increasingly recognized as important causes of severe lower respiratory tract disease in immunocompromised hosts, especially in patients treated for hematological malignancies [95, 96]. In these patients, PCR is more sensitive for detection of respiratory viruses than are viral culture, antigen detection, and serological testing [97], and PCR offers the possibility of early diagnosis and treatment. PCR testing of bronchoalveolar lavage samples may also be a useful tool for diagnosis of cytomegalovirus pneumonia in bone marrow and solid-organ transplant recipients [98, 99].

CONCLUSIONS

PCR has many attributes that make it a useful diagnostic test for pneumonia. However, no single diagnostic test for pneumonia is likely to appear, and clinicians will need to remain abreast of the strengths and weaknesses of new diagnostic tests for specific pathogens. An increasing number of commercial PCR assays for use with respiratory samples will be available in the next few years, including some that will be able to detect multiple pathogens via a multiplex format. However, standardized protocols will first need to be established before PCR and other nucleic acid amplification methods become routine diagnostic tools.

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References


