Laboratory Diagnosis of Pneumonia in the Molecular Age

Antoni Torres[1], Nelson Lee[2], Catia Cilloniz[1], Jordi Vila[3] and Menno Van der Eerden[4]

Affiliations: [1]Dept of Pneumology, Institut Clinic del Tórax, Hospital Clinic of Barcelona, Institut d'Investigacions Biomèdiques August Pi i Sunyer (IDIBAPS), University of Barcelona, Ciber de Enfermedades Respiratorias (CIBERES), Barcelona, Spain. [2]Division of Infectious Diseases, Dept of Medicine and Therapeutics, Faculty of Medicine, The Chinese University of Hong Kong, Hong Kong, China. [3]Servei de Microbiologia, Hospital Clinic, ISGlobal, Barcelona Ctr. Int. Health Res. (CRESIB), School of Medicine, Universitat de Barcelona, Barcelona, Spain. [4]Dept of Respiratory Medicine, Erasmus Medical Centre, Rotterdam, The Netherlands.

Correspondence: Antoni Torres, Dept of Pneumology, Hospital Clinic of Barcelona, C/ Villarroel 170, 08036 Barcelona, Spain. E-mail: atorres@clinic.ub.es

ABSTRACT   Pneumonia remains a worldwide health problem with a high rate of morbidity and mortality. Identification of microbial pathogens which cause pneumonia is an important area for optimum clinical management of pneumonia patients and is a big challenge for conventional microbiological methods. The development and implementation of molecular diagnostic tests for pneumonia has been a major advance in the microbiological diagnosis of respiratory pathogens in recent years. However, with new knowledge regarding the microbiome, together with the recognition that the lungs are a dynamic microbiological ecosystem, our current concept of pneumonia is not totally realistic as this new concept of pneumonia involves a dysbiosis or alteration of the lung microbiome. A new challenge for microbiologists and clinicians has therefore arisen. There is much to learn regarding the information provided by this new diagnostic technology, which will lead to improvements in the time to antibiotic therapy, targeted antibiotic selection and more effective de-escalation and improved stewardship for pneumonia patients. This article provides an overview of current methods of laboratory diagnosis of pneumonia in the molecular age.

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Introduction

In 1930, pneumonia was the third most frequent cause of death in the USA [1]. Now, almost a century later, and despite the introduction of antibiotics in the 1950s, pneumonia and other respiratory infections remain the fourth most common cause of death worldwide [2]. Although the implementation of vaccination programmes throughout the world has reduced the incidence of pneumonia, especially in at-risk populations [3, 4], pneumonia still represents a major burden in terms of morbidity, mortality and health costs, as well as days of work lost [5–8].

Microbiological diagnosis of pneumonia is fundamental to ensure appropriate antibiotic therapy, which is associated with decreasing mortality [9]. However, aetiological diagnosis is only achieved in half of patients and the initial antibiotic regimen should be chosen empirically in order to avoid the delay in instating appropriate therapy that is associated with significant mortality [10, 11].

The Infectious Disease Society of America/American Thoracic Society (IDSA/ATS) guidelines for community-acquired pneumonia (CAP) recommend an optional microbiological diagnostic test in low-level to mild cases of pneumonia, which should be selected as clinically indicated [12]. However, routine microbiology should be performed in the case of pathogens that would significantly alter empirical decisions, such as the influenza virus, *Mycobacterium tuberculosis*, *Pseudomonas aeruginosa* and especially methicillin-resistant *Staphylococcus aureus* (MRSA), because the current guideline recommendation for severe CAP is empirical therapy with β-lactam with macrolide or fluoroquinolone, which may not provide adequate protection against MRSA [12]. Sputum cultures are only recommended for patients ill enough to require intensive care unit (ICU) admission. This means that, depending on the epidemiology of microbial aetiology, empirical treatments are chosen and given to patients as soon as possible after culture sample collection.

In current practice, most CAP patients can be treated following guidelines. The use of scores can help to detect those patients with potential risk for multidrug-resistant pathogens or patients who require non-standard treatment. [13–15] In these patients, molecular information, in addition to conventional techniques, may be very useful. Current clinical practices have three main consequences. 1) The adequacy of the initial antibiotic is the key factor for prognosis. It is known that mortality increases with each hour of delay in initiating the correct therapy for pneumonia [16–19]. 2) Many patients receive broad-spectrum antibiotic therapies until cultures are available and de-escalation cannot be performed quickly; inappropriate use of antibiotics is thus more likely [20, 21]. 3) The emergence of antibiotic-resistant pathogens related to selection pressure by over use of antibiotics worldwide [22, 23].

Antimicrobial resistance among Gram-positive cocci and Gram-negative bacilli is a major problem worldwide [23–25]. The chapter in the State of the World's Antibiotics report 2015 [26] on the human use of antibiotics reports that consumption continues to increase (comparative data 2000–2010), and that antibiotic treatment is inappropriate in between 20 and 50% of cases. The data on consumption per capita by class and country reveal considerable variability between geographical locations, and this underlines how much countries' responses to the problem can vary [26]. Public campaigns encouraging the implementation of specific measures have been successful [27, 28]. Most campaigns of this kind include calls and warnings for better and more rapid diagnostic testing in order to reduce antibiotic administration and to increase the use of microbe targeted therapies. The problem of microbial resistance is so serious that, last year, President Obama drew attention to the threat it poses and called for greater research funding [29]. However, the discovery of novel classes of antibiotics is more or less at a standstill, as the costly and inefficient process is a challenge for pharmaceutical companies, who have abandoned their investment in developing new antimicrobials. The Generating Antibiotic Incentives Now (GAIN) is a new regulation of the Food and Drug Administration (FDA) aimed at providing economic incentives for antibiotic drug discovery by offering market exclusivity and accelerated drug approval processes. The regulation extends the exclusivity with which antibiotics that treat serious or life-threatening illnesses can be sold without generic competition by 5 years. It also provides an additional 6 months of exclusivity to the pharmaceutical or biotechnological company that identifies a companion diagnostic test. Additionally, the FDA grants the option of accelerating the approval for the new antimicrobial [30].

Rapid molecular techniques (figure 1 and table 1) promise to be an effective tool to help guide appropriate therapy and de-escalation from broad-spectrum antibiotic therapy. However, like any new medical technology, they need to be validated in clinical practice.

In this article, we assess the most recent information on non-molecular and molecular methods for detecting respiratory pathogens such as bacteria and viruses. The conclusions of this review are that for most of the pathogens that cause pneumonia, there is the possibility of using rapid methods that may help clinicians to use a better-targeted antibiotic and antiviral treatment. Point-of-care testing will be the best way to implement these methods in clinical practice. However, before implementing these methods, well-designed studies must be performed to determine their cost-effectiveness.
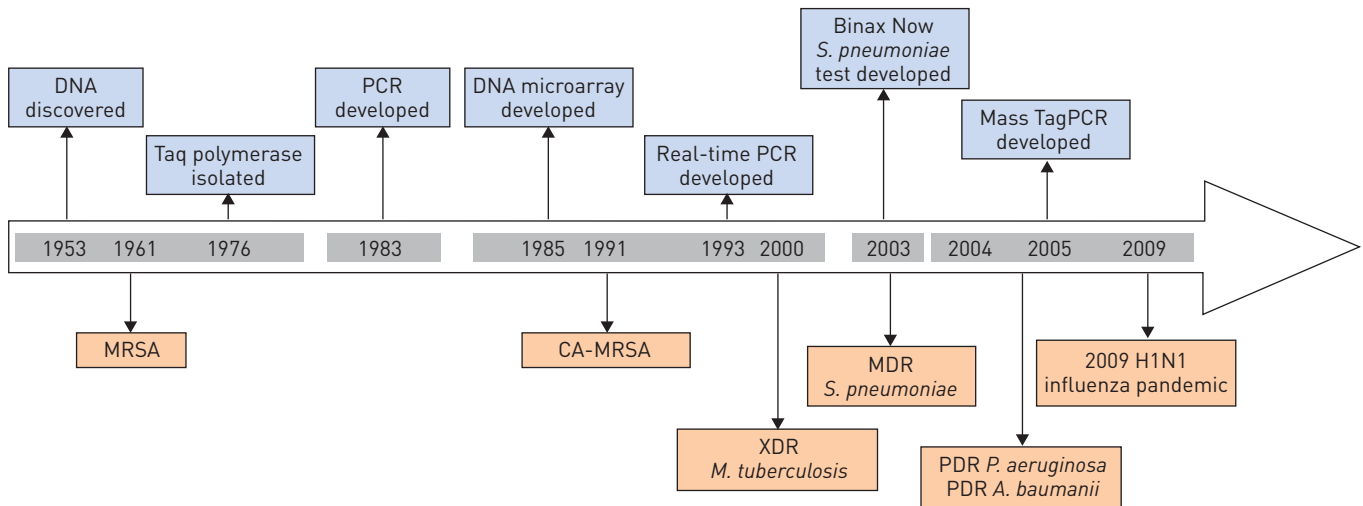
FIGURE 1 Timeline for diagnostic test development and emergence of resistant pathogens. MRSA: methicillin-resistant *Staphylococcus aureus*; CA-MRSA: community-acquired methicillin-resistant *Staphylococcus aureus*; MDR: multidrug-resistant: PDR: pan drug-resistant; XDR: extensively drug-resistant.

Diagnosis of lower respiratory tract infections caused by conventional bacteria
Conventional microbiological diagnosis

In patients with CAP, microbiological investigation should consist of at least a sputum culture, a urinary antigen test for *Streptococcus pneumoniae* and *Legionella pneumophila*, and a blood culture [12]. Additional serological investigation for atypical bacterial pathogens may be performed [12]. In studies involving intensive microbiological investigation in patients with CAP, an aetiology was identified in 53–75% of the sample [31–33]. The most common pathogens identified in these studies were *S. pneumoniae*, *Mycoplasma pneumoniae* and *Haemophilus influenzae*.

Blood cultures performed before administration of antibiotic therapy have a very high specificity but are positive in less than 20% of cases [12, 34]. Blood cultures from patients with severe CAP have a higher yield because pathogens such as *S. aureus* and Gram-negative bacilli are frequently isolated and are not affected by empirical therapy [12].

Pleural effusion is present in approximately 40% of CAP cases [35]. The specificity of pleural exudate culture is very high, although the sensitivity is low because of the low incidence of invasion of the pleura [35]. The sensitivity of the Gram stain is approximately 80% in cases of pneumococcal pneumonia [36] and 78% for staphylococcal pneumonia, with a specificity of 93–96% [37].

Detection of S. pneumoniae *using PCR*

PCR is a molecular diagnostic technique based on DNA detection and offers the advantage of providing results within a few hours. Furthermore, as PCR does not require viable bacteria, it is less influenced by antimicrobial therapy. In a study by JOHANSSON *et al.* [38], in 184 consecutively admitted patients with diagnosis of CAP, 80% of cases with a positive PCR test and negative sputum culture had been treated with antibiotics prior to sputum sampling. In another study on the effect of antibiotic treatment on the accuracy of diagnosing invasive pneumococcal disease using culture or molecular methods, an aetiological diagnosis was established using molecular methods after 4 days or more of antibiotic treatment, and only in the first 2 days of treatment using conventional diagnostic methods [39].

PCR-based detection of *S. pneumoniae* depends on the amplification of pneumococcus-specific genes. Specific characteristics for this species are the production of toxins such as pneumolysin (*Ply*), as well as the presence of surface antigens such as pneumococcal surface adhesin A (*PsaA*) and pneumococcal autolysin A (*LytA*) [40]. However, studies investigating PCRs directed at the *ply* gene showed a lack of sensitivity and specificity for pneumococcal disease [41]. It has also been shown that the *ply* gene was present in some species of *Streptococcal* mouth flora such as *Streptococcus mitis* and *Streptococcus oralis* [42]. To investigate whether gene targets other than the *ply* gene might be more specific, CARVALHO *et al.* [42] compared two other targets: the *lytA* gene and the *psaA* gene. They showed that a PCR designed to target the *lytA* gene was 100% specific, while that for the *psaA* gene was 98% specific. Another study confirmed the high specificity for the *lytA* gene, in which no positive results were found in healthy control

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TABLE 1 Rapid microbiological tests for pneumonia diagnostics.

Platform	Pathogens	Technology	Sensibility/ specificity %	Clinical evidence	Time/cost	Sample
Curetis Unyvero system (P50 pneumonia) [122]	Bacterial/fungal pathogens (18 types) Antibiotic resistance markers (22 types)	Multiplex-PCR (cartridge system)	81/99	Potential for accurate and timely detection of pathogens and their resistance in severe pneumonia	4 h/€280–300	Sputum BAL BAS
Gene Xpert MRSA/SA [117, 118]	MRSA MSSA	Multiplex-PCR	99/72	Rapid, accurate tool for detecting MRSA and MSSA in blood and respiratory samples	1 h/€40	Blood Nasal swabs
MALDI-TOF MS [108, 109, 110]	Microorganisms (200 types)	MS Identification directly from bacterial/fungal colonies	99–100/97–100	Rapid identification of microorganisms in BAL was associated with adjustment of antibiotic therapy and a shorter ICU stay for ventilated patients with pneumonia Detects certain types of antibiotic resistance mechanisms	30 s–1 min/ €0.50–1.00	Colonies Positive blood cultures Direct samples (*e.g.* urine)
Gene Xpert Flu Assay [91]	Influenza A/B (A/2009 H1)	Multiplex-PCR	97–100/100	Rapid identification of influenza virus in outbreaks	1 h/€40	Nasopharyngeal swabs Nasal aspirates Nasal washes
Gene Xpert Flu/RSV Assay [91]	Influenza A/B/RSV	Multiplex-PCR	97–100/100	Rapid identification of influenza virus in outbreaks	1 h/€40	Nasopharyngeal swabs Nasal aspirates Nasal washes
eSensor Respiratory Viral Panel [121]	Influenza A/B (seasonal H1, H3, 2009 H1) RSV A/B Parainfluenza 1, 2, 3 Human metapneumovirus Rhinovirus Adenovirus B/E/C	Multiplex-PCR	98–99/99	Rapid identification of respiratory viruses Co-infection detection	8 h/no data	Nasopharyngeal swabs
FilmArray Respiratory Panel [119, 120]	Adenovirus Coronavirus 229E, OC43, NL63, HKU1 Metapneumovirus Influenza A, H3, H1, 2009 H1 Parainfluenza virus 1, 2, 3, 4, RSV Rhinovirus/enterovirus *Bordetella pertussis* *M. pneumoniae* *C. pneumoniae*	Nucleic acid purification, reverse transcription, high-order nested multiplex-PCR and DNA melting curve analysis (unprocessed biological/clinical sample needed)	84–100/98–100	Detection of several respiratory pathogens in one test Significant impact on the care of patients with respiratory infections	1 h/€100–120	Nasopharyngeal swabs

MALDI-TOF: matrix assisted laser desorption/ionisation time-of-flight; MS: mass spectrometry; MRSA: methicillin-resistant *S. aureus*; MSSA: methicillin-sensitive *S. aureus*; RSV: respiratory syncytial virus; BAL: bronchoalveolar lavage; BAS ▪▪▪; ICU: intensive care unit

subjects [43]. The diagnosis of pneumococcus using PCR tests can be applied to respiratory samples, nasopharyngeal swabs and blood.

Sputum: PCR versus culture

Several studies have investigated the ability of PCR to detect *S. pneumoniae* in sputum [38, 44–46]. In two Scandinavian studies, sputum was collected to perform PCR and culture. In the first study, 128 and 127 patients with CAP had at least one sputum sample analysed by culture and real-time PCR, respectively; the PCR targeted the pneumolysin (*ply*) gene of *S. pneumoniae* [38]. Sputum PCR was positive in 34 patients (27%) while sputum culture was positive in 19 patients (15%) (p<0.001). Sixteen of the 19 patients with a positive culture (84%) also had positive results with real-time PCR and 17 of the 34 patients with positive real-time PCR (50%) had negative cultures. Fourteen of these 17 patients (82%) had been treated with antibiotics before sputum sampling. In the second study, a sputum culture was performed in 112 patients and PCR in 103 patients: 36 patients (32%) had positive cultures and 55 patients (53%) had positive PCR results [47]. These studies suggest that sputum PCR is a more sensitive method than sputum culture for detecting *S. pneumoniae* in patients hospitalised with CAP, especially in those previously treated with antibiotics.

Blood/plasma: PCR versus culture

Blood cultures have a low sensitivity for detecting *S. pneumoniae* infections [31]; bacteraemia is found to be present in about 20% of CAP cases where conventional blood cultures are used [34]. PCR may be able to increase this sensitivity. One study, which assessed the role of PCR in whole blood when targeted at the *ply* gene, showed a positive result in 22 of 40 patients (55%) with pneumococcal pneumonia, while blood culture had a sensitivity of 28% (11 of 40 patients). The PCR was negative in all 30 patients with non-pneumococcal pneumonia, giving a specificity of 100% [48]. Two studies in adult patients determined the sensitivity of positive PCR results compared to positive blood cultures as a gold standard. In the first study, 10 CAP patients had pneumococcal bacteraemia, PCR for *lytA* had a sensitivity of 70% (seven out of 10) [49]. The second study showed a positive PCR *lytA* result for 10 out of 13 patients (77%) with *S. pneumoniae* bacteraemia [50]. Furthermore, in a review assessing the value of PCR for diagnosing pneumococcal bacteraemia, a sensitivity of 57.1% was reported (95% CI 45.7–67.8%). The PCR specificity of 98.6% (95% CI 96.4–99.5%) was determined in a population of "patients with no disease", defined as healthy people or patients with bacteraemia caused by other bacteria [51].

Other PCR studies targeted at the *lytA* gene showed better performance with PCR than with blood culture. In a prospective study including children younger than 15 years of age and adults with CAP, significantly more cases of definite pneumococcal pneumonia were detected by PCR than by blood culture (27.1% *versus* 5.0%; p<0.005). PCR in plasma was negative in a control group of 50 adults [43]. In another study, including children aged 0–16 years, invasive pneumococcal disease was diagnosed in 47 of 292 patients (16%). Of these patients, 45 (15.4%) tested positive with PCR and 11 (3.8%) tested positive with culture. PCR was thus significantly more sensitive than culture in revealing bacteraemic pneumonia (OR 30.6, 95% CI 5.8–97.5; p<0.001) [52]. The results of these two studies showed that molecular methods had higher sensitivity than blood cultures in bacteraemic patients. One of the explanations may be that PCR can also detect DNA from non-viable bacteria. In the latter case, the sensitivity of blood cultures may be influenced by the fact that antibiotic treatment was started prior to culture collection. In one study, in patients with CAP who received antibiotic treatment before hospital admission, PCR sensitivity was 7.0 times higher than that of culture (p=0.043) [39].

Once a positive result is obtained from a PCR test in respiratory samples it is important to determine whether this result reflects bacterial colonisation or infection. Recently, quantitative PCR assays have been performed in different clinical samples (whole blood, sputum and nasopharyngeal specimens) that may differentiate between colonisation and infection. In these studies, a cut-off of 10^4 to 10^5 colony forming units per mL of pneumococcal DNA was described in non-HIV patients [49, 53, 54]. Furthermore, there is some evidence that a quantitative approach can also be used to predict disease presentation and severity, which may correlate with levels of pneumococci DNA in clinical PCR samples. In a prospective study of 353 patients with a diagnosis of CAP, RELLO *et al.* [55] showed that the median number of copies per mL of *S. pneumoniae* DNA in whole blood samples was significantly higher in patients in whom septic shock developed. In two other studies, a higher *S. pneumoniae* DNA load in blood samples was also associated with the presence of more severe disease [50, 56]. Another beneficial application of PCR is that this diagnostic method can be used for pneumococcal serotyping in cases of bacteraemia [52, 57]. This is important for the identification of serotypes that may be responsible for severe CAP cases. These new tests may also be useful for identifying the circulating pneumococcal serotypes in order to assess the effect of pneumococcal vaccination.

Additionally, PCR has the potential to recognise genes inducing antibiotic resistance [58]. At present, however, conventional cultures remain the gold standard for determining resistance to antibiotics.

Diagnosis of viral and atypical pneumonia

Viral and atypical pathogens are common causes of pneumonia worldwide, representing 10–22% and 11–28% of all CAP cases, respectively [59–63]. In a recent study by JAIN et al. [59] analysing 2320 cases of pneumonia where an intensive microbiological diagnosis was applied, particularly viral molecular techniques, microbial aetiology was identified in 853 (38%) cases. The three main causal agents found were respiratory viruses (23%), bacterial aetiology (11%) and co-infections (3%). This study was particularly important in putting two issues into context. 1) The isolation of viruses in CAP is much more frequent than was previously thought, thanks to molecular techniques; and 2) Although molecular techniques are better than microbiological cultures, these methods are not perfect in terms of operational values.

Diagnosis commonly involves antigen tests, culture, serology and molecular assays, all of which have their own advantages and limitations (table 2), such as: specimen type, site and time of collection with respect to illness onset, and the pretest probability (e.g. seasonal peaks), which may significantly affect test performance [64–66]. Testing is generally recommended in hospitalised patients because specific antimicrobial treatments (e.g. neuraminidase inhibitors for influenza, or macrolides, fluoroquinolones, or doxycycline for atypical pathogens) are available and diagnosis may assist effective implementation of isolation precautions [61, 64, 65, 67, 68].

Influenza (seasonal, pandemic, avian) is the most important cause of viral pneumonia, leading to high morbidity and mortality; however, clinical diagnosis is unreliable [64, 65]. Rapid Influenza Diagnostic Tests (RIDT, antigen immunoassays) are convenient, commercially available and highly specific (90–95%). The major drawback is their low sensitivity (40–70%), which means that a negative result cannot rule out influenza infection [65, 69–71]. The tests' performance does improve if they are applied within 48–72 h of illness onset, before significant viral-load drop occurs [65, 66]. Similar considerations apply to immunofluorescence antigen assays, although they are generally more sensitive (50–85%), can be used in upper and lower respiratory specimens (e.g. tracheal aspirates, bronchoalveolar lavage) and can detect a range of respiratory viruses (e.g. respiratory syncytial virus (RSV), parainfluenza virus) if included in the panel. Laboratory expertise is essential, as is the availability of quality specimens with abundant epithelial cells for staining [64, 65]. Generally, serology and culture cannot assist patient care because of their retrospective or slow results, but they are useful for surveillance and research purposes (e.g. for strain identification and phenotypic resistance testing) [65, 72, 73].

Where available, PCR is now considered the test of choice, due to its high sensitivity and specificity, greater time window for detection and rapid turnaround time [64, 65, 71]. It can detect all influenza A subtypes with the universal primers (targeting the M-gene, as for influenza B), or individual subtypes using specific primers (e.g. H1, H3, H5, H7) [74]. This information may have implications for treatment (e.g. pre-pandemic H1N1 is oseltamivir-resistant) and may identify possible novel or avian strains (e.g. H1N1pdm09 and H7N9 are initially "untypable" using existing primers) [69, 72, 74, 75].

Even with PCR, negative results may occur with nasopharyngeal samples in the case of influenza pneumonia due to differential viral kinetic changes along the respiratory tract. Additional testing with a lower respiratory sample (in which viral loads are generally higher) should therefore be considered [66, 69, 75, 76].

Notably, PCR does not provide information regarding infectiousness and dead virus RNA fragments may be detected, albeit at a low level, even upon clinical resolution [74]. Semi-quantitative, real-time assays may be useful for the monitoring of virologic response (cycle-threshold values have been used as alternative estimates) [66, 73, 76]. Rapid, direct detection of genotypic resistance is also possible with PCR for known mutations (e.g. H275Y in H1N1pdm09) [72–74].

As manifestations of influenza are indistinguishable from other respiratory pathogens, multiplex PCR platforms that detect a range of common viruses (e.g. influenza, RSV, human metapneumovirus, parainfluenza virus, rhinovirus) and atypical pathogens (e.g. M. pneumoniae, Chlamydophila pneumoniae) are increasingly being used in the clinical setting [59, 63, 71, 77]. More recently, molecular-based point-of-care tests (loop-mediated isothermal amplification (LAMP) of nucleic acids, real-time PCR) have become available for detecting influenza and other viruses at the bedside, offering a degree of accuracy comparable to conventional laboratory PCR assays [68, 71]. However, the impact of these molecular assays on clinical outcomes and their cost-effectiveness remain unclear and they deserve further evaluation [78].

RSV causes major morbidity in children and older adults, with burdens, manifestations and severity comparable to influenza. Specific antivirals (e.g. fusion inhibitors) are in the pipeline [79, 80]; however,

TABLE 2 Laboratory diagnosis of *S. pneumoniae*, atypical pathogens and respiratory viruses in the clinical setting.

Pathogen	Diagnostic method			
	Culture	**Antigen test**	**Blood culture**	**PCR**
S. pneumoniae	Positive in 60–80% of good quality sputum samples Between 17 and 35% of CAP patients can expectorate sputum of adequate quality [3, 43, 51]	Results in <30 min ICT: sensitivity 60–85% and specificity 99% UAD: sensitivity 98% and specificity 99% [51]	Definitive diagnosis Positive in less than 10% of pneumococcal CAP cases Results in 1–2 days [43, 51]	Results within hours High sensitivity/specificity Allows diagnosis of early, low bacterial load disease Lower influence of previous antibiotic treatment Accessibility, technical demands and cost are practical concerns, as is lack of standardisation [3, 51, 62]
L. pneumophila	Sensitivity 50–80% with high specificity ("gold standard") Allows detection of different *L. pneumophila* strains Technically demanding-requires optimal sampling and post-sampling processing conditions False-negative results may occur in late or treated infections Results are too slow to guide patient care [9]	Results in <30 min Sensitivity 75-80%; specificity 99–100% Unreliable for non-serogroup 1 infections Positive results may indicate recent infection "False-negatives" may occur in early infection Concomitant culture of respiratory secretions in selective media is recommended, especially in immunocompromised patients [98, 99]		Results within hours High sensitivity and specificity Allows diagnosis of early, low bacterial load, and non-serogroup 1 infections Accessibility, technical demands and cost are practical concerns Lack of standardisation Respiratory specimens may be unavailable [48]
	RIDT [82]	**DFA or IFA** [74, 75, 82]	**Viral cell culture** [75, 80, 85]	**RT-PCR** [67, 71, 81, 83]
Influenza virus	Immunoassay for antigens Results in <30 min Applicable to a range of upper respiratory samples Differentiates between influenza A/B Specificity 90–95% Sensitivity 40–70% Unable to distinguish between virus subtypes	Direct or indirect immunofluorescence antibody staining Results within hours Applicable to upper and lower respiratory samples Detects influenza A, B and other viruses simultaneously if included in the panel Specificity 90–95% Sensitivity 50–85% A quality specimen containing adequate epithelial cells together with laboratory expertise is essential Unable to distinguish between virus subtypes	Conventional or shell-vial viral cell culture High specificity Sensitivity 7–20% Allows virus subtyping, strain identification, titre assay/quantification and resistance testing Provides information on infectiousness Requires optimal sampling and post-sampling processing conditions Results too slow to guide patient care (conventional culture: >3–10 days; shell-vial culture: >2–3 days)	Reverse-transcription polymerase chain reaction Results within hours High sensitivity and specificity ("gold standard") Applicable to a wider range of specimen types Able to distinguish between virus subtypes and detect genotypic resistance Multiplex PCR methods may allow simultaneous detection of other respiratory pathogens Accessibility, technical demands and cost are practical concerns Unable to distinguish non-viable from viable viruses "False-negatives" may result from site-differential viral kinetic changes in pneumonia cases

Continued

TABLE 2 Continued

Pathogen	Diagnostic method	
	Serology [95, 96]	**Molecular assay [96]**
M. pneumoniae	Includes ELISA, CFT and particle agglutination A four-fold rise in titre confirms the diagnosis of acute infection A single, elevated IgM/IgA titre may indicate recent infection Specificity 70–90% Sensitivity 20–60% Paired acute and convalescent serum (2–4 weeks apart) are required. Results are thus retrospective "False-positives" may occur with IgM tests and with previous infection	Results within hours High sensitivity, moderate-to-high specificity Accessibility, technical demands and cost are practical concerns Lack of standardisation Specimen of choice is uncertain Cannot distinguish acute infection from asymptomatic carriage, especially in children

CAP: community-acquired pneumonia; ICT: immunochromatographic assay; UAD: serotype-specific urinary antigen detection assay; RIDT: rapid influenza diagnostic test; DFA: direct immunofluorescence antibody staining; IFA: indirect immunofluorescence antibody staining; ELISA: enzyme-linked immunosorbent assay; CFT: complement fixation test.

diagnosis can be difficult because of the low sensitivity of antigen assays (20–60%, higher in children), and culture is slow and technically challenging [78, 79]. The more sensitive PCR assays (singleplex, multiplex) are becoming the tests of choice, especially in adults who generally present with lower viral loads [71, 78, 81]. Similarly, in RSV pneumonia, lower respiratory samples including sputum should also be considered if available; use of a quality sputum sample and methods to overcome the difficulties in its processing have been suggested [63, 76, 78]. Testing for other viruses (commonly with multiplex assays) is gaining importance because of the increasing recognition of their roles in causing lower respiratory infections, hospitalisations and ICU admissions; clinical trials of broad-spectrum or virus-specific agents are underway (*e.g.* nitazoxanide-NCT01227421, favipiravir-NCT01728753 and DAS181-NCT01644877) [59, 63, 68, 74, 79]. Lessons from epidemics caused by emerging pathogens suggest that careful laboratory evaluation of undiagnosed pneumonia with molecular (*e.g.* H1N1pdm09, H7N9) and culture (*e.g.* severe acute respiratory syndrome coronavirus, Middle East respiratory syndrome coronavirus) methods, together with application of advanced techniques such as next-generation sequencing, are important for their prompt detection [69, 79, 82].

Traditionally, atypical bacterial pathogens are not routinely diagnosed due to difficulties in performing cultures and the lack of reliable tests [60, 61]. Single-titre IgM assays provide quicker results but are limited by their moderate sensitivity (*e.g.* early disease, attenuated IgM response in adults) and specificity (*e.g.* past infection, other infectious/non-infectious diseases) [83, 84].

Recent studies indicate high sensitivity for rapid molecular tests (*e.g.* LAMP, singleplex PCR, multiplex PCR) which can be applied to a range of respiratory specimens including sputum and bronchoalveolar lavage [71, 84]. Nevertheless, the methods have not been standardised (*e.g.* primer targets) and the optimal specimen types remain uncertain. Improvements in diagnosis are urgently needed to guide specific therapy because of the emergence of macrolide resistance worldwide (Asia 30–100%, USA/Europe 10–30%) and the challenge this poses for empirical regimens against CAP (typically a β-lactam plus a macrolide) [83]. Similar issues exist for the serological and molecular diagnosis of acute *C. pneumoniae* infection, including prolonged asymptomatic carriage and lack of standardisation [85]. In contrast, diagnostic methods for *L. pneumophila* infection are well established and are described in current pneumonia management guidelines [85, 86]. Briefly, urine antigen tests have high sensitivity (75–80%) and specificity (99–100%), but only reliably diagnose the commonest serogroup (serogroup 1). More recent data suggest that PCR of lower respiratory specimens, including sputum, allows the diagnosis of early, low bacterial load and non-serogroup 1 infections. However, the presence of a dry cough may limit its use. Combining PCR with urine tests may maximise the diagnostic yield [59, 85, 87].

Diagnosis of pneumonia caused by potentially multidrug-resistant bacteria

The most frequent multidrug-resistant bacteria involved in pneumonia are MRSA, *P. aeruginosa*, *Acinetobacter baumannii* and various *Enterobacteriaceae* [23, 25, 88–90]. The reference diagnostic techniques used to identify the bacteria causing respiratory tract infections remain the Gram stain and semi-quantitative conventional culture from direct respiratory samples, followed by bacterial identification using MALDI-TOF (matrix-assisted laser desorption/ionisation time-of-flight) mass spectrometry [91–93] and susceptibility testing of the potential pathogen [94]. In addition to the difficulty of differentiating between colonisation and infection, this process requires a minimum of 2 days and has low sensitivity, especially if the sample is taken after the start of antibiotic treatment [40]. As a consequence, many patients receive inappropriate antibiotic treatment, which may increase morbidity and mortality [16, 95–97]. This statement is also valid for conventional bacteria.

Current guideline recommendations for multidrug-resistant pathogens (MRSA, *P. aeruginosa* and drug-resistant *S. pneumoniae*) include the use of respiratory fluoroquinolone or ß-lactam plus macrolide in outpatients. For inpatients, ICU patients and suspected *P. aeruginosa* infections, the use of an anti-pneumococcal, anti-pseudomonal ß-lactam plus either ciprofloxacin or levofloxacin; ß-lactam plus an aminoglycoside; or ß-lactam plus an aminoglycoside and an anti-pneumococcal fluoroquinolone are recommended. For community-acquired MRSA, the recommendation is to add vancomycin or linezolid.

There are several scoring systems to identify CAP patients with risk factors for acquiring multidrug-resistant pathogens. Of these, two systems, the clinical scores by SHORR *et al.* [14] and ALIBERTI *et al.* [13] categorise risk factors into groups: In the system devised by SHORR *et al.* [14], points are allocated for recent hospitalisation (4 points), nursing home residence (3 points), haemodialysis (2 points) and ICU admission (1 point). Patients with less than 3 points have a 20% prevalence of multidrug-resistant pathogens whereas patients with more than 5 points have a prevalence of 75%. The system developed by ALIBERTI *et al.* [13] assigns points to hospitalised patients with CAP involving chronic renal failure (5 points), prior hospitalisation (4 points), residence in a nursing home (3 points) and other risk factors (0.5 points each). Patients with at least 3 points have a prevalence of multidrug-resistant

pathogens of 38% whereas patients with a score of ⩽0.5 points have a prevalence of 8%. Molecular diagnosis, such as with dedicated PCR assays, has partly overcome the difficulties mentioned [98]. In recent years, molecular techniques based on multiplex PCR have also been developed in order to simultaneously identify and quantify multiple respiratory pathogens from different types of samples in a single procedure (table 3) [99–103].

Another challenge for the rapid diagnosis of respiratory infections is early detection of the antibiotic resistance profile of the various bacteria [104]. The biggest obstacle in the use of molecular techniques for detecting resistance is the discrepancy between genotype and phenotype [105]. New resistance mechanisms are continually being discovered and, as a result, the potential presence of unknown mechanisms may lead to false-negative results using molecular techniques [106]. An additional problem is the detection of genotypic markers which phenotypically do not show clinically significant resistance [105].

Despite the drawbacks of molecular techniques, early information on the resistance profile is more likely to improve empirical treatment than no information at all [106]. An assay based on multiplex PCR is currently under development, which can detect some bacterial resistance markers involved in causing respiratory infection, including the genes related to β-lactam resistance ($mecA$, bla_{TEM}, bla_{SHV}, bla_{CTX-M}, bla_{DHA}, bla_{EBC}, bla_{OXA-51} and bla_{KPC}), macrolides/lincosamides ($ermA$, $ermB$, $ermC$, $msrA$ and $mefA$), fluoroquinolones (detection of changes in the amino acid codons gyrA83and gyrA87 of GyrA and in ParC) and markers of class 1 integrons (int1 and sul1) [107].

Recently, a new group of molecular techniques has emerged based on LAMP[108]. Unlike conventional PCRs, in which a series of temperature change cycles are required for the amplification of nucleic acids, these techniques allow amplification at a constant temperature. ZHANG et al. [109] applied this technique to the rapid diagnosis of major pathogenic bacteria (S. pneumoniae, S. aureus, Escherichia coli, Klebsiella pneumoniae, P. aeruginosa, A. baumannii, Stenotrophomonas maltophilia and H. influenzae) in 120 sputum samples from patients with suspected chronic obstructive pulmonary disease exacerbations. LAMP obtained more positive results than conventional cultures and a higher correlation with clinical symptoms as well.

In a recent study by KONCAN et al. [110], a new methodology called "respiFISH" was developed that combined classical fluorescence in situ hybridisation (FISH) technology with fluorescence-labelled DNA

TABLE 3 Laboratory diagnosis of pneumonia caused by potentially multidrug-resistant bacteria.

Diagnostic method	Advantages	Disadvantages
Culture [3, 42, 60]	Easy to perform and cheap Isolation and identification of bacteria whose detection by other methods has not been standardised (i.e., S. maltophilia) The susceptibility of the isolated pathogens can be assessed	Turnaround time (1–2 days) Low sensitivity, especially if sample is taken after starting antibiotic treatment Sometimes difficult to differentiate infection from colonisation
Molecular assay (Singleplex) [3, 48, 52, 60, 66, 69]	High turnaround time High sensitivity and specificity Allows diagnosis of early, low bacterial load disease Lower influence of antibiotic pretreatment compared to conventional culture Allows detection of resistance markers (i.e., mec gene for MRSA)	Cost is a concern Only detects one pathogen Does not determine susceptibility to all antibiotics-a conventional antibiogram remains the "gold standard"
Molecular assay (Multiplex) [9, 56, 93, 113, 117]	High turnaround time (1–6 h) High sensitivity and specificity Allows diagnosis of early, low bacterial load disease Lower influence of antibiotic pretreatment compared to a conventional culture Detection of some resistance markers	Cost is the main current concern Not all pathogens causing respiratory tract infections are included in the multiplex panel Does not determine susceptibility to all antibiotics-a conventional antibiogram remains the "gold standard"

MRSA: methicillin-resistant S. aureus

molecular beacons as probes. This was used to analyse 165 respiratory samples, including 85 sputa, 79 bronchial secretions (75 bronchial aspirates and four bronchial lavages) and one tracheal aspirate. The sensitivity of this methodology was 94.39% and its specificity was 87.93%. The hands-on time of the assay was approximately 30 min.

VINCENT et al. [111] described a PCR followed by electrospray ionisation mass spectrometry, which could detect more than 800 infection-relevant bloodstream pathogens in a single assay in patients with sepsis and or healthcare-associated pneumonia, ventilator-associated pneumonia, or severe CAP. This study showed that applying this diagnostic method resulted in a three times higher identification of organisms in blood than using standard cultures and, more importantly, changed the antibiotic treatment in 57% of the included patients.

The lung microbiome

In recent years, new culture-independent methods have shown that the lungs, previously thought to be sterile, have a diversity of microbial communities [112, 113]. These communities exist in the absence of infection and they are modified in the event of an acute or exacerbated infection (figure 2) [113]. The pathogenic micro-organisms found in pneumonia (*e.g. S. pneumoniae*) are only some of those that have access to the lower respiratory tract but can also be identified in the lung microbiota of patients without acute pneumonia [114].

CHEN et al. [114] studied the microbiome in sputum samples from 45 patients with CAP. They found similarities with healthy controls at phyla level, and at genera level they found stability for *Streptococcus* spp. and *Neisseria* spp. but changes in *Moraxella spp.* and *Rothia* spp. They concluded that *Rothia* spp. may be an endogenous pneumonia-causing pathogen. In elderly patients with pneumonia, WOUTER et al. [115] observed three microbiota profiles strongly associated with pneumonia: lactobacilli, *S. pneumoniae* and *Rothia* spp. They suggested that pneumonia in elderly patients with dysbiosis of the upper respiratory tract microbiome had bacterial overgrowth of a single species and distinct anaerobic bacteria.

ABELES et al. [116] studied the effect of the two most commonly prescribed antibiotics in the USA (amoxicillin and azithromycin) to discern whether short-term antibiotic use had any prolonged effect on human microbiota. As antibiotics are often absorbed across the gastrointestinal tract and distributed to the tissues *via* the bloodstream, they expected that each would affect the microflora of each body surface tested (gut, skin and mouth). The authors found that as few as 3 days of treatment with the most commonly prescribed antibiotics could result in sustained reduction in microbiota diversity, which may have implications for the maintenance of human health and resilience to disease.

Although there is still a long way to go, we are sure that, in the coming years, better knowledge and understanding of the lung (and gut) microbiome will change our view of the physiopathology, treatment and prevention of pneumonia.

Molecular techniques in clinical practice

Rapid diagnostic tests identify a specific pathogen or help to distinguish between bacterial and viral infection; provide information about antibiotic susceptibility; monitor response to antibiotic therapy; assess prognosis; aid antimicrobial stewardship; and give information for disease surveillance.

These technologies can be applied to different patient populations ranging from outpatients (allowing safe discharge) to patients that need ICU treatment. The quick results (1–2 h) may be useful in the decision management of critical patients especially in the prompt initiation of appropriate antimicrobial therapy, a factor associated with mortality. Also, rapid identification of antibiotic resistant pathogens is central to timely isolation of patients [19]. However, there are still two major challenges to face. 1) The differentiation between colonisation and infection is still an issue for clinicians, except probably in the case of *S. pneumoniae* and we do not have enough evidence for all these techniques to draw conclusions regarding this. 2) The relative costs and outcomes of diagnostic testing must be considered when the decision to implement these rapid tests is taken by healthcare systems-further studies on cost-effectiveness are needed.

Conclusions and implementation

The combination of new and old techniques will improve our ability to detect the microbes that cause pneumonia more precisely and rapidly. This strategy will ensure that patients receive the most appropriate antimicrobial therapy and may reduce the use of broad-spectrum antibiotics, thereby reducing antibiotic resistance (figure 1). Since the majority of studies described in this review show the potential advantages of molecular techniques, such as improved sensitivity and improved speed in establishing a microbiological diagnosis, more studies are needed to systematically and rigorously evaluate their performance characteristics, determine how these new technologies will improve diagnostic testing for respiratory pathogens and effect patient management during their future implementation in daily practice. To

distinguish between colonisation and infection, the use of a quantitative cut-off for the number of colony-forming units ($\geqslant 10^{-5}$ CFU·mL^{-1}, as described in previous studies) is recommended [49, 53, 54, 117]. For implementation of these techniques it is necessary to discuss with the microbiology department and the treating specialist which molecular tests need to be used and which pathogens should be targeted. Although recent publications regarding the apparent utility of multiplex molecular procedures in the detection of CAP pathogens are encouraging, significant concerns remain [49, 53, 54]. A protocol addressing these issues should be developed and evaluated. A key question in this evaluation is whether our current clinical practice needs to change. Will there be a change in prescribing antibiotics and, if so, would it improve antibiotic stewardship? Furthermore, a close evaluation of costs needs to be implemented. Finally, the concepts regarding the lung microbiome are very promising and are currently the focus of many studies. We believe that these concepts will change our understanding of pulmonary infection, how to prevent it and how to improve its treatment.

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Author Queries

Q1   Figure 2 was not received. Is it acceptable to delete reference to this figure?

Q2   Reference 25 is a duplicate of reference 18, but with more up to date citation details. Can another reference be substituted for 25 or should this be deleted?

Q3   Ref. 26-Details added-please confirm if acceptable.

Q4   Ref-28-Details added-please confirm if acceptable.

Q5   Ref 29-Details added-please confirm if acceptable.

Q6   Ref 30-Details added-please confirm if acceptable.

Q7   Ref 62-Details amended. Please confirm if acceptable.

Q8   Refs 118–122 from table 1 are not listed in the reference list. Please provide these references.

Q9   What does the abbreviation BAS stand for?