

Laboratory-based diagnosis of pneumococcal pneumonia: state of the art and unmet needs

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Abstract

In view of the increasing use of pneumococcal vaccines, especially in the developing world, there is a need for appropriate diagnostics to understand the aetiology of pneumonia, to define the burden of pneumococcal disease, and to monitor vaccine efficacy and effectiveness. This article summarizes a meeting on the diagnosis, detection and serotyping of pneumococcal disease organized by PATH and Fondation Mérieux (18–20 October 2009, Fondation Mérieux Conference Centre, Les Pensières, France). Workers and experts met to discuss the gaps in the microbiology-based diagnosis of *Streptococcus pneumoniae* disease, with special emphasis on pneumonia. The meeting was designed to evaluate the state of the art of pneumococcal diagnostics and serotyping methodologies, identify research and development needs, and propose new guidelines to public health authorities to support the introduction of vaccines. Regarding detection, the main recommendations were to encourage chest X-rays and antigen detection in urine. Large-scale studies are needed to evaluate the diagnostic utility of test algorithms that associate chest X-rays, antigen detection in urine, *S. pneumoniae* quantitative PCR in nasopharyngeal aspirates and sputum, and C-reactive protein or procalcitonin measurement in blood. Efforts should be focused on proteomics to identify pneumococcus-specific antigens in urine or host markers in blood expressed during pneumonia. It was recommended to develop *S. pneumoniae* typing capacities, to understand the epidemiology of pneumococcal disease, and to evaluate vaccine effectiveness. Simple and effective approaches are encouraged, and new technologies based on beads, microarrays or deep sequencing should be developed to determine, in a single test capsular serotype, resistance profile and genotype.

Keywords: Pneumococcal pneumonia, diagnosis, pneumococci, detection, serotyping

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Introduction

Pneumonia remains the leading cause of mortality resulting from infectious disease worldwide. In 2008 alone, it killed nearly 1.6 million children <5 years of age [9]. In Bangladesh, for example, pneumonia is responsible for filling 32% of paediatric hospital beds [56]. The term 'pneumonia' is often

used in reference to respiratory disease resulting from a variety of infectious causes, but often carries an unspoken aetiological assumption that the disease is probably bacterial in origin and therefore requires antibiotic therapy.

Although it is true that *Streptococcus pneumoniae* is the most common aetiological agent of pneumonia, *Moraxella catarrhalis*, *Haemophilus influenzae*, *Mycoplasma pneumoniae*, *Bordetella pertussis* and other bacterial species also cause the disease. It is also true that non-bacterial agents can be responsible for causing pneumonia, including more than 20 viruses and several fungal agents. A number of parasites have also been associated with pneumonia [21,78]. In addition, recent data have shown associations between severe respira-

tory disease in children and co-infection by two or more viruses [65] or with synergistic interactions between bacterial pathogens such as pneumococci and a number of respiratory pathogens such as influenza virus, respiratory syncytial virus [44,45,52,81], and human metapneumovirus [48]. Tuberculosis can also be responsible for a significant proportion of pneumonia diagnoses, and it too can act synergistically with pneumococcal infection [53]. For the purposes of this review, we will use the term 'pneumonia' in reference to the clinical disease, regardless of aetiology. We will use the term 'pneumococcal pneumonia' when *S. pneumoniae* is the specific aetiology.

The most common causes of severe pneumonia are largely vaccine-preventable. Vaccines against *S. pneumoniae* and *H. influenzae* type b are available and being increasingly used worldwide to provide protection. Following the introduction of the seven-valent pneumococcal conjugate vaccine (PCV-7), the Active Bacterial Core surveillance program, covering 9.3% of the US population, demonstrated a sustainable 76% reduction among children <5 years of age in the incidence of invasive pneumococcal disease (IPD), which includes pneumonia, bacteraemia, and meningitis [5], and the number of pneumonia hospitalizations has dropped by about 40% in the USA among children <2 years old [27]. However, vaccine efficacy is probably lower in developing countries and for clinical syndromes that are less often pneumococcal, e.g. in South Africa, where a study on 39 836 children reported a reduction in incidence of only 16%, with clinical pneumonia as an outcome measure [46].

S. pneumoniae vaccine probe studies have shown that the real burden of pneumococcal pneumonia is much higher than originally suspected [46]. Studies have also demonstrated that vaccination reduces the rates of hospitalization of patients with culture-positive tuberculosis, suggesting that pneumococcal co-infections may also play a role in tuberculosis. Vaccination against pneumococcus has been shown to prevent hospitalization for influenza [45], and is also likely to prevent co-infections with pneumococcus. Prevention of viral infections may also contribute to the reduction in pneumonia incidence, although this has not been demonstrated, and no vaccines against major respiratory viruses apart from influenza virus are available.

Improved methods for diagnosing pneumonia are needed to better inform health policy-makers. Traditional diagnosis of pneumonia relies on clinical examination, including chest X-rays (CXRs), and physiological and host response examinations. Although CXR is considered to be the reference standard for pneumonia diagnosis today, CXR evaluation cannot specifically determine pneumonia aetiology. Wider use of and improvements in microbiological techniques are possible,

and warrant further exploration. In Bangladesh, for instance, blood culture testing increased from 5% to 37%, increasing the number of diagnosed pneumococcal cases (*S. Saha*, unpublished data). However, vaccine probe studies have shown that blood culture sensitivity should be improved, because it fails to detect an aetiology in most pneumonia cases. Antigen detection in urine has also come into more frequent use in adults, while researchers explore other innovative ways to detect *S. pneumoniae*.

Epidemiological surveys of IPD in the USA have shown that cases remaining after vaccine introduction (23 cases per 100 000) are primarily attributable to non-PCV-7 serotypes [61]. Serotype replacement potentially threatens to erode the tremendous benefits of the pneumococcal conjugate vaccines, with reports in the literature indicating an increase or change in non-vaccine-related pneumococcal serotypes in the nasopharynx following vaccine introduction [30]. Understanding multiple serotype carriage in addition to disease is therefore critical for predicting and monitoring conjugate vaccine effectiveness, measuring changes in disease epidemiology, and determining the impact of serotype replacement. *S. pneumoniae* serotype 19A has emerged among carriage and invasive cases in the USA, and seems to have reached a plateau; however, the proportion of penicillin-resistant serotype 19A strains is increasing [5]. The role of other pathogens in replacement is still not clearly understood.

In 18–20 October 2009, 40 experts on pneumococcal disease diagnosis from research organizations, hospitals, the vaccine and diagnostic industry and funding agencies met in Les Pensières, France, to address the needs and the state of the art of clinical diagnosis of pneumococcal disease, laboratory-based identification of *S. pneumoniae* identification, and molecular epidemiology. New technologies and research needs were extensively discussed. This article summarizes the main points and recommendations discussed by the speakers, and some recent developments in these fields.

Unmet Needs for Pneumonia Diagnosis, Laboratory-based Surveillance, and Epidemiological Surveys

The capacity to detect pneumococcal pneumonia is extraordinarily limited, in large part because of problems in obtaining an optimal specimen for diagnosis. Blood cultures from pneumococcal pneumonia cases are often negative, and respiratory specimens such as sputum or nasopharyngeal samples can be confounded by the presence of normal flora. Because a comprehensive aetiological evaluation of all pneumonia cases by syndrome-based laboratory diagnosis would be too costly and

technically challenging, researchers do not have a full understanding of the major burden of non-bacteraemic pneumococcal disease. As a result, it is not known which tests are needed to enable a real syndrome-based aetiological diagnosis of pneumonia. More accurate tests are needed to eliminate wasteful and non-productive treatments and to limit the development of antimicrobial resistance. Enabling earlier diagnosis is also important for starting optimal therapy sooner, improving treatment effectiveness, and reducing the spread of disease. Simpler, easy-to-use tests may also increase access to and expand the use of diagnostics.

Pneumococcal IPD surveillance has several important objectives. Information on disease burden can build local awareness of disease, enable evaluation of health impacts resulting from interventions, and inform treatment priorities and prevention strategies. Surveillance can also help in the identification of high-risk groups, description of syndrome distribution, identification of the predominant disease-causing serotypes, and monitoring of antibiotic susceptibility patterns. Additionally, it can provide valuable information to the vaccine researchers and policy-makers on disease burden, vaccine cost effectiveness, and vaccine development.

Unfortunately, hospital-based surveillance frequently underestimates the burden of pneumococcal disease. Rates of specimen collection from pneumonia patients are relatively low, particularly in non-research settings. Specific detection of *S. pneumoniae* classically relies on blood culture, which is insensitive and most often not available at the point of care; also, its results are typically not available until several days after specimen collection, and it may be performed at another institution. Also, current culture-based tests for pneumococcal pneumonia require viable organisms, posing challenges to specimen collection and handling. Another factor to consider is that hospital-based surveillance will miss milder cases treated in the outpatient setting.

All available tests for pneumococcal detection and serotyping have limitations and variable performance in different settings, leading to underestimation of pneumococcal pneumonia disease rates in comparison with illnesses with more robust surveillance, such as meningitis.

Although *S. pneumoniae* is the major cause of pneumonia, it is also important to consider surveillance of other bacterial, viral and parasitic causes of pneumonia, including testing methods that differentiate bacterial from non-bacterial infections and that detect all pathogens in a syndrome-based approach. Ideally, tests should be able to detect all pneumococcal diseases—pneumonia, meningitis, septicaemia, and others—and, in addition, determine antimicrobial resistance patterns and serotypes of strains causing disease. To achieve this will probably require testing methods using pleural fluid,

blood, cerebrospinal fluid (CSF), nasopharyngeal swabs, urine, and high-quality sputum samples. The ability to quantitatively detect multiple serotypes within nasopharyngeal specimens would be ideal for a better understanding of carriage and its relationship with pneumococcal disease. However, the positive predictive value of quantitative PCR for diagnosing pneumococcal pneumonia using sputum and nasopharyngeal specimens is recognized to be low, especially in children [84].

In general, ideal diagnostic tests should not necessarily rely on the culture of viable organisms, must have high specificity and sensitivity, and should not be affected by prior antibiotic treatment. In addition, they should be inexpensive and easy to use without extensive training. Finally, pneumococcal testing methods should not jeopardize surveillance for antimicrobial resistance or other pathogens.

Laboratory-based Technologies for Diagnosis and Surveillance

Identification techniques

The main recommendations of the expert group for the diagnosis of pneumococcal pneumonia are detailed in Table 1. Laboratory-based diagnosis of pneumonia currently considers *S. pneumoniae*, largely because of the exclusion of other potential aetiologies. Although PCR from nasopharyngeal specimens for multiplex detection of atypical bacteria, viruses and parasites in a syndrome-based approach is promising, it is only slowly becoming part of hospital routines in industrialized countries. In addition, the detection of *S. pneumoniae* in nasopharyngeal specimens is indicative of carriage, but its use for the determination of pneumonia aetiology is currently of little interest.

No true reference standard exists for the diagnosis of pneumococcal pneumonia. Clinicians today typically use a combination of patient medical history, examinations, CXR and other selected tests to diagnose pneumonia in their practices. To determine pneumonia aetiology, they may also use blood culture, antigen detection in urine, C-reactive protein (CRP), and procalcitonin (PCT). Serology is of little help in these acute settings, as it requires serum from the convalescent phase, but the advent of platforms for simultaneous determination of multiple serotype-specific antibody responses may make these assays more useful in study settings. A consensus regarding the sensitivity of PCR from blood as compared with other diagnostic methods seems promising, although large-scale confirmation is still required. The use of PCR on nasopharyngeal specimens and sputum appears, at this time, to be limited, because of concerns that a sample may be positive with either

TABLE 1. Recommendations for diagnostic test use and development for pneumococcal pneumonia

Chest X-ray (CXR) remains the reference standard for diagnosis of pneumonia syndrome, and should be used when available, especially for hospitalized patients. Blood culture is insensitive but can provide a specific aetiological diagnosis and should be used when available.

When blood culture is not available, *Streptococcus pneumoniae* teichoic acid antigen detection in urine (Binax) may be an alternative, although its specificity is low in children. The use of BINAX NOW also cleared by the Food and Drug Administration for use with cerebrospinal fluid in the USA. Data supporting the use of antigen detection have been also published for presumptive Spn identification in bronchoalveolar lavage fluid, pleural fluid, and blood culture.

Quantitation of DNA loads by using real-time PCR in nasopharyngeal specimens is promising as a diagnostic method; however, the practical use of this measurement for differentiation of invasive pneumococcal disease (IPD) from carriage must be evaluated, as many children without pneumonia have high bacterial densities in nasopharyngeal specimens.

The detection of *S. pneumoniae* in blood by PCR needs to be further explored. A high bacteraemia load detected by quantitative PCR may be predictive of pneumonia severity.

The measurement of C-reactive protein (CRP) or procalcitonin (PCT) in serum may be a useful adjunct to improve the specificity of CXR, antigen detection in urine, and *S. pneumoniae* PCR in nasopharyngeal specimens.

Large-scale studies are needed to evaluate or confirm the diagnostic utility of test algorithms that associate CXR, antigen or DNA detection in urine, *S. pneumoniae* or pneumonia PCR in nasopharyngeal specimens with CRP or PCT.

Multiplex syndrome-based diagnoses of respiratory pathogens would enable accurate aetiological diagnosis of pneumonia besides IPD.

Research efforts should focus on proteomics to identify new, more specific antigens in urine or host markers in blood. They should also explore using transcriptomics to identify new pathogens or host genes expressed differently in blood during IPD.

Development should focus on ASSURED (affordable, sensitive, specific, user-friendly, rapid, equipment-free, delivered to those who need it) tests for *S. pneumoniae* antigen in urine, host markers in blood, and *S. pneumoniae* and pneumonia syndrome-based assays in nasopharyngeal specimens.

carriage or disease. PCR also has high costs and the need for sophisticated instruments, infrastructure requirements, and technical skills among personnel.

Detection of S. pneumoniae in blood. Vaccine probe studies have shown that blood culture methods for detecting *S. pneumoniae* capture only approximately 10–20% of suspected pneumococcal pneumonia cases, with the sensitivity in infants being even lower [50]. Possible explanations for these low rates of success could be the inherently low density of the organism in blood, prior antibiotic treatment, contamination of blood samples and cultures, insufficient blood volumes, incorrect ratios of blood to broth, delays in transporting blood culture bottles to the laboratory, and delays in performing subcultures of blood cultures.

One way of increasing the success of blood culture as a method of detecting pneumococci is to build skills among nurses and laboratory staff through training in blood culture standard protocols, which include critical procedures for hospitalized patients, outpatients, and emergency room patients. Protocols are widely available in the literature, and should be made more accessible, especially through e-learning. Currently, from a cost and practicality standpoint, PCR or antigen-based assays may have the potential for assessing 'beep-positive' or chocolate, culture-negative culture bot-

les. In these cases, pneumococcal DNA or antigen concentrations are high, although pneumococci have reached the stationary phase and subsequently lysed. In a study on 2739 patients, the use of the immunochromatographic antigen test on chocolate blood culture bottles led to the identification of eight invasive pneumococcal cases in addition to the 1605 cases detected by blood culture alone [69].

Matrix-assisted laser desorption ionization time-of-flight mass spectrometry (MALDI-TOF MS) is starting to be used in clinical microbiology laboratories for routine bacterial identification from colonies (for a review, see ref. [74]). MALDI-TOF MS has also been used for diagnosis on positive blood cultures [23,43,79]. This technology is promising, as it reduces the cost and time to result of bacterial identification. However, the performance of MALDI-TOF MS for *Streptococcus* spp. is generally weak, and further studies are required to improve differentiation between *S. pneumoniae* and closely related viridins species such as *Streptococcus mitis* [74].

In addition to the building of skills among medical and microbiology personnel, the development of more sensitive and specific assays is also needed. The use of *S. pneumoniae* PCR in blood for the diagnosis of IPD, for example, is one method whose true value has yet to be fully explored. Although several reports have shown *lytA* (autolysin), Spn9802 or pneumolysin gene (*ply*) PCR in serum to be less sensitive than blood culture in adults [1,15,59], more recent studies, using more sensitive quantitative assays, have shown the opposite for *lytA* [4,36,64]. *LytA* PCR also been shown to be more sensitive than detection of teichoic acid (Binax-NOW; Inverness Medical, Princeton, NJ, USA) in urine [61]. *Ply* PCR, in particular, is not specific and shows cross-reactions with other streptococcal species. In addition, not only is the gene target important, but exactly which segment of this gene is targeted is apparently also important, owing to allelic variation in the target gene between closely related species.

Serotype-specific real-time PCR assays are also showing promise for low-concentration DNA extracts prepared from whole blood and other clinical specimens [4,54] (Carvalho, Mda G. *et al.*, unpublished data). Moreover, when positive, a high bacterial DNA load has been associated with increased mortality and higher risk of septic shock [14,61].

The properties of the antigen pneumococcal choline-binding protein A (PcpA) could be used to design and validate an antigen assay in blood that could be specific for pneumococcal pneumonia. PcpA is expressed only in the presence of a low manganese concentration, which is the case in blood ($<0.1 \mu\text{M Mn}^{2+}$) as compared with nasopharyngeal specimens ($40 \mu\text{M Mn}^{2+}$) [34]. In addition, studies have shown that

PcpA is expressed in the lungs, but not in the nasopharynx [26], which makes this target promising for distinguishing pneumococci causing carriage from those causing pneumonia.

Antigen detection in urine. Extensive efforts to validate the BinaxNOW assay have shown the assay to be more sensitive than blood culture, and to be able to identify more cases of pneumococcal pneumonia than traditional methods in both inpatient and outpatient settings [2,17,25,28,31,39,80]. It is easy to use and non-invasive, but the assay lacks specificity in children, because it can give positive results in healthy children with carriage of pneumococci and of other closely related *Streptococcus* species. Increasing the cut-off at which the assay result is determined to be positive could improve the specificity, but doing so would require extensive validation to determine the impact on sensitivity. Although there is an enormous literature on the use of BinaxNOW in urine specimens, little is known about its use in serum.

Assays based on other antigens, including proteins (PpmA, Ply, or PcpA) or polysaccharides that may also allow typing, are less sensitive or specific than BinaxNOW, but this approach warrants further research to search for better antigens and methodologies. Proteome profiling in urine could help to identify new antigens that could be useful in the development of new diagnostic assays.

Serotype-specific antigen detection in urine might be a promising approach to detect serotype-specific pneumonia in adults [75], but may also be confounded by carriage in children. *Plasmodium* and mycobacterial DNA can be detected in urine and inform diagnoses, possibly making it worthwhile to look for *S. pneumoniae* DNA in urine and to explore the sensitivity and specificity of a urine PCR-based assay for the detection of invasive pneumococcal disease, including pneumonia.

S. pneumoniae PCR in nasopharyngeal specimens or sputum. PCR in nasopharyngeal specimens and sputum is more sensitive than culture, especially in patients receiving antibiotics [1,33,35]. It must be mentioned here that the classic high predictive value of the Gram stain and pneumococcal culture from a high-quality sputum specimen should be fully appreciated in the modern era [55].

A broth enrichment step of 4–6 h in conjunction with conventional culture and PCR techniques increased the sensitivity of pneumococcal detection in nasopharyngeal specimens by 15–20% in carriage prevalence studies [16]. Broth enrichment in combination with conventional PCR frequently allows the detection of multiple serotypes, which is rarely achieved with the current WHO-recommended culture-based approach [57]. However, culture-based amplification (broth enrichment) of nasopharyngeal specimens would not

be predictive of the relative concentrations of the different serotypes originally present. For this purpose, a quantitative PCR approach without broth enrichment might be most applicable; however, this would be suboptimal for the detection of low-density serotypes [16].

Ply quantitative real-time PCR (qRT-PCR) in sputum appears to be promising: the sensitivity and specificity as compared with a composite reference standard comprising Gram staining of sputum samples and sputum/blood cultures were as high as 90% and 80%, respectively, using a cut-off value of 3.7×10^4 genomic equivalents of *S. pneumoniae* per millilitre of sputum [84]. However, obtaining good-quality sputum from infants can be difficult, unless it is induced sputum. Spn9802 qRT-PCR in nasopharyngeal specimens also performed well with a cut-off value of 10^4 genomic equivalents of *S. pneumoniae* per millilitre of nasopharyngeal specimen [1]. Large clinical studies are required to validate the use of pneumococcal qRT-PCR on nasopharyngeal specimens in children with suspected pneumonia in developing countries.

The bacterial load in nasopharyngeal specimens by quantitative PCR may be predictive of pneumonia. A recently published study on 550 hospitalized children in Vietnam showed that the median nasopharyngeal bacterial load of *S. pneumoniae* was substantially higher in children with CXR-confirmed pneumonia than in healthy controls or children with other lower respiratory tract infections (LRTIs) [81]. This was also true for *H. influenzae* and *M. catarrhalis*. However, the diagnostic usefulness of bacterial load in nasopharyngeal specimens is still unclear, as many children without pneumonia carry a high density and the quantities may be serotype-specific.

Experimental approaches to the use of nasopharyngeal specimens for diagnosing pneumonia may also rest on the idea that there may be differences in patterns of gene expression in nasopharyngeal pneumococci between carriage and pneumonia. Many virulence genes are differentially expressed in bacteria isolated from blood or CSF as compared with nasal secretions in mice [50]. Different quantities of capsular polysaccharide are observed between virulent and avirulent pneumococcal phenotypes associated with IPD and carriage, respectively [37]. Host immune protein gene expression is also different in niches linked to IPD. A collaborative transcriptomic research project should be encouraged to identify genes and proteins that are differentially expressed in IPD and carriage.

However, to be widely used in developing countries, PCR techniques need to be simpler and more robust. The GeneXpert system of Cepheid (Sunnyvale, CA, USA; <http://www.cephheid.com/>) or the Film Array system from Idaho

Technology (Salt Lake City, UT, USA; <http://www.idahotech.com>) integrate reagents in a sealed device that prevents contamination of the whole process, from sample preparation to multiplex detection of PCR products, reducing infrastructure and competence needs. The Enigma platform for integrated real-time PCR (Enigma Diagnostics, Salisbury, UK; <http://www.enigmadiagnostics.com>) is fully portable, and uses self-contained reagents that are stable at ambient temperature. Nevertheless, instrument platforms that require limited maintenance are still to be developed for field applications. In addition, costs per test may remain an obstacle for many years.

Serology. The detection by immunoassay of pneumolysin, C-polysaccharide or mixed capsular polysaccharide antibodies trapped in immune complexes has been evaluated as a diagnostic tool for pneumococcal pneumonia in Kenyan adults, but its sensitivity and specificity were insufficient [71]. An immunoassay that detects IgG against pneumococcal surface adhesin A, as compared with blood culture, increased the sensitivity for diagnosis of pneumococcal pneumonia in adults from 23% to 56%, with good specificity [72]. However, the sensitivity of this assay for detecting pneumonia in children is not sufficient [73]. Although serology is not affected by prior antibiotic use, its use is limited to epidemiological studies, and it has drawbacks, such as the potential for detection of antibodies induced by carriage and the difficulty in obtaining acute and convalescent sera from the patient.

Assays of antibody functionality (Opsonophagocytic activities), such as those that detect IgM or low-avidity IgG, are used to diagnose acute infections caused by, for example, cytomegalovirus, rubella virus, dengue virus, or *Toxoplasma gondii*. This approach may be worth exploring for IPD diagnosis, although it may be necessary to use conditions that disrupt immune complexes.

Host markers. *S. pneumoniae* vaccine probe studies have shown that serum CRP concentrations higher than 40 ng/mL and PCT concentrations higher than 5 ng/mL add specificity to the CXR diagnosis of pneumococcal pneumonia [47,49]. Studies involving limited numbers of children hospitalized with LTRIs have demonstrated that elevated PCT is correlated with positive PCR, serology and CXR confirmation, but not with positive BinaxNOW test results in urine. PCT has a sensitivity of 86% and a specificity of 88% for the differentiation of bacterial and viral LRTIs. The specificity of CRP and the sensitivity of interleukin-6 are both lower than those of PCT. Studies in large cohorts of children in developing countries should be encouraged to further document the clinical utility of PCT in pneumonia diagnosis.

A recent study on 835 children from Mozambique with clinically severe pneumonia, 87 with viral pneumonia and 89 with invasive bacterial pneumonia, showed that, in the absence of infection with malarial parasites, levels of PCT and CRP were lower in the viral group than in the invasive bacterial group (PCT, median = 0.21 ng/mL vs. 8.31 ng/mL, $p < 0.001$; CRP, 18.3 mg/L vs. 185.35 mg/L, $p < 0.001$). However, in the presence of malarial parasites, the distribution between clinical groups overlapped, and neither of the two markers could predict mortality [19].

Syndrome-based pneumonia aetiology. Despite an obvious need to establish more comprehensive ways of diagnosing pneumonia aetiology, particularly for the improvement of patient management, viral and bacterial pathogen multi-detection methods are unlikely to be widely available in the near future in developing countries. Developed countries, however, are gradually starting to introduce a complete diagnostic approach in hospitals for the most severe pneumonia cases, but further studies are needed to determine their utility and cost-effectiveness.

Studies to broadly evaluate pneumonia aetiologies should be introduced into surveillance schemes. Some commercial laboratory tests, all based on PCR, are emerging for the detection of some of the major causes of pneumonia. These tests are generally multi-detection assays that identify mostly viruses and atypical bacteria, although some of them also include *S. pneumoniae* and other respiratory bacteria. The formats for these tests include the following: multiplex real-time PCR (Fast-Track Diagnostics, Luxembourg); PCR followed by detection with beads (xMAP; Luminex, Austin, TX, USA); mass spectrometry (MassTag); size fractionation on a capillary electrophoresis system (RespiFinder; Life Technologies, Carlsbad, CA, USA); or microarrays such as TaqMan Low Density Array (Life Technologies) or INFINITI (AutoGenomics, Carlsbad, CA, USA).

Adaptation of these platforms to the needs of clinics at district or local levels has yet to occur, even in hospitals located in major cities. Platform technologies, such as Cepheid's GeneXpert, reduce dependence on highly skilled technologists, but still require significant maintenance and involve high costs per test. For wide use in developing countries, these multi-detection assays must satisfy the same criteria as assays for *S. pneumoniae* detection described previously.

Typing techniques

The main recommendations of the expert group for *S. pneumoniae* typing are detailed in Table 2.

The serotyping reference standard is microbiological culture followed by the Quellung reaction, which is labour-

TABLE 2. Recommendations for typing assays used for pneumococcal surveillance

Bead assays can automate antibody-based and DNA-based serotyping assays and replace the labour-intensive Quellung technique, which is the present reference standard for serotyping.

Sequential multiplex PCR tests are less labour-intensive and expensive than the Quellung reaction, making implementation more feasible for public health laboratories in developing countries. However, until we have a full resolution of the specific DNA signatures essential for expression of each individual serotype, some reference laboratories should continue the use of Quellung tests for quality control.

Real-time multiplex PCR serotyping should be developed and evaluated for diagnostic purposes. For routine serotyping, newly developed nanofluidic platforms should prove practical and inexpensive.

New analytical techniques such as mass spectrometry, microarrays, microfluidics or deep sequencing may allow complete genotyping of strains from various specimens and the determination of serotype, resistance profile, antigen profiles and multilocus sequence types in a single test. Detailed clonal dissection and tracking of recombinant events in real time is feasible with current genomic sequencing technology.

intensive and used by only a few laboratories worldwide. Descriptions of attempts to simplify traditional antibody-based techniques such as the latex agglutination and/or sweep methods are available in the scientific literature. Latex agglutination has proved to be an invaluable tool for serotyping of pneumococci [76]. Other options for serotyping include alternative techniques using molecular approaches, which may also have the potential to provide further information such as the multilocus sequence type or antimicrobial resistance patterns. In the text below, we review some of the serotyping technologies, with a particular focus on recently developed methods, including possible improvements, and an overview of innovative approaches under exploration to fulfil unmet needs.

Antibody and molecular-based serotyping. The current reference standard for serotyping of pneumococci is culture followed by a capsular reaction test (Quellung reaction or Neufeld test). Only a few laboratories in the world have a comprehensive, self-maintained set of typing antisera corresponding to the currently known 92 capsular serotypes. Accurate use of this methodology is particularly challenging and requires experienced laboratory technicians, and is therefore not easily transferable to laboratories in developing countries. Other serotyping methods include latex agglutination assays, which use beads coated with antisera to type to the serotype or group level [29] and, in some cases, to the serotype level (Pneumotest-latex) [75]. Although much faster than the Quellung reaction, this technique is often not as exacting and is also labour-intensive. Home-made latex reagents are also widely used; however, caution is needed in conducting proper quality control testing.

Descriptions exist in the scientific literature for alternative antibody-based technologies such as ELISA (seven serotypes) [77], colony blot [10], dot blot assays using intact pneumo-

cocci (15 serotypes) [22], or immunoblot [11]. These assays have global performance characteristics similar to those of the Quellung reaction, but they have not been developed to test a large number of serotypes. Multibead inhibition immunoassays using a flow cytometer, Luminex or BioRad Bio-plex platforms allow for high multiplexing and throughput, and have a high concordance with the Quellung assay [24,75,85]. Evaluation of the usefulness of the Bio-plex assay as a tool for identifying pneumococcal serotypes directly from CSF specimens or isolates from children with meningitis in an African setting will take place in the near future (MRC, Fajara, The Gambia). Gas chromatography/mass selective detection for the analysis of *S. pneumoniae* capsular polysaccharides [38], nuclear magnetic resonance spectroscopy [83] and Fourier transform infrared spectroscopy [70] may also be used for *S. pneumoniae* serotyping.

Serotyping by multiplex PCR has also been described by a number of researchers [8,58,66,67]. In such cases, several panels of primers are applied to determine the serogroup or type. For the CDC, these protocols are regularly updated at the US Centers for Disease Control and Prevention website. The current CDC assay efficiently resolves 40 different serotypes or serogroups (<http://www.cdc.gov/ncidod/biotech/strep/pcr.htm>). Usually, a first set of primers covers the most prevalent serotypes, and subsequent sets cover less common serotypes. For the typing to be most efficient, each set may require adaptation to local serotype prevalence or the specimen set under evaluation, particularly because of geographical differences, different serotypes in carriage and pneumonia cases, and culture-positive and culture-negative CSF from patients with meningitis. For example, in a multiplex strategy developed in Bangladesh, the first primer set covered 31% of pneumonia/sepsis isolates (48% of meningitis isolates), but the use of seven sets covered 99% of all invasive isolates [68]. In this scenario, the first set of the Bangladeshi algorithm covers only 18% of African isolates and 9% of US isolates. Surveillance in Bangladesh has detected only 45 serotypes causing invasive disease. Serotypes 13, 9L, 17F, 17A, 28A, 35C and 36 do not appear as invasive strains, whereas 12 serotypes make up 80% of isolates. The database of capsular gene sequences for all serotypes, available at <http://www.ncbi.nlm.nih.gov/Genbank>, has proven to be a valuable resource for assay development [6].

Multiplex PCR also improves serotyping in carriage studies. When applied on colonies obtained through culture from nasopharyngeal specimens, PCR is able to identify the putative serotype in more nasopharyngeal specimens than the Quellung reaction. In addition, PCR allows the detection of multiple serotypes from mixed cultures or non-viable specimens, although the entire set of reactions should be applied

to avoid bias in serotype detection. Prior identification of pneumococci in specimens with a real-time assay (*lytA*-based) allows the targeting of only positive specimens for the more cumbersome PCR-based serotyping assays [16].

Sequential multiplexed real-time PCR assays are also currently being developed to identify 21 capsular serotypes [4], 22 serotypes (B. Beall, unpublished data), or 29 serotypes (G. Vernet, G. Baccala, J.-N. Telles, unpublished data). The last of these, based on 4-plexes, has been shown in a preliminary evaluation to be able to identify a serotype in more than 90% of 520 nasopharyngeal or blood specimens from South Africa, Brazil, and Paraguay. Real-time multiplex PCR has been shown to be more sensitive than conventional sequential multiplexed PCR, as demonstrated on a panel of 67 nasopharyngeal specimens and sterile fluids [4].

Although real-time PCR serotyping assays are undoubtedly more sensitive, a major attractive feature of the conventional sequential multiplex PCR assay is that it allows laboratories with the modest capability for conventional PCR to perform quite comprehensive capsular serotype surveillance of pneumococcal isolates, and to identify pneumococci and serotypes within certain clinical specimens such as CSF, pleural fluid, and nasopharyngeal specimens. In general, CDC *Streptococcus* laboratory researchers have found that the conventional sequential multiplexed PCR assay is reliable for identifying serotypes in DNA extracts that display C_t values ≤ 30 in a *lytA* real-time PCR assay, and that 1–3-plex real-time PCR assays are reliable for C_t values of 30–35. They consider *lytA* assay C_t values of >35 –40 to be subjective and C_t values of >40 to be negative.

For pneumococcal strains causing disease, discrepancies between antibody and sequential PCR approaches employing serotype-specific gene sequences are rare. In a documented example, however, a strain identified as serotype 19F with the Quellung test yielded a false-positive serotype 19A result when tested with sequential multiplex PCR, because the strain's *cps* locus target had more similarities with known serotype 19A sequences [62]. Problems also arise in differentiating certain serotypes. For example, only one base pair dictates the structural difference between serotype 6A and 6B capsular polysaccharides; however, a reliable PCR assay has recently been developed to resolve these serotypes [32]. Also complicating serogroup 6 resolution into its composite serotypes is the recent discovery of two additional serotypes created by the recombinational replacement of the *wciN-alpha* gene within serotypes 6A and 6B with the unrelated *wciN-beta*, resulting in serotypes 6C and 6D, respectively. To ensure against error, at least some reference laboratories should continue using the Quellung reaction in parallel with sequential PCR-based serotype

determination until biologically based sequence signatures can be identified for each serotype. Discrepancies between molecular methods and Quellung results are more common. The non-encapsulated phenotype is not uncommon among carriage isolates, many of which apparently contain non-functional serotype-specific gene sequences. Alternative molecular technologies have been described for *S. pneumoniae* typing, including multiplex PCR-based reverse line-blot hybridization [13] or PCR and capillary electrophoresis [42]. Restriction fragment length polymorphism analysis and terminal restriction fragment length polymorphism analysis for mixed-serotype samples have also been described; however, they use a non-*cps* target to predict serotype [30]. Similarly, some techniques are based on *cps* polymorphisms, but employ genes that are functionally interchangeable between different serotypes. To achieve the maximum resolution, another strategy is to combine a multibead assay, in which two antibody panels are sequentially used to cover the serotypes of the 13-valent vaccine, and then the 23-valent vaccine (and six cross-reactive serotypes), with three primer panels that achieve complete coverage of this limited number of serotypes. This assay is currently under validation (M. Nahm, unpublished data).

Another technology for *S. pneumoniae* serotyping includes microarray analysis. Bentley *et al.* [6] have designed a microarray (Agilent Technology) to determine *S. pneumoniae* serotypes in nasopharyngeal specimens on the basis of serotype-specific oligonucleotides within the *cps* locus. The array can potentially be used directly on nasopharyngeal specimens, without culture and DNA amplification. It can detect multiple serotypes and determine their relative abundance. It can also identify novel serotypes (re-assortants) and help to characterize non-typeable *S. pneumoniae* strains. After initial validation on 90 isolates from the Serum Staten Institute, testing of the array took place using 100 nasopharyngeal specimens from The Gambia. DNA was extracted after overnight growth, labelled, and hybridized on the microarray. The microarray identified a serotype in four additional nasopharyngeal specimens as compared with culture-dependent Quellung testing, and the presence of multiple serotypes in 11 additional specimens (M. Antonio, unpublished data).

Electrospray ionization mass spectrometry can discriminate between PCR products with very similar sequences through base composition analysis, derived from determination of the mass/charge ratio of each DNA strand and comparison with a known library of PCR fragments obtained with the given primer set [20]. Forty primer sets (32 amplifying *cps* locus sequences and eight amplifying multilocus sequence typing (MLST) loci) are used in a 8-plex assay on a

96-well format. This technology was shown to resolve 43 different serotypes within a very highly diverse sample of 229 strains (B. Beall and R. Sampath, unpublished data). Of the 197 strains expressing serotypes included in the assay, only two discrepancies (owing to amplification failure) were observed. All 32 strains expressing one of 22 rare serotypes not included in the assay yielded the expected negative results. The MLST-based genotyping component was also highly concordant for strains with genotypes highly related to those included within the T5000 genotyping database. This T5000 kit now has proven potential for characterization of isolates, but needs to be validated on clinical specimens and assessed for its capacity to detect multiple serotypes in carriage specimens. Overall, the performance of these technologies is promising, and will allow for higher throughput at lower cost, as they are less labour-intensive and require fewer skills. However, despite this promise, to be applicable in developing countries these approaches should be further standardized and validated, including the use of more automation. These techniques could ultimately be independent of isolates and work directly on pleural fluid, whole blood, blood culture bottles, CSF, or nasopharyngeal specimens [3]. Whereas conventional PCR approaches generally work well for deducing serotypes from *lytA*-positive CSF specimens, pneumococcal DNA concentrations are generally lower in extracts prepared from whole blood, so more sensitive DNA-based assays such as real-time PCR are more applicable [4].

MALDI-TOF MS may prove valuable for pneumococcal serotyping, and deserves further study. By use of the MALDI BioTyper and its associated software for automated data analysis (Bruker Daltonics, Billerica, MA, USA), it has been possible to classify 109 of 110 *Streptococcus agalactiae* strains at the serotype level [41]. Williamson et al. [82] have been able to differentiate, at the genus, species and, to a certain extent, strain level, bacterial isolates of *S. pneumoniae*—including 13 non-typeable strains, seven vaccine strains, R6 and TIGR4, as well as four clinical isolates—and 18 isolates of other *Streptococcus* spp. or of other genera.

A recent initiative, the PneuCarriage project, coordinated by C. Satzke (Murdoch Childrens Research Institute, Australia), will compare approximately 15 different antibody-based and molecular-based methods for detecting multiple serotypes within carriage specimens, using both laboratory-prepared (spiked) and field swabs from children in developing countries. The sensitivity and specificity of these methods in detecting multiple serotype carriage will be determined, in comparison with each other and also with traditional serotyping methods.

Antibody response to vaccine serotypes. The direct comparison of antibody responses to the serotypes used for different vaccines is important for the evaluation of their efficiency. The most common format for antibody testing is ELISA [18,40]. Commercial assays also exist, such as the Luminex Pneumococcal Immunity Panel (xMAP PneumoI4), which enables the quantitative assessment of IgG against 14 serotypes following the administration of a pneumococcal vaccine [7,60]. An evaluation of a panel of 80 sera showed IgG concentration values obtained with this US Food and Drug Administration-approved and European conformity-marked product to be well correlated with those assigned to the 89SF standard serum [63]. A new version of the product is currently under development to measure antibodies for all PnPV23 serotypes.

An instrument platform that uses electroluminescence chemistry, developed by Merck, has also been used for a multiplex antibody assay for eight serotypes [51].

Comprehensive molecular typing techniques. The objective of the MLST and Pneumococcal Molecular Epidemiology Network (PMEN) databases (<http://www.sph.emory.edu/PMEN> and <http://pneumoniae.mlst.net/>) is to better understand the molecular epidemiology of *S. pneumoniae* at a regional and global level. The PMEN specifically aims to expand its Global Strain Bank with invasive and non-invasive strains from children in developing countries, using genotyping, susceptibility testing, and serotyping for characterization. Information on the Global Strain Bank is available at http://www.cdc.gov/ncidod/biotech/strep/global_pneumo_strain_bank.htm for commercial and academic vaccine researchers.

Comprehensive and integrated assays covering all of these needs in a single test would greatly improve strain characterization capacity. The introduction of genotypic techniques has improved the capacities of laboratories to detect and monitor the emergence and spread of clones that are resistant to antibiotics. The combination of genotyping and serotyping techniques allows insights into the origin and emergence of serotype switch and multiresistant strains.

Today, understanding the molecular epidemiology of *S. pneumoniae* largely relies upon pulsed-field gel electrophoresis and MLST. MLST has defined nearly 6000 different sequence types on the basis of the combination of seven housekeeping gene sequences. This technique has been useful to demonstrate the emergence of specific clones; for example, the post-conjugate vaccine emergence of a serotype 19A capsular switch variant produced through an event involving a common serotype 4 genetic recipient and a prevalent serotype 19A capsular locus donor [12]. The development of new analytical technologies makes possible the simultaneous

detection of multiple genotypic characteristics that are useful for serotyping, resistance detection, genotyping, and evolutionary analysis of strains.

Added to the microarray designed by Hinds *et al.* (see above) are probes to identify other pathogens, such as *H. influenzae* and *Clostridium difficile*, that co-colonize the nasopharynx and to detect six antibiotic resistance genes. Finally, the array has also been designed to study *S. pneumoniae* gene expression.

Deep sequencing by technologies such as Solexa (Illumina), 454 or AB SOLiD allows a massive increase in sequence output as compared with older methods. For example, Solexa generates 30 000 Mbp per run, an amount approximately 15 000 times the genome size of *S. pneumoniae*. Even if 96 samples are sequenced in a single run, coverage is still 166 times the genome size. This capacity enables identification and comparisons of all known pneumococcal genetic determinants, including those that encode serotype, known virulence factors, and protein antigens, multilocus sequence type, and antimicrobial resistance phenotypes. Costs of approximately US\$100 per bacterial genome may currently be possible through higher sample multiplexing.

Conclusion and Recommendations

Pneumonia is still killing millions of people each year, despite it being a largely vaccine-preventable disease. Patient management, surveillance and vaccine effectiveness evaluations require significant improvements in laboratory tools for optimal identification and characterization of the major respiratory pathogens. There is a strong need to support research initiatives, from basic science to clinical validation. Proteomic and genomic technologies must be used to identify new markers for diagnosis (e.g. markers of infection in urine, and markers of host response to infection). New technologies for specimen processing, marker detection and typing (mass spectrometry, bead-based assays, microarrays, and sequencing) could facilitate the implementation of diagnostics in laboratories and ultimately in the fields, for patient management and pathogen surveillance. Existing assays must be carefully evaluated in clinical settings to establish their utility for disease diagnosis. In particular, the use of a combination of assays in parallel must be carefully evaluated, including cost-effectiveness aspects. Collaborative projects must be encouraged, between country infectious disease surveillance centres, research centres (academic and commercial-technological), and clinicians in the countries most affected by the diseases.

Transparency Declaration

The authors have declared no conflicts of interest.

Appendix I

List of participants

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