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REVIEW

How recent advances in molecular tests could impact the diagnosis of pneumonia

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ABSTRACT

Molecular diagnostic tests have been the single major development in pneumonia diagnostics over recent years. Nucleic acid detection tests (NATs) have greatly improved the ability to detect respiratory viruses and bacterial pathogens that do not normally colonize the respiratory tract. In contrast, NATs do not yet have an established role for diagnosing pneumonia caused by bacteria that commonly colonize the nasopharynx due to difficulties discriminating between pathogens and coincidental carriage strains. New approaches are needed to distinguish infection from colonization, such as through use of quantitative methods and identification of discriminating cut-off levels. The recent realization that the lung microbiome exists has provided new insights into the pathogenesis of pneumonia involving the interaction between multiple microorganisms. New developments in molecular diagnostics must account for this new paradigm.

Pneumonia continues to provide a huge global burden of disease [1]. Although the incidence of pneumonia increases with increasing age, it is not widely appreciated that pneumonia is also the world's biggest killer of young children [2].

A wide variety of microorganisms are listed as pneumonia pathogens [3], and identification of pneumonia etiology is useful for both patient management and surveillance purposes. However, despite increased recognition of the burden of disease and advances in effective vaccines against major pneumonia pathogens, we continue to struggle in our efforts to identify the pathogens that cause pneumonia in individual patients [4]. Indeed, historically, we have been unable to define a causative pathogen in a significant proportion of pneumonia episodes, even with the best methods. Determining the microbial etiology of pneumonia in children has been a particular challenge [5]. The implications of poor pneumonia diagnostics extend to the population level as well; assessment of interventions, such as vaccines, is hindered by suboptimal measures of disease impact that often rely on accurate surveillance data [6].

Why is it so difficult to determine the microbial etiology of pneumonia? The inability to obtain good quality specimens from the lower respiratory tract is one fundamental problem with pneumonia diagnostics. Whereas, it is relatively easy to get specimens from the site of infection with diseases such as meningitis, gastroenteritis, and endocarditis, obtaining representative and uncontaminated specimens from the lungs in pneumonia is a challenge. Sputum and bronchoscopic specimens may be contaminated by normal respiratory flora, and transthoracic lung aspirates are rarely performed despite a good safety profile [7]. Instead, we are currently reliant on testing more distant clinical specimens, particularly from the upper respiratory tract, blood, or urine. In general, testing these specimens has suboptimal sensitivity and/or specificity

for determining the microbial etiology of pneumonia with confidence. A second important issue hindering our ability to determine pneumonia etiology is the fact that some major pneumonia pathogens, such as *Streptococcus pneumoniae, Haemophilus influenzae*, and *Staphylococcus aureus*, may also asymptomatically colonize the upper respiratory tract as part of normal oropharyngeal flora. Consequently, the detection of these microorganisms is insufficient by itself in order to attribute pneumonia causation.

This perspective reviews the current use of molecular diagnostics for determining the microbial etiology of pneumonia and future prospects for this purpose. The focus is on recent advances and their clinical applications rather than detailed description of specific technologies.

Current pneumonia diagnostics

Presently, we are still reliant on traditional diagnostic tools that have been used for decades to determine the microbial etiology of pneumonia. Current guidelines for the management of community-acquired pneumonia in adults typically recommend that microbiologic testing should be largely restricted to patients with more severe disease, and give guidance about the judicious use of blood cultures, sputum microscopy and culture, urinary antigen tests, and serology [8–10]. Guidelines for the management of community-acquired pneumonia in children are even more restrictive, again recommending that tests should mainly be used on patients with severe disease, with a focus on blood cultures and detection of respiratory viruses [11,12]. Common to these guidelines is a cautious approach to the use of molecular diagnostics, although this may change with ongoing reviews. This caution is driven partly by the perceived and real lack of commercial and standardized assays and partly by lack of good data on

diagnostic accuracy. There is also a lingering perception that molecular diagnostics are expensive tests and that use should be restricted on this basis. Cost is not the barrier it used to be, and the relative price of molecular diagnostics has actually fallen over recent years. Indeed, many molecular assays are now comparable in cost to conventional culture-based methods.

Molecular diagnostics for pneumonia

Molecular methods have been a particularly welcome addition to the pneumonia diagnostic toolbox. Collectively, they represent the single biggest recent advance in the field. While not exactly new (polymerase chain reaction (PCR) assays for respiratory pathogens have been around for over 20 years), the widespread adoption of nucleic acid detection tests (NATs) by diagnostic laboratories has been relatively slow. This contrasts to NATs for other infectious diseases, which have been more quickly embraced by laboratories and clinicians [13]. A major reason for the slow uptake and acceptance of respiratory NATs has been the lack of commercial assays. This situation is changing rapidly, undoubtedly spurred on by recent outbreaks of global concern, such as pandemic influenza, Middle East respiratory syndrome (MERS), and Ebola [14–16]. Indeed, the increased availability of commercial assays is the major change in the area since I first started reviewing the role of molecular diagnostics in pneumonia over 10 years ago [17,18].

To date, molecular tests for pneumonia have mainly focused on detection of specific known pathogens by NATs. NATs have several advantages over other existing diagnostic tools. They potentially detect low levels of all-known pneumonia pathogens in clinical specimens, do not depend on the viability of the target microbe, and can provide results within a clinically relevant time frame. They are also probably less affected by previous antimicrobial therapy than are culture-based diagnostic methods. There are now a wide variety of user-friendly platforms that have enabled NATs to be deployed in laboratories outside of specialist tertiary referral centers. Although NATs have mainly focused on detecting the presence of a particular microorganism, they may also provide additional information, such as data on antimicrobial resistance and strain typing.

The NATs that are most widely used in diagnostic laboratories are those that detect potential pneumonia pathogens that are not part of the normal flora, namely respiratory viruses and selected non-colonizing bacteria. For these microbes, simply detecting their presence in a respiratory sample has been regarded as sufficient evidence to assign causation. In contrast, NATs for other bacteria, including some of the most important pneumonia pathogens, have struggled for a defined role outside research laboratories. NATs for detection of the following respiratory pathogens now have established roles.

Respiratory viruses

NATs have revolutionized the diagnosis of viral respiratory tract infections [19,20]. With high sensitivity and specificity, rapid turnaround time, and availability as commercial assays, NATs are now the testing method of choice for respiratory viruses. Respiratory viruses commonly detected by NATs, often in large multiplex panels, include influenza A and B viruses, respiratory syncytial virus, parainfluenza viruses, human metapneumovirus, human rhinoviruses, enteroviruses, adenoviruses, human bocavirus, and several coronaviruses (OC43, 229E, NL63, and HKU1). NATs are also established diagnostic tools for detection of severe acute respiratory syndrome-coronavirus and MERS-coronavirus [16,20].

In the context of pneumonia, the detection of a respiratory virus in an upper respiratory specimen by a NAT has been regarded as sufficient to assign causation in both children and adults [21]. However, this assumption is not always reliable. There is still debate about the exact role (if any) of some viruses in the pathogenesis of pneumonia, including human rhinoviruses and human bocavirus [22]. This has led some to question the wisdom of using large multiplex NAT panels as first-line tests for respiratory pathogens given potential problems with interpretation of positive results [23]. In addition, when control groups are used in pneumonia etiology studies, respiratory viruses are often detected in a similar proportion of both subjects with and without pneumonia, especially in children [24,25]. These findings vary by specific virus, with influenza A and B viruses, respiratory syncytial virus, and human metapneumovirus being typically detected in a significantly higher proportion of cases with pneumonia than controls.

Legionella species

Environmental bacteria of the genus *Legionella* are the cause of Legionnaires' disease, a pneumonia that requires specific antimicrobial treatment and is often associated with outbreaks [26]. Of the many *Legionella* species that can cause human infection, *Legionella pneumophila* is the most common cause globally, with infection usually acquired from water sources. Other species predominate in some geographic locations, particularly *Legionella longbeachae* which is an inhabitant of soil and compost. There is no human-to-human spread of Legionnaires' disease, and human infection follows environmental exposure to the causative microorganism.

Legionella spp. are fastidious organisms and the traditional reliance on culture, serology, and urinary antigen tests has led to underdiagnosis of Legionnaires' disease globally [27]. Indeed, the almost sole reliance on the urinary antigen test, which can only detect *L. pneumophila* serogroup 1, in some parts of the world has created a 'blind spot' for Legionnaires' disease caused by other species and serogroups [27]. NATs have long been used to detect *Legionella* infection [27] and are well-suited for this purpose. *Legionella* are not regarded as human colonizers [28], and the detection of any amount of legionellae in a clinical specimen is regarded as diagnostic for infection, assuming contamination has not occurred during the testing process. Furthermore, all species and serogroups can be detected. *Legionella* DNA has been detected in both upper and lower respiratory samples, urine, and blood from patients with Legionnaires' disease [29–34], although sputum and other lower respiratory samples are regarded as the specimens of choice [31]. Recently, the systematic use of PCR [31]

and collection of induced sputum from patients unable to expectorate [35] have uncovered a hidden burden of Legionnaires' disease and have demonstrated the diagnostic utility of NATs for this disease. Arguably, NATs are now the test of choice for Legionnaires' disease.

Being an environmental organism, there has been concern about *Legionella* contamination during testing, highlighted by the occasional documented contamination of nucleic acid extraction kits [36]. This problem can be overcome by strict adherence to good laboratory practice.

Mycoplasma pneumoniae

Mycoplasma pneumoniae is associated with a variety of both upper and lower respiratory tract infections and is an important cause of pneumonia, frequently occurring in outbreaks. NATs are now widely regarded as the methods of choice for detection of *M. pneumoniae* infections [37,38], and a *M. pneumoniae* target has been incorporated alongside viral targets in many large respiratory multiplex panels. In practice, NATs have also been useful for *M. pneumoniae* outbreak identification and management [39]. Both upper and lower respiratory tract specimens are suitable for testing by NATs, and a positive result in the context of pneumonia is regarded as being diagnostic, as asymptomatic carriage of *M. pneumoniae* is uncommon [40].

Chlamydophila pneumoniae

Chlamydophila pneumoniae is a relatively common cause of community-acquired pneumonia in some geographic regions, but is not typically associated with severe disease [41,42]. NATs have long been used for diagnostic purposes and, indeed, *C. pneumoniae* NATs were the focus of some of the earliest efforts to standardize NATs for respiratory pathogens [43]. Perhaps because of the association with milder disease, *C. pneumoniae* targets have not been a priority for incorporation into multiplex respiratory panels.

Pneumocystis jirovecii

Colonization with *Pneumocystis jirovecii* is common among the general population and is associated with pneumonia in immunocompromised individuals. NATs have advantages over traditional microscopy-based diagnostic tools, detecting *P. jirovecii* in induced sputum, bronchoscopic, or oropharyngeal specimens with high sensitivity [44]. Indeed, it was PCR-based methods that provided some of the early evidence of the existence of *P. jirovecii* colonization [45]. The use of quantitative methods has been necessary in order to discriminate between colonization and disease; thus, at least partially overcoming the problem of false-positive results. Although promising, the cut-off values remain to be standardized for quantitative NATs and may vary with patient population [46].

Mycobacterium tuberculosis

Microscopy and culture of lower respiratory samples remain the standard diagnostic tools for tuberculosis. Despite improvements in culture-based methods, the slow turnaround time for laboratory diagnosis and global concern about disease burden and multidrug resistance has provided the impetus for the development of rapid testing methods [47]. Considerable effort has been put into the development of NATs for *Mycobacterium tuberculosis*, and several commercial assays are now widely available [48]. Interestingly, tuberculosis is unusual among infectious diseases in that NATs are less sensitive than culture, a possible consequence of difficulty with DNA extraction. Sensitivities are typically 92–100% for smear-positive specimens and 40–93% for smear-negative specimens [48]. Importantly, small, user-friendly platforms (such as the Xpert MTB/RIF assay) have been developed to an extent that they have been successfully deployed in many resource-poor locations where rapid diagnostics for tuberculosis are most needed [47,49]. This is a real success story and highlights what can be achieved with molecular diagnostics outside major laboratories.

Bordetella pertussis

NATs are now established tests for the detection of the causative agents of pertussis [50–52]. While *Bordetella pertussis* is the major cause of pertussis in humans and can be complicated by pneumonia [53,54], *Bordetella bronchiseptica*, *Bordetella holmesii*, and *Bordetella parapertussis* have all been occasionally associated with (often milder) pertussis-like illnesses [50]. NATs are the most sensitive methods for the detection of *B. pertussis* and, with rapid turnaround times, have been invaluable tools in outbreak management [55,56]. The main limitation of *B. pertussis* NATs is specificity, particularly the ability of the most widely used (IS481-based) assays to also detect *B. holmesii*, which is generally considered a false positive result [50]. The use of dual target assays has been advocated to overcome this potential problem [52].

Current limitations of molecular diagnostics

What are the limitations of molecular diagnostic tests for pneumonia and what needs to be addressed in order to progress with development? Common to all pneumonia diagnostic testing, the inability to obtain good quality specimens from the lower respiratory tract is a major problem that will only be overcome through new innovative and safe methods of specimen collection and a greater understanding of the relationships between changes in the lung and in more distant specimens in pneumonia.

A second-major limitation of NATs is their inability to distinguish pathogens from innocent bystanders. In essence, we need two pieces of information. First, is a particular microorganism present in the clinical specimen? Second, if the microorganism is present, is it causing this episode of pneumonia? To date, diagnostic efforts have focused almost exclusively on the first question and have ignored the second, which is usually much more difficult to answer unless the microorganism is never present as a colonizer (e.g. *Legionella* spp.). The efforts to use NATs as a diagnostic for pneumococcal pneumonia illustrate some of the key issues.

S. pneumoniae is regarded as the most common cause of pneumonia in all age groups and yet, molecular tests do not have an established diagnostic role [57]. *S. pneumoniae* is also a common nasopharyngeal colonizer, with carriage prevalence exceeding 50% among children in some regions of the world [58]. Earlier pneumococcal PCR assays, such as those targeting the pneumolysin gene, had problems of poor specificity due to detection of other, closely related streptococci [59], although false-positive results are less likely with some of the currently more widely used targets (e.g. autolysin gene) [60]. *S. pneumoniae* is frequently detected by NATs in both upper and lower respiratory specimens from patients with pneumonia [59], but the clinical implications of a positive result are uncertain given the relative high prevalence of pneumococcal carriage. Testing of blood for *S. pneumoniae* by PCR has been promoted as an alternative approach that can potentially avoid issues of contamination. Among Italian children, blood PCR showed promise as a diagnostic for invasive pneumococcal disease [61–64] with high specificity [65]. However, in other populations, positive results have been reported in control participants who do not have suspected pneumococcal disease [66], raising concerns about the broader utility of this approach. In particular, false-positive results are relatively common in children from developing countries where pneumococcal carriage is common [67]. Consequently, a positive pneumococcal NAT result alone is usually not enough to diagnose pneumococcal pneumonia. The possible exception is the use of NATs to detect *S. pneumoniae* in pleural fluid [68,69].

Another important limitation with current NATs is their focus, by nature, on specific known or suspected pneumonia pathogens. Implicit with this focus is the assumption that we know the full range of key potential pathogens that cause pneumonia. This may well be correct, but there is increasing interest in the use of modern techniques, such as next generation sequencing, which provide the opportunity for discovering new or unexpected pathogens [70,71]. The broader approach of these technologies may provide novel insights into pneumonia etiology, but have yet to show added advantage as a routine diagnostic tool [72]. No major new pneumonia pathogens have been uncovered over recent years.

A key reason for the slow adoption of respiratory NATs by diagnostic laboratories is the lack of commercial assays that have been approved by regulatory bodies. The impact has probably been felt more in the USA given the strict requirements of the US FDA. The level and importance of regulation have been debated [73,74], and it is clear that a balance is needed between appropriate regulation of new diagnostics, while avoiding unnecessary barriers to the deployment of useful tests. The use of molecular tests for Legionnaires' disease is a good example of how assay regulation has had an impact on diagnostic strategies. Despite NATs being the diagnostic test of choice for Legionnaires' disease, the shortage of FDA-approved assays has severely limited the use of these tests in the USA, resulting in the continued use of suboptimal diagnostics and underdiagnosis of this disease [27]. Moreover, the absence of a prominent role in diagnostic algorithms has possibly led to an incorrect perception that the performance of NATs is inadequate. Hopefully, increased experience with these tests will reverse this perception.

New insights into pneumonia pathogenesis

A major recent revelation in respiratory medicine has been recognition of the lung microbiome [75]. Until recently, the lungs in health were regarded as sterile. The use of modern culture-independent techniques has not supported this concept, consistently finding evidence of bacteria in the lower airways [75]. This important realization has challenged our traditional paradigm of pneumonia pathogenesis. The tradition view that pneumonia is caused by a single invasive pathogen in a normally sterile site is likely wrong. Increasing recognition that bacteria and viruses frequently interact in the causative pathway to pneumonia [76,77] adds additional complexity, as does the frequent finding of polymicrobial infections [78]. Under the new paradigm, dominant species emerge from the lung ecosystem in pneumonia through uncertain mechanisms, and the bacterial versus viral pneumonia concept is too simplistic. Consequently, we probably need to use more sophisticated approaches to pneumonia diagnosis than assays that simply target single specific putative pathogens.

We have a lot to learn and are only just beginning to understand changes in the microbiome during acute infections [75,79,80]. Analysis of the lung microbiome may provide insights into pneumonia etiology and reveal novel markers for pneumonia prognosis and for treatment guidance [81]. Molecular diagnostic techniques clearly have a central role in these metagenomic analyses [82,83], providing another opportunity for next generation sequencing technology.

Future directions

Any future developments in pneumonia diagnostics must be cognizant of new knowledge about the lung microbiome and about changes in the lungs during the pathway to pneumonia. There is likely to be less focus on just the detection of specific known pathogens, with more interest in the search for markers of change in the lung microbial ecology in the diseased state. The potential application of metagenomics in the diagnostic laboratory is still uncertain and will dependent on the emergence of improved sequencing technology and bioinformatics software. Whole genome sequencing of bacterial isolates is already being increasingly used for strain characterization and epidemiological analyses [84].

For detection of specific pathogens, existing molecular tests can detect very low microbial loads with high analytical specificity. It is unlikely that further test developments will lead to significantly improved performance than we have now. Indeed, the interpretation of low-level positive results from NATs already provides a dilemma for diagnostic laboratories. Instead, the focus of developers should be on making test platforms that are more user-friendly and that allow shorter turnaround times.

We also need to place greater attention on the meaningful interpretation of positive results in order to determine which microorganisms are actually causing individual episodes of pneumonia. One approach to help distinguish infection from

contamination or colonization is to quantify microbial load by molecular methods. The situation is analogous to culture-based methods, whereby organisms isolated in greater quantities from certain cultures are regarded as more likely to be clinically significant. This approach depends on the determination of cut-off microbial load levels that will provide sufficient diagnostic accuracy to distinguish infection from colonization/contamination. However, given the lack of suitable comparator 'gold' standards, these cut-off values are very difficult to identify in a scientific manner. They may also vary for different microorganisms.

Quantitative multiplex PCR has been used to determine the etiology of community-acquired pneumonia in adults using cut-offs developed for interpretation of culture results from lower respiratory tract specimens [85,86]. Not surprisingly, the addition of PCR targets for common bacterial pneumonia pathogens, such as *S. pneumoniae*, resulted in an etiological diagnosis being made in a high proportion of cases. However, these cutoffs have been determined by expert opinion and, although generally intuitive, might be quite misleading. Despite the challenges in doing so, we need more objective assessment of cut-off values using good scientific and epidemiologic principles.

Quantitative approaches have also been applied to nasopharyngeal specimens. Among HIV-infected adults in South Africa, quantitative PCR testing of nasopharyngeal samples distinguished between pneumococcal pneumonia and asymptomatic pneumococcal colonization with reasonable diagnostic accuracy [87,88]. Data from other populations are needed in order to assess whether this method can be used in clinical practice. Microbial load in pneumonia can also be a prognostic marker. For example, pneumococcal density in the nasopharynx [89] and blood [90,91] are associated with disease severity in adults.

We can probably also be more innovative with statistical methods to help interpret molecular test results. Statistical modeling techniques can be used to help overcome the limitations of diagnostic testing for pneumonia, particularly the lack of good comparator standards. Latent class analysis has been used to determine the prevalence of pneumonia caused by specific pathogens in epidemiologic studies [92,93] and for the assessment of diagnostic tests in tuberculosis [94,95]. Possibly the most sophisticated use of statistical modeling techniques in the context of pneumonia etiology is currently being undertaken as part of the Pneumonia Etiology Research for Child Health (PERCH) study [96]. PERCH is a large 7-country case-control study focused on the causes of severe pneumonia in young children from developing countries, the findings of which will be published in 2016. In this study, partially latent class models have been designed for estimating the population etiology distribution and the individual etiology probabilities for specific pneumonia pathogens, with a major focus on molecular diagnostic test results [97]. The findings of PERCH are awaited with great anticipation, as this approach has never before been so extensively applied to the etiology of an infectious disease.

We have entered a new age in pneumonia diagnostics that needs to look beyond the targeting of a limited number of potential pathogens. New knowledge about the lung microbiome and pneumonia pathogenesis, together with emerging developments in sequencing technology, provide opportunities for novel diagnostic tools to help better guide the management of pneumonia.

Expert commentary

Molecular tests are now mainstream diagnostics for many respiratory infections, although we still have much to learn about using them more effectively to assist with the management of pneumonia. Those involved in molecular diagnostic test development for pneumonia need to look beyond the simple detection of specific known pathogens and also provide means to accurately interpret the clinical significance of a positive result. It is not good enough to simply assess a new test by measuring analytical sensitivity and making comparisons to the performance of other similar assays. Diagnostic tests must be evaluated properly following good epidemiological principles. New developments in molecular diagnostics must also be cognizant of emerging information about the lung microbiome, which will likely provide opportunities for the discovery of novel markers to help guide pneumonia management.

Five-year view

The recent expansion in the number and variety of commercial NATs is likely to continue, leading to greater uptake by diagnostic laboratories and greater incorporation into diagnostic test algorithms and guidelines for the management of pneumonia. The provision of even more user-friendly platforms will enable deployment of these tests in laboratories that have not traditionally used molecular diagnostics. Further efforts are needed to evaluate the clinical application of microbial load measurement for the purpose of distinguishing pathogens from colonizing microorganisms and as a prognostic marker.

It is still unclear about how new knowledge about the lung microbiome will affect the development of pneumonia diagnostics, but the impact may be profound. While it will be some time before next generation sequencing becomes a standard tool in diagnostic laboratories, this technology may provide valuable insights into the lung microbial ecology in health and disease and a greater understanding of the pathogenesis of pneumonia. With this comes a shift away from the targeting of a limited number of putative pneumonia pathogens toward the identification of signal patterns characteristic of different etiologies and stages of pneumonia. The hope is that these signals will have sufficient diagnostic accuracy to help guide pneumonia management.

Financial and competing interests disclosure

Key Issues

- Identifying the microbial etiology of pneumonia is challenging, largely due to difficulty in obtaining uncontaminated specimens from the site of infection and in discriminating between colonizing microorganisms and true pathogens.
- Molecular tests, particularly NATs, have been the major advance in pneumonia diagnostics over recent years.
- Although uptake has been relatively slow, NATs now have established roles (often as the diagnostic of choice) for the detection of respiratory viruses and several non-colonizing bacteria (e.g. *Legionella* species). In contrast, NATs have yet to have an established role for several important bacterial pneumonia pathogens (e.g. *S. pneumoniae*) that also are asymptomatic colonizers of the upper respiratory tract.
- Further developments in molecular tests need to focus on methods to help interpret the significance of positive results. The use of quantitative NATs and microbial load cutoffs has shown promise as one means to discriminate between colonizing and pathogenic microorganisms.
- The recent revelation that the lung microbiome exists and new knowledge about the interaction between bacteria and viruses has changed the traditional view of pneumonia pathogenesis. New diagnostics need to account for this new paradigm and be less focused on just detecting specific known pathogens.

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