Interpreting Assays for the Detection of *Streptococcus pneumoniae*

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***Streptococcus pneumoniae* is both an aggressive pathogen and a normal part of the human respiratory microbiome. Clinicians and microbiologists have struggled to develop tests that can identify pneumococcal respiratory infection and accurately distinguish colonization from invasive disease. Molecular methods hold the promise of an improved ability to rapidly detect microorganisms in respiratory secretions and to make an accurate diagnosis; however, interpretation of diagnostic testing for *S. pneumoniae* remains problematic. Molecular assays, such as those targeting the pneumolysin gene, may cross-react with other streptococcal species, confounding detection and quantification. Assays that target the autolysin gene appear to be more specific. Even when accurately identified, however, the significance of *S. pneumoniae* DNA detected in clinical samples is difficult to determine. Here we will discuss the challenges faced in the interpretation of molecular testing for *S. pneumoniae*, and some strategies that might be used to improve our ability to diagnose pneumococcal respiratory infection.**

Streptococcus pneumoniae is an important human pathogen that causes a wide range of disease. Respiratory tract illness is one of the primary manifestations of pneumococcal infection; however, microbiologic confirmation can be difficult. Traditionally, diagnoses of pneumococcal community-acquired pneumonia (CAP) and other lower respiratory tract infections (LRTIs) have been made through conventional culture of respiratory secretions, including sputum, bronchoalveolar lavage, or pleural fluid, or the detection of pneumococcal bacteremia, but yields are low [1]. Current CAP guidelines discourage the pursuit of a microbiologic diagnosis for pneumonia in non-hospitalized patients [1]. This stems from the lack of sensitive, specific, and cost-effective diagnostics. While

retrospective studies have shown favorable outcomes with empiric therapy of CAP, failure to specifically identify *S. pneumoniae* as the causative agent has consequences for the individual patient and the population at large. The individual patient may not receive adequate antimicrobial therapy or may receive excessively broad-spectrum and expensive treatment. At the population level, the lack of a pathogen-based diagnosis decreases our ability to accurately assess disease burden, to evaluate the effects of interventions such as immunization, to track antimicrobial resistance, and to investigate new therapeutic agents.

THE IMPORTANCE OF DIAGNOSTIC TESTING FOR *S. PNEUMONIAE* LRTI

Worldwide, *S. pneumoniae* causes an estimated 1.6 million deaths each year [2]. Pneumococcal disease is the leading cause of vaccine-preventable death in children less than 5 years old and is also an important cause of morbidity and mortality in the elderly. Available vaccines target the serotype-specific capsular protein or the polysaccharide capsule, and thus are dependent on a detailed understanding of pneumococcal epidemiology.

The most common invasive pneumococcal disease (IPD) is pneumonia. *S. pneumoniae* is the most frequent cause of bacterial pneumonia due to a known pathogen, and vaccine studies have shown that it is likely the most common pathogen in culture-negative disease as well [3, 4]. The true proportion of CAP due to *S. pneumoniae* is difficult to determine, as there is no gold standard for diagnosis. In addition to CAP, *S. pneumoniae* is a common pathogen (along with *Haemophilus influenzae* and *Moraxella catarrhalis*) in other LRTIs such as acute bacterial exacerbations of chronic bronchitis and exacerbations of chronic obstructive pulmonary disease.

Although empiric therapy for community-acquired LRTI is directed primarily at *S. pneumoniae,* other pathogens may often be involved. Many cases of CAP are due to respiratory viruses or atypical bacterial pathogens [3, 4]. Accurate and reliable detection of *S. pneumoniae* would thus be beneficial for both pneumococcal and nonpneumococcal disease—directed therapy in confirmed pneumococcal disease could lead to the use of more narrow-spectrum agents for *S. pneumoniae*, and for nonpneumococcal disease, there could be a more focused use of other antibacterials and antiviral agents. Molecular and antigen-based testing has the advantage of rapid results that could be available before therapy is started. Despite the potential benefits of diagnostic tests to identify *S. pneumoniae*, however, there are a number of hurdles that must be overcome. There are issues surrounding both sensitivity and specificity for disease that depend on the clinical setting and the type of specimen tested. In considering the interpretation of diagnostic testing for *S. pneumoniae* for LRTI, there are 2 fundamental questions to be asked: (1) does the test identify *S. pneumoniae* specifically and (2) does this detection adequately implicate *S. pneumoniae* as the causative pathogen of disease?

IDENTIFICATION OF *S. PNEUMONIAE*: CURRENT METHODS

S. pneumoniae was first identified in the late 1800s, and early on was recognized as the most common cause of lobar pneumonia [5]. In the conventional laboratory, identification of *S. pneumoniae* from culture is achieved by accurate observation of both its morphologic appearance and four main phenotypic characteristics, including α-hemolysis of blood agar, catalase negativity, optochin susceptibility, and bile solubility. The finding of optochin-resistant pneumococci [6] has decreased the utility of this characteristic as a distinguishing feature, but overall these phenotypic markers are quite reliable. Culture-based methods have a number of advantages, including the ability to implement them worldwide with low cost and high specificity, as well as the ability to provide both antibiotic susceptibility and serotype data. There are difficulties, however, in recovering *S.*

pneumoniae in culture, including the tendency of *S. pneumoniae* to autolyse when reaching the stationary phase of growth, antibiotic treatment prior to specimen collection, and in the case of LRTI, difficulty with adequate specimen collection and the low prevalence of detectable bacteremia in CAP. For these reasons, newer tests have been developed that use antigen-based or molecular detection methods.

ANTIGEN-BASED DETECTION

The most widely used indirect detection method for *S. pneumoniae* is the detection of pneumococcal antigen in urine [2, 7]. Tests based on the capsular polysaccharide antigens of *S. pneumoniae* were the first commercialized assays; however, these had poor sensitivity and specificity when compared with the standard Gram stain and culture [2]. More recently, a rapid immunochromatographic test detecting the group C polysaccharide cell wall antigen common to all pneumococcal strains (NOW *S. pneumoniae* urinary antigen test, Binax) has shown good utility for the diagnosis of pneumococcal pneumonia in adults [2, 8]. Sensitivity and specificity of this test in the diagnosis of CAP due to *S. pneumoniae* are reported in the 77%–88% (sensitivity) and 67%–100% (specificity) range [7, 8]. Urine antigen-based testing has some limitations. In all studies, a proportion of patients with positive blood or sputum cultures have negative antigen tests [2, 9], the antigen test may cross-react with other closely-related streptococci [7], and the urine antigen can be positive for weeks after the onset of disease [10]. Thus, it is necessary to use this test in conjunction with other diagnostic modalities. In children, excretion of pneumococcal antigen in the urine can as likely be due to pneumococcal carriage as disease; therefore, when used on urine, this test lacks adequate specificity in this population to be useful [11].

While primarily designed and marketed as a urinary antigen test, studies have also shown the utility of the group C polysaccharide antigen test in the identification of *S. pneumoniae* from pleural fluid [12, 13], bronchoalveolar lavage (BAL) [14], and blood culture media [15] in both adult and pediatric patients with CAP. The use of antigen detection from sterile sites may be a better alternative than urine detection, especially in children.

MOLECULAR DETECTION

Molecular detection of *S. pneumoniae* has primarily been achieved through use of the polymerase chain reaction (PCR). PCR-based detection depends on the amplification of species-specific genes that are unique to the pneumococcus. Finding specific pneumococcal genes, however, has been challenging.

One widely used PCR target for the identification of *S. pneumoniae* in clinical specimens is the pneumolysin gene (*ply*). Pneumolysin was first used to identify *S. pneumoniae* in an agglutination assay [16] and was initially described as highly specific. Based on this and other publications [17, 18], PCR-based assays were developed for use with clinical specimens. A number of studies of pneumococcal disease using the pneumolysin target for detection of *S. pneumoniae* from patients with LRTI were published, and showed relatively poor sensitivity and specificity for invasive disease overall [2, 19–23]. Over the last several years, it has become clear that *ply* can be detected in nonpneumococcal Viridans-group streptococci, particularly *S. pseudopneumoniae* and *S. mitis* [24, 25]. These findings lead to the question of whether the poor performance of pneumolysin-based PCR for the detection of pneumococcal disease is related to limitations of molecular testing itself, or to the poor specificity of the assay.

More recent studies have shown that other targets, including the autolysin gene (*lytA*) ([26]), the pneumococcal surface adhesion gene (*psaA*) [27], and the spn9802 gene fragment [28] may be more specific. Carvalho et al [24] compared 3 gene targets: *ply, lytA,* and *psaA*. These studies demonstrated that autolysin was the most specific, although *psaA* was a close second. Autolysin primers did not amplify the DNA from any of the nonpneumococcal bacteria tested, including 21 strains of pneumococcus-like Viridans-group streptococci, of which 18 were positive by *ply*-PCR and two were positive using *psaA*. Autolysin in particular has been shown to be quite specific for *S. pneumoniae* in other studies [29], and the use of this target may solve the issues of misidentification that have hampered the success of PCR-based pneumococcal studies to date.

INTERPRETATION OF TESTS: SENSITIVITY AND SPECIFICITY FOR DISEASE

Even when detected accurately, the question of significance remains difficult to answer for *S. pneumoniae* in many cases of LRTI. In other types of invasive pneumococcal disease, the site of infection is readily accessible, and isolation of *S. pneumoniae* provides conclusive evidence of disease. This is true for bloodstream infection, meningitis, and joint infection. LRTI is more problematic as the lung, the primary site of infection, is difficult to sample directly, and thus surrogate specimens must generally be used. Unfortunately, the most readily available surrogates have either very low yields in terms of positive culture (blood) or are not normally sterile (sputum, nasal pharyngeal samples). Consequently, for each specimen type examined in LRTI, either the sensitivity or specificity of pneumococcal detection as an indicator of invasive disease provides a challenge for the clinician.

ISSUES OF SENSITIVITY

A number of studies have addressed PCR-based detection of *S. pneumoniae* in blood for the diagnosis of pneumococcal LRTI. While the detection of *S. pneumoniae* from blood is considered definitive evidence of disease, it is detected by conventional blood culture in less than 20% of pneumonia cases diagnosed as pneumococcal using other criteria [1, 2]. For children, this rate is even lower [30]. It was hoped that a PCR-based detection of pneumococcal DNA would show increased sensitivity; however, initial studies using pneumolysin as a target were disappointing [2, 19]. Recently, however, Rello et al [31] published data showing detection of pneumococcal DNA in the blood of 62% of adult patients with confirmed or probable pneumococcal pneumonia. This study used the autolysin gene as the target, and PCR performed better than blood culture, which was positive in only 37%. The study by Rello et al excluded patients who had been pretreated with antibiotics prior to sample collection, but other studies have demonstrated the superiority of *lytA* PCR over blood culture in pretreated patients [32, 33]. Other small studies have also shown relative success with autolysin PCR in blood [34]; testing with this target deserves further study. In addition to blood specimens, a number of studies have demonstrated the successful PCR-based detection of pneumococcal DNA from pleural fluid samples in patients with parapneumonic effusion. These studies showed significantly increased detection of pneumococcal empyema when compared with conventional culture using *ply* or *lytA* and the capsular *wzg/cpsA* gene targets [20, 33, 35, 36]. In addition to identification, Tarrago et al [33] as well as Azzari et al [36] performed pneumococcal serotyping directly from the clinical sample, obviating the need for a cultured isolate to gather serotype data. Unfortunately, pleural fluid is only available from a small percentage of patients with pneumonia.

ISSUES OF SPECIFICITY

Respiratory specimens provide a challenge in specificity due to the presence of *S. pneumoniae* at varying levels in the upper respiratory tract of healthy persons. Some general guidelines for conventional testing have been established; for example, the presence of gram-positive diplococci as the predominant morphotype on a Gram stain of "high-quality" sputum (>10 white blood cells per epithelial cell at a magnification of 400X) is considered good evidence of pneumococcal pneumonia [37]. However, because of the difficulty of obtaining a high-quality specimen, the overall diagnostic yield is low. While molecular testing may have the ability to increase the sensitivity of detection of *S. pneumoniae* in sputum or other respiratory secretions, there is a significant issue of specificity to be overcome. Issues with particular sample types are discussed below.

A sample type that is easy to obtain and available in both adult and pediatric patients is the nasopharyngeal aspirate or nasopharyngeal swab. This upper respiratory specimen has not been adequately studied with conventional culture to know its true utility as a predictive site for identifying the causative agent of LRTI; however, with new molecular testing for viral URTI using NPA or NPS as the sample type rapidly coming into common use, it is reasonable to consider whether there is a role for *S. pneumoniae* detection here. Due to issues with pneumococcal carriage in the nasopharynx, particularly among children [38], the significance of a positive detection is unclear, and it may not be possible to interpret when nasopharyngeal detection is related to invasive disease. Quantification may be helpful, but this has issues of its own (see below). A recent publication did demonstrate a correlation between the detection of *S. pneumoniae* in the nasopharynx and severe disease associated with the 2009 H1N1 influenza virus [39]. In contrast to decision making based on a positive detection of *S. pneumoniae*, however, a negative nasopharyngeal test might be useful to exclude pneumococcal CAP, which could lead to a more rapid investigation of other pathogens.

Sputum is the most well-studied sample type for the diagnosis of LRTI, and will likely be the focus of molecular diagnostic development. As discussed above, obtaining high-quality sputum can be challenging, and will not be addressed specifically here. It is likely that there will need to be some conventional or molecular quality measures used to identify adequate sputum samples in conjunction with molecular testing in order to reliably interpret results.

When a high-quality specimen is examined under the microscope, the diagnostic accuracy of Gram stain can be up to 63%, with cultures positive in almost 90% of adult patients [37]. Prior studies have not demonstrated a significant increase in either sensitivity or specificity when using molecular testing, even when quantification was included [19, 40]; however, most studies used pneumolysin as the target gene and may have been confounded by the detection of oral streptococci. Studies using autolysin may show better specificity [41]. The detection of pneumococci in the sputum of patients previously treated with antibiotics is improved by molecular methods, and PCR may have a significant role here [42]. A major potential benefit of molecular detection of *S. pneumoniae* in sputum is the ability to use multiplex formats to detect many important pathogens in one assay [41, 43], to apply molecular serotyping [33, 36, 44], and/or to detect both the pathogen and determine its susceptibility penicillin [45]. Additionally, as discussed for nasopharyngeal samples, the reliable finding of no detectable *S. pneumoniae* in the sputum may be useful as a method to rule out this organism and trigger a more detailed work up.

IMPROVING ACCURACY

Accurately diagnosing pneumococcal disease depends on distinguishing patients infected with *S. pneumoniae* from those that are merely colonized or are infected with other pathogens. As discussed, simple detection of pneumococcal DNA or the absence thereof may not suffice for this purpose. Are there ways to improve the accuracy of *S. pneumoniae* diagnostics for LRTI unrelated to the sensitivity and specificity of the actual diagnostic test? *S. pneumoniae* detection is already used in conjunction with clinical information such as fever, cough, and respiratory distress to make the diagnosis of pneumonia. It may also be beneficial to combine *S. pneumoniae* detection with either quantification or other biomarkers.

Quantification has been suggested as a mechanism by which pneumococcal colonization could be distinguished from infection. Real-time PCR is inherently quantitative, and thus would seem to be a relatively straightforward proposition. However, the actual implementation may prove more complicated for several reasons. First, the variability of quantitative assays makes it difficult to establish standards and a ''cutoff'' definition for disease that is consistent across laboratories. This is best described for viral testing where quantitative assays for Epstein-Barr virus, cytomegalovirus, and hepatitis C virus (HCV) performed in various laboratories have been shown to vary by up to 4 $\log_{10}$-fold in quantification of the same specimen [46, 47]. Second, while specific laboratories may use culture-based levels at which *S. pneumoniae* is considered pathogenic when found in a respiratory specimen (ie, sputum with *S. pneumoniae* found at $>10^5$ colony-forming units [cfu] per milliliter, protected brush specimens with $>10^3$ cfu/ml, etc), these cutoffs are based on limited data and their performance is not clear. Molecular quantification will likely differ due to the presence of dead bacteria with detectable DNA. A definition of a ''significant'' level of *S. pneumoniae* detected by PCR would have to be determined though well-designed research studies for each specimen type. Initial studies, though, have shown promise. Yang et al [48] evaluated receiver operating characteristic (ROC) curves for the prediction of disease using pneumolysin PCR in the sputum of adult patients with definite or probable (by IDSA guidelines [1]) pneumococcal pneumonia. This study reported good sensitivity (90%) and negative predictive value (96%) at a cutoff value of approximately 4×10^4 genomic equivalents per milliliter of sputum. Specificity and positive predictive value were lower; however, the ''gold standard'' probably underestimates disease. Other studies have shown a positive correlation between either blood [31, 49] or sputum [42] bacterial load and the severity of disease.

The specificity of molecular detection of *S. pneumoniae* in sputum could be improved by combining it with other markers of bacterial pneumonia. Clinical indicators already in use will

undoubtedly be combined with molecular detection, and it may be possible to better evaluate their specificity when combined with newer tests. An additional specific biomarker that may be promising in the diagnosis of pneumococcal LRTI is procalcitonin. Procalcitonin is the peptide precursor of calcitonin and is released in a cytokine-like manner in response to proinflammatory mediators such as interleukins and TNF-α, as well as in response to bacterial toxins. Procalcitonin release is attenuated in response to viral infection and thus the measurement of procalcitonin has been proposed as a method to distinguish viral vs bacterial disease [50]. Published studies have evaluated procalcitonin as a marker for the initiation or cessation of antibiotic therapy, and shown noninferiority when compared with standard guidelines [50, 51]. By combining the measurement of procalcitonin levels with the detection of *S. pneumoniae* and/or other bacterial and viral pathogens (see below), the significance of bacterial detection may be clearer.

Multipathogen testing for bacterial LRTI has received less discussion, but could be very useful. While *S. pneumoniae* remains the most common cause of microbiologically diagnosed LRTI, a number of pathogens are emerging. Methicillin-resistant *S. aureus* (MRSA) has become a significant cause of severe CAP and complicated pneumonia. Antibiotic-resistant gram-negative pathogens are becoming more important among adults with pneumonia, particularly in chronically ill or hospitalized patients. *Legionella, Mycoplasma pneumoniae,* and *Chlamydophila pneumoniae* are readily detected by PCR. By testing for multiple pathogens, particularly if quantitative detection is available, the relative contribution of each could be considered, and a more accurate diagnosis made. Similarly, the combination of viral testing with bacterial detection might help better define the significance of *S. pneumoniae* detection. In children, coinfection with viruses and bacteria has been demonstrated in up to 23% of patients hospitalized with pneumonia [4]. Recent studies of CAP in adults also suggest a much greater role for respiratory viruses than expected [3, 52, 53]. Among 259 evaluable patients hospitalized with CAP, de Roux [52] identified respiratory viral infection alone in 10% and mixed bacterial and viral infection in 10%. In a prospective study of 105 patients using conventional methods, paired sera, and PCR, Templeton [53] showed an increased yield for an etiology from 49.5% to 76% of patients. *S. pneumoniae* was detected in 21% of patients and viral infection in 40%. Most recently, Johansson et al [3], using conventional culture, antigen testing, and PCR-based testing for *S. pneumoniae, H. influenzae, M. catarrhalis*, atypical bacterial pathogens, and viruses found a definite or probable microbial etiology of CAP in 67% of patients. *S. pneumoniae* infection was found in 38% of patients, of which almost 50% had a second pathogen identified. A viral pathogen was identified in 29% of all patients.

CONCLUSIONS

The interpretation of diagnostic testing for *S. pneumoniae,* particularly for patients with LRTI, remains complicated. Diagnostic assays and methods are improving, however, and the outlook is promising. Inaccurate identification likely hampered early studies of molecular diagnostic testing for pneumococcal CAP; these issues may be solved by the use of more specific gene targets. Using these newer targets, particularly autolysin, both the sensitivity and specificity of pneumococci detection from sterile body fluids have improved. In particular, molecular detection of *S. pneumoniae* from the blood of patients with CAP may be more valuable than previously thought.

Establishing the significance of *S. pneumoniae* detection from respiratory specimens will continue to be a challenge. Either quantitative assays or the adjunctive use of biomarkers will likely be necessary to more accurately identify patients with true pneumococcal disease. In addition, multipathogen testing, including assays for other pathogenic bacteria of the lower respiratory tract, as well as viral testing, should be strongly encouraged.

Development of rapid, accurate, and sensitive molecular diagnostics for *S. pneumoniae* will involve a detailed evaluation of many patients and different sample types. The cost of these studies will be sizeable, and may require cooperation between the diagnostics and pharmaceutical industries to test drugs and diagnostics together. Of course, the National Institutes of Health and Food and Drug Administration should also be involved in the funding process. Cost-effectiveness will need to be evaluated, and will depend on both the savings generated by more accurate diagnosis and the costs of the testing itself. These costs can be quite high for molecular assays; however, further development and more players in the field may bring them down significantly. Overall, despite the hurdles and complexities involved, the clinical and epidemiologic necessities for accurate diagnosis of pneumococcal LRTI are substantial, and the continued pursuit of improved testing strategies essential.

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