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Laboratory Diagnosis of Invasive Pneumococcal Disease

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The laboratory diagnosis of invasive pneumococcal disease (IPD) continues to rely on culture-based methods that have been used for many decades. The most significant recent developments have occurred with antigen detection assays, whereas the role of nucleic acid amplification tests has yet to be fully clarified. Despite developments in laboratory diagnostics, a microbiological diagnosis is still not made in most cases of IPD, particularly for pneumococcal pneumonia. The limitations of existing diagnostic tests impact the ability to obtain accurate IPD burden data and to assess the effectiveness of control measures, such as vaccination, in addition to the ability to diagnose IPD in individual patients. There is an urgent need for improved diagnostic tests for pneumococcal disease—especially tests that are suitable for use in underresourced countries.

Streptococcus pneumoniae (the pneumococcus) is one of the most important human pathogens. It is a major cause of pneumonia, meningitis, bacteremia, sinusitis, and otitis media, and it occasionally infects tissues at other sites. The collective term invasive pneumococcal disease (IPD) refers to pneumonia, meningitis, bacteremia, and infections of other normally sterile sites with *S. pneumoniae*. The World Health Organization estimates that ~1.6 million people, including up to 1 million children aged <5 years, die of IPD every year [1], with developing countries bearing the greatest burden [2]. With the availability of an effective conjugate vaccine [3–6], IPD is also the leading cause of death among vaccine-preventable infectious diseases [7].

Despite its importance, IPD (particularly pneumococcal pneumonia) can be surprisingly difficult to confirm microbiologically. In a recent editorial commentary, Bartlett [8] pointed out the decrease in microbiological testing in the context of pulmonary infections. Instead of having developed more rapid, definitive, and conclusive tests to diagnose pneumonia, we seem to be doing less well now than scientists did 70 years ago. With regard to pneumococcal pneumonia, Bartlett's conclusion was that "either the pneumococcus is disappearing or microbiology is disappearing" [8, p. 170]. At least part of this phenomenon is likely due to a change in emphasis on microbial detection, with a higher threshold for performance of microbiological tests and less attention to obtaining high-quality samples. The emergence of multidrug-resistant pneumococci is a fundamental reason for enhanced efforts toward culturing pneumococci.

Isolation of *S. pneumoniae* from a normally sterile body site provides conclusive evidence of pneumococcal infection, but this is achieved for only a minority of cases of IPD. Pneumococcal pneumonia, the most common manifestation of IPD, can be particularly difficult to diagnose. This is largely a result of the problems associated with obtaining high-quality lower respiratory tract samples for testing and with uncertainty regarding the differentiation of infection from colonization. Prior antibiotic use will also significantly reduce the ability to isolate *S. pneumoniae* from clinical samples. Despite the global importance of pneumococcal disease, there have been surprisingly few recent developments in laboratory diagnostics. The difficulties in diagnosing IPD have ramifications that extend well beyond the ability to diagnose IPD in individual patients. The capacity to obtain accurate data on IPD burden and to assess the effectiveness of vaccination are hindered by the limitations of existing diagnostic tests. The laboratory diagnosis of IPD currently relies on methods (or variations of methods) that have been around for many decades.

MICROSCOPY AND CULTURE

The laboratory identification of *S. pneumoniae* isolates relies on the recognition of typical morphological characteristics and on the results of a few phenotypic tests. During microscopic evaluation, *S. pneumoniae* appear as lancet-shaped, gram-

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positive diplococci or chains of cocci (figure 1). If observed by experienced microscopists, these features have high specificity for the presence of *S. pneumoniae* and should be reported as such. However, typical appearances can be altered by antimicrobial therapy, and over-decolorization of the stain can give the false impression that they are gram-negative diplococci. Although infrequently used nowadays, the quellung reaction is a more specific method for pneumococcal detection from pure cultures or sputum samples [9, 10]. After reaction of the pneumococcus with streptococcal anticapsular antisera, the pneumococcal capsule becomes visually enhanced, and the bacterial cell appears to be surrounded by a halo (figure 2). Although the quelling reaction is generally regarded as being highly specific for pneumococcus, cross reactions have been reported with other streptococcal polysaccharides [11], and unencapsulated strains will produce false-negative results.

After overnight incubation at 35°C with 5% CO_2 on 5% sheep blood agar or chocolate agar, *S. pneumoniae* colonies appear to be small, grayish, and mucoid and are surrounded by a greenish zone of α-hemolysis. After 24–48 h of incubation, the colonies become centrally depressed ("draughtsman" colonies) (figure 3). Further identification is important to confirm the identity. Laboratory differentiation between *S. pneumoniae* and other viridans streptococci is usually accomplished by 2 key reactions: optochin susceptibility and bile solubility. Optochin (ethylhydrocupreine) is an antibacterial agent that is not used therapeutically but is used for the laboratory identification of streptococci. The bile solubility test is based on the autolysis of *S. pneumoniae* in the presence of the surfactant sodium deoxycholate. *S. pneumoniae* isolates are typically susceptible to optochin and are bile soluble, whereas other viridans streptococci are typically resistant to optochin and are bile insoluble. Although bile solubility is generally regarded as being very sensitive and specific for identification of *S. pneumoniae,* the finding that up to 10% of *S. pneumoniae* isolates can be resistant to optochin has reduced reliance on the latter test [12]. Consequently, suspicious isolates that have reduced susceptibility to optochin should also be tested for bile solubility. Commercial slide agglutination, coagulation, and DNA probe hybridization tests are alternative methods for rapid identification of *S. pneumoniae* isolates [12, 13]. All of these methods are highly sensitive but occasionally produce positive results with other viridans streptococci (specificity range, 85%–95%) [12, 14].

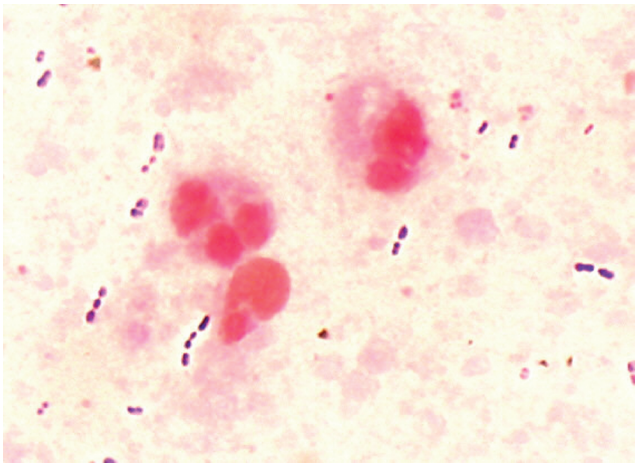

Figure 1. Gram stain of a sputum sample showing *Streptococcus pneumoniae* as gram-positive diplococci.

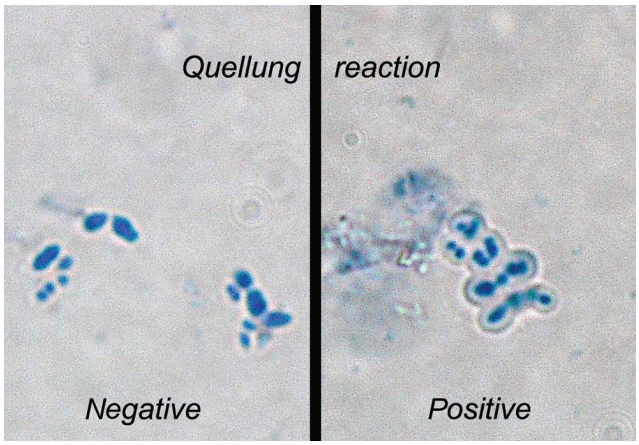

Figure 2. Quellung reaction, showing both positive (for *Streptococcus pneumoniae*) and negative results.

The laboratory identification of *S. pneumoniae* has been further complicated by the recent description of *Streptococcus pseudopneumoniae* [15]. *S. pseudopneumoniae* is phenotypically and genetically distinct from *S. pneumoniae* and other viridans streptococci, but it has the potential to be incorrectly identified as *S. pneumoniae.* Some key characteristics of *S. pseudopneumoniae* are the absence of a pneumococcal capsule, insolubility in bile, resistance or indeterminate susceptibility to optochin when incubated in 5% CO_2 but susceptibility to optochin when incubated in ambient air, and positive reactions with DNA probe hybridization and antigen detection tests [15, 16]. The clinical relevance of *S. pseudopneumoniae* is still uncertain, although there may be an association with chronic obstructive pulmonary disease [16].

Blood cultures. The isolation of *S. pneumoniae* from blood culture provides a definite diagnosis of pneumococcal disease. However, documented bacteremia occurs in only a minority of cases of IPD. Although *S. pneumoniae* is regarded as the most common cause of community-acquired pneumonia in all age groups, rates of positive blood culture results for adults hospitalized with pneumonia are typically only 3%–8% [17–20] and are lower in children [21, 22]. In pneumococcal meningitis, documented bacteremia occurs more frequently than in pneu-

Figure 3. *Streptococcus pneumoniae* growing on sheep blood agar as "draughtsman" colonies. The disk contains optochin and is surrounded by a zone of inhibition.

monia, and reported rates of positive results are often >50% [23, 24]. The relatively low documented rates of bacteremia in patients with IPD involve several factors, including prior administration of antimicrobials and the intermittent nature of bloodstream invasion by *S. pneumoniae*. In addition, *S. pneumoniae* releases autolysin during the stationary growth phase, resulting in cell death and making traditional methods of bacterial growth on media, such as blood culture, difficult [25].

CSF examination. For the diagnosis of pneumococcal meningitis, the combination of Gram stain and bacterial culture of CSF samples will identify most cases. In one large review, Gram stain smears of CSF samples detected *S. pneumoniae* with a sensitivity of 84% and specificity of 98%, but prior administration of antibiotics significantly reduced the yield for both Gram stain smear and culture [23]. Processing a CSF specimen for culture as soon as possible is vital for optimal culture performance, because bacterial viability decreases over time.

Sputum examination. In the absence of documented bacteremia, the diagnosis of pneumococcal pneumonia can be challenging, especially in children who may not produce sputum. The microscopic demonstration of numerous gram-positive diplococci in a sputum sample containing <10 squamous epithelial (SEC) cells and >25 polymorphonuclear (PMN) cells per low-power field (magnification, ×100) [26] or ⩾10 leukocytes for each SEC [27] for a patient with pneumonia is strongly suggestive of pneumococcal pneumonia. This is further supported if *S. pneumoniae* is the predominant isolate in cultures of sputum specimens. Poor-quality sputum samples, which contain relatively low numbers of PMN cells and high numbers of SEC cells, should not be processed, because they are likely to represent commensal oropharyngeal flora. Having a sputum quality assessment system in place is a valuable and cost-effective tool that allows the microbiology laboratory to maintain clinically relevant results. In this context, it is useful to remember that intra- and intertechnologist variability in the specimen quality assessment process have been reported and might account for some of the varied sensitivities of Gram-stained sputum specimens for the detection of pneumococci [28, 29].

Several clinical studies have shown that sputum culture and Gram stain are still useful for the diagnosis of pneumococcal pneumonia, as long as specimens are of high quality and, ideally, were obtained before the administration of antibiotic therapy or up to 24 h after the initiation of therapy [27, 30, 31]. One prospective study revealed that high-quality sputum samples can be obtained from a substantial proportion of adults with community-acquired pneumonia and that the sputum Gram stain had sensitivity of 57% and specificity of 97% for the diagnosis of pneumococcal pneumonia [31]. For bacteremic pneumococcal pneumonia in adults, sputum Gram stain and culture have sensitivities of 80% and 93%, respectively, if an adequate specimen has been produced before therapy [27]. The reason why sputum culture has been shown to have variable sensitivities in different studies is not necessarily the inadequacy of the microbiological tool itself; it can be the result of various factors, such as delayed processing of a sputum sample or processing of an inadequate sample, the patient's failure to produce a sputum sample, and the administration of antimicrobial therapy before obtaining a specimen.

Lung aspirate examination. Transthoracic needle aspiration has the potential to improve the diagnostic yield of pneumococcal pneumonia, especially in individuals with large peripheral lesions, including in children who may not produce sputum [32–34]. This is a relatively safe procedure if it is performed by experienced staff, but it has yet to be widely adopted because of its invasive nature and concerns about complications. The less compliant lungs of older adults are more prone to pneumothoraces after the procedure, and patients receiving anticoagulation may bleed.

ANTIGEN DETECTION ASSAYS

The detection of pneumococcal antigen in clinical samples—particularly urine samples—dates back at least to 1917 [35]. Over the past few decades, commercial latex agglutination tests targeting capsular polysaccharide antigens of *S. pneumoniae* have been widely used, although their use has been controversial. Systematic evaluations have cast doubt on their clinical usefulness over and above standard Gram stain and culture methods. In one large study, the sensitivity of a latex agglutination assay was high for detection of *S. pneumoniae* in CSF samples, but all samples that yielded positive results also demonstrated the causative organism on Gram stain [36]. Moreover, false-positive results were common, especially for urine sam-

ples, for which most positive results were erroneous. However, latex agglutination tests have still found a role for diagnosing pneumococcal pneumonia and meningitis in communities with limited laboratory facilities [37, 38].

The recent development of a rapid immunochromatographic test (ICT) that detects the C polysaccharide cell wall antigen common to all strains of *S. pneumoniae* (NOW *S. pneumoniae* urinary antigen test; Binax) has renewed interest in antigen detection. Arguably, this test has been the only major advancement in pneumococcal diagnostics over recent years. If applied to urine samples, this test has a sensitivity of 70%–80% and a specificity of >90%, compared with conventional diagnostic methods for detection of pneumococcal pneumonia in adults [39–44]. In all studies, a proportion of patients with positive blood or sputum culture results have negative NOW test results. Consequently, the NOW test should be used in conjunction with other testing methods. The test result can remain positive for several weeks [41, 45], and pneumococcal vaccination may produce false-positive reactions [46]. The utility of the NOW test for children is still being defined because of the high rate of false-positive results in children, which results from nasopharyngeal colonization with *S. pneumoniae* [47–49]. Other limitations of the test are its relatively high cost and the inability to provide antimicrobial susceptibility data. Recent reports have shown that the NOW test can be used to support the initiation of treatment with narrow-spectrum β-lactam antibiotics for pneumonia in adults, thereby preventing the unnecessary use of broad-spectrum therapy [50, 51].

Although originally designed for testing urine samples, the NOW test has been successfully used with specimens of other body fluids. The test is particularly useful for the rapid diagnosis of pneumococcal meningitis with use of CSF samples, with a sensitivity of 95%–100% and a specificity of 100% [52, 53]. It has also been successfully used with pleural fluid specimens obtained from children and adults with pneumonia [54, 55]. With bronchoalveolar lavage fluid samples, pneumococcal antigen could be detected with a sensitivity of 95% and a specificity of 87% [56]. The NOW test can also provide a rapid provisional identification of *S. pneumoniae* in blood cultures with positive results [25].

Other pneumococcal antigens—in particular, pneumolysin—have been investigated as potential diagnostic targets. Pneumolysin antigen detection has been applied to urine [57–59] and CSF [60] specimens. The results have been promising, but they have yet to be demonstrated as superior to the cell wall C polysaccharide (NOW) assay [57]. It is possible that the combination of a pneumolysin-specific antigen detection ELISA together with the NOW test would result in a better diagnostic yield, because of the higher specificity of the pneumolysin detection ELISA [58].

ANTIBODY DETECTION ASSAYS

Detection of pneumococcal antibodies or immune complexes has been used for diagnosis of pneumococcal disease in some research settings [61–64], but they have never been widely used. These assays suffer from problems inherent in most serological tests, including suboptimal sensitivity and specificity, as well being limited by the time it takes to demonstrate seroconversion.

NUCLEIC ACID AMPLIFICATION TESTS

Nucleic acid amplification tests, such as PCR, have established themselves as important diagnostic tools and are now representative of the current microbiological zeitgeist. Their attractiveness in a diagnostic laboratory setting stems from the following attributes: they can detect minute amounts of nucleic acid from potentially all pathogens, they do not depend on the viability of the target microbe, they are probably less affected by prior antimicrobial therapy than are culture-based methods, and they provide results within a short time frame.

To date, nucleic acid amplification tests have had variable performance for diagnosing IPD. In the setting of pneumonia, PCR has a sensitivity for detecting *S. pneumoniae* in blood samples ranging from 29% to 100% [65], although there is a tendency for the performance to be better in children than in adults. The generally poor performance of PCR in blood samples may be because of the rapid clearance of the *S. pneumoniae* from the blood stream and sampling errors resulting from the small sample volumes used in PCR reactions. In addition, positive pneumococcal PCR results have also been recorded from asymptomatic control subjects [66–68], and these findings are not readily explained.

When testing sputum samples, reported PCR positivity rates have ranged from 68% to 100% for samples from patients with pneumonia [65], although it is unclear how often this reflects colonization of the upper respiratory tract rather than infection [69]. This is a particular concern given the presence of the pneumolysin gene, a common pneumococcal PCR target, in some nonpneumococcal viridans streptococci [16]. Further refinement of PCR assays, including the use of multiple targets, have increased the specificity [70], with *lytA* assays potentially offering advantages over other assays [71]. Some investigators have suggested that quantitative PCR may help distinguish colonization from infection, with a higher bacterial burden in IPD than in a carrier state. Although this has not been systematically evaluated, initial data suggest that this might be worth exploring further [72]. A recent study from Malawi showed that high pneumococcal DNA loads in blood and CSF were associated with fatal outcome in children with IPD [73].

Unlike for pneumococcal pneumonia, detection of pneumococcal DNA in CSF specimens can be useful for diagnosis

of pneumococcal meningitis. This is perhaps unsurprising, given the high bacterial concentration in CSF in the presence of meningitis and the lesser concerns about contamination with colonizing bacteria. Although they have yet to be extensively evaluated, the sensitivity and specificity of PCR applied to CSF samples is high for diagnosis of pneumococcal meningitis (92%–100% and 100%, respectively), and this finding has been demonstrated in a variety of field settings [74–78]. PCR has also been successfully used with other samples obtained by invasive means, such as pleural fluid [79, 80] and lung aspirate [81] specimens.

FUTURE PERSPECTIVES

What does the future hold for pneumococcal diagnostics? There is still no easy way to establish the diagnosis of IPD, and there have been relatively few major developments in laboratory diagnostics during the past few decades. The promise of nucleic acid amplification methods has not yet been realized for IPD, and the problem of differentiating between colonization and infection for pneumonia remains predominant. The NOW antigen detection assay is a welcome addition, although it still has limitations, especially for diagnosis of pneumonia in children. It is essential to look again at existing diagnostic tools with the intention to optimize their use. There has been a tendency to downplay the role of diagnostic tests for pneumonia, and undoubtedly traditional microscopic and culture-based methods would have an increased yield if greater care was given to collecting appropriate samples at the right time.

Increased efforts should be directed toward development of new diagnostic tools for IPD. The search should continue for other pneumococcal antigens to be used for diagnostic purposes. Considerable efforts have gone into determining good vaccine targets for IPD. These targets often encompass the same criteria necessary for a diagnostic target. The trend in vaccine development is to move away from the inherent difficulties that are associated with the pneumococcal capsule as a result of its variability. The same holds true for diagnostic targets, and this view is somewhat supported by the success of the cell wall C polysaccharide as a useful antigen in the NOW test.

We have not yet fully explored the use of established diagnostic tools, such as antigen detection tests and molecular methods, in the framework of novel diagnostic strategies or in combination with more-experimental techniques, such as PCR–mass spectrometry. Developments in the field of bacterial identification by detection of volatile organic compounds in breath samples or mass profiling by mass spectrometry may open up new diagnostic avenues in the future.

Developments in diagnostics for IPD should occur in the context of pneumonia and meningitis in general, recognizing that other pathogens that cause pneumonia and meningitis are also difficult to identify. Finally, it is of immense importance to never lose sight of the fact that the major burden of pneumococcal disease is in the developing world. To successfully diagnose IPD in underresourced countries, the microbiological test needs to be rapid, cheap, and easy to use, and it should be useful for surveillance purposes.

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