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REVIEW

Emerging strategies for the noninvasive diagnosis of nosocomial pneumonia

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ABSTRACT

Introduction: Hospital-acquired pneumonia is a common and therapeutically challenging diagnosis that can lead to severe sepsis, critical illness, and respiratory failure. In this review, we focus on efforts to enhance microbiological diagnosis of hospital-acquired pneumonia, including ventilator-associated pneumonia.

Areas covered: A systematic literature review was conducted by searching Medline from inception to December 2018, including hand-searching of the reference lists for additional studies. The search strategy comprised the following common search terms: hospital pneumonia OR nosocomial pneumonia OR noninvasive OR molecular diagnostic tests (OR point-of-care systems OR VOC [i.e. volatile organic compounds]) OR rapid (or simple or quick test), including brand names for the most common commercial tests.

Expert opinion: In recent years, the microbiological diagnosis of respiratory pathogens has improved significantly by the development and implementation of molecular diagnostic tests for pneumonia. Real-time polymerase chain reaction, hybridization, and mass spectrometry-based platforms dominate the scene, with microarray-based assays, multiplex polymerase chain reaction, and MALDI-TOF mass spectrometry capable of detecting the determinants of antimicrobial resistance (mainly β-lactamase genes). Introducing these assays into routine clinical practice for rapid identification of the causative microbes and their resistance patterns could transform the care of pneumonia, improving antimicrobial selection, de-escalation, and stewardship.

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1. Introduction

Hospital-acquired pneumonia (HAP) remains an important cause of morbidity and mortality (range, 20%–50%) despite advances in diagnosis and treatment. Ventilator-associated pneumonia (VAP) is the subtype that develops in 10%–40% of patients on mechanical ventilation for at least 48 h [1,2]. Together, these are the second most common and frequent nosocomial infections among hospitalized patients [3]. Identifying causative pathogens is key to antibiotic choice, differential diagnosis, and epidemiological monitoring. The relative prevalence rates of specific causative pathogens in HAP and VAP vary considerably by patient population, duration of hospitalization and mechanical ventilation, prior antibiotic therapy, and the diagnostic methods and criteria [4].

HAP is often caused by multidrug resistant (MDR) pathogens that pose therapeutic challenges, with both gram-positive and gram-negative bacterial MDR pathogens common in patients with HAP or VAP [3]. The most common gram-positive bacteria are methicillin-resistant *Staphylococcus aureus* (MRSA) and vancomycin-intermediate *S. aureus*. The most common gram-negative bacteria are *Pseudomonas aeruginosa*, Acinetobacter species, and *Enterobacteriaceae* which may be either extended-spectrum β-lactamase (ESBL)-producing or carbapenem-resistant, including *Klebsiella pneumoniae* carbapenemase (KPC)-producing variants [3,5]. Interest has increased in gram-negative pathogens over recent years because they are associated with a higher mortality [6,7]. Moreover, patients treated in intensive care units (ICUs) with an MDR pathogen prevalence of >25% have an increased risk of developing MDR VAP, regardless of other risk factors [7–9]. For this reason, determining the frequency of MDR pathogens in ICUs is essential [3,5].

The choice of antimicrobial treatment is based on recommendations from guidelines and on local and/or regional epidemiological data on frequency of occurrence and antimicrobial susceptibility patterns [2,6]. To minimize the risks of inappropriate empirical antimicrobial therapy, including excessive broad-spectrum β-lactam use, it is recommended that broad-spectrum empirical antimicrobial therapy be initiated early followed by de-escalation as soon as the results of antibiotic susceptibility tests are available [2,6].

In this review, we focus on VAP as a major diagnostic challenge for which delays in microbiological identification hinder the ability of clinicians to streamline therapy. Such diagnostic delay results in excessive exposure to broad-spectrum antimicrobials and risk of perpetuating the problem of antibiotic resistance [2], efforts are needed to address the issue.

2. Diagnosis

There is no consensus on a gold standard for the diagnosis of HAP/VAP. Clinical suspicion of VAP is often confirmed through

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Article highlights

- Current clinical approaches for pneumonia surveillance, early detection, and conventional culture-based microbiology in critically ill patients are inadequate for targeted antibiotic treatment and stewardship.
- Molecular diagnosis in hospital-acquired pneumonia and ventilator-associated pneumonia can provide accuracy and rapidity in pathogen detection.
- Rapid microbiological diagnostics, including multiplex polymerase chain reaction, nucleic acid amplification, mass spectrometry, fluorescence microscopy-based technologies, and exhaled breath biomarkers (including volatile organic compounds) represent promising approaches for the future.
- Molecular tests can help to detect multiple bacterial pathogens simultaneously, but novel approaches are needed to distinguish infection from colonization. This may include the use of quantitative methods and the identification of discriminating cut-off levels.
- We anticipate further studies of molecular tests to focus on the methods required to help interpret the significance of positive results and their application in clinical practice.
- The research agenda should place greater focus on next-generation sequencing and microbiome research.

microbiological culture of lower respiratory samples, but controversies persist about the optimal methodology for collecting lower respiratory tract samples when diagnosing VAP [10]. Obtaining such lower respiratory tract samples from distal or proximal sites can narrow the spectrum of initial empirical antimicrobial therapy [4]. However, there is limited evidence to support the use of invasive or noninvasive methods to obtain respiratory samples in HAP/VAP [11].

Invasive techniques, such as bronchoalveolar lavage (BAL) or protected specimen brush with quantitative culture, require qualified clinicians. Less invasive strategies, such as endotracheal aspirates or nasotracheal suctioning, can lead to over-identification of bacteria [9,10]. Current guidance from the American Thoracic Society and Infectious Diseases Society of America therefore states that there is limited evidence to support invasive techniques to obtain respiratory samples in patients with HAP/VAP, instead recommending noninvasive sampling and semiquantitative cultures for most patients [6]. However, the use of clinical criteria and a reliance on semiquantitative cultures can result in false-positive VAP diagnoses and unnecessary antibiotic use [12,13]. For this reason, the European guidelines of 2017 suggest that accuracy could be improved by obtaining distal samples, with quantitative cultures based on bacterial growth of $>10^5$ colony-forming units (CFU)/mL for an endotracheal aspirate, $>10^4$ CFU/mL for a BAL specimen, and $>10^3$ CFU/mL for a protected specimen brush sample [2]. Thereafter, traditional gram staining, cultures, and antibiotic susceptibility testing is necessary for 48–98 h. This may be too long for patients treated with inappropriate empirical antimicrobial therapy [14] and may be sufficient for the emergence of carriage of carbapenem-resistant bacteria to [15].

Non-Ventilated -HAP poses several challenges for lower respiratory sample collection. The initial approach starts with sputum collection, which requires a coordinate patient effort for a good quality sample [16]. If the patient cannot expectorate we can prescribe nebulized sodium chloride 0.9% can help to loosen secretions before specimen collection.

There are 4 sampling methods for upper respiratory samples collection: naso- pharyngeal swabs (NP), nasal aspirates, nasal washes, and throat swabs. Compared to NP swabs, nasal aspirates and nasal washes are more technically challenging, and because of aspiration risk, are not practical in very severely ill children & adults in resource-poor settings. So, NP swab as the preferred method of URT sampling for detection of viruses. Quantification of baterial load in NP specimens may help differentiate colonization from disease in the context of pneumonia, but only very limited information is available at present.

Issues with existing methodologies have led to the development of several alternative microbial culture techniques that can achieve rapid and accurate diagnosis of nosocomial pneumonia. Although molecular techniques have been under development for the last 30 years, progress in the last decade has been particularly rapid, with quantitative polymerase chain reaction (PCR), nucleic acid arrays, and mass spectrometry (MS) all available to improve the speed and accuracy of laboratory results.

In this review we intent to perform a systematic review of the existing literature on diagnosis of HAP/VAP. According to the recommendations of the new guidelines for HAP/VAP, we explain the difficulties in its diagnosis, the suggested method of collection of respiratory samples for testing and then the new microbiologic techniques. At present, a number of rapid molecular tests are being developed that identify pathogens and the presence of genetic determinants of antimicrobial resistance. We attempt to describe the most validated and useful of them into clinical practice. The results of our systematic review of emerging diagnostic methods for HAP/VAP are presented in detail below and highlight novel radiologic and mainly microbiologic approaches for expediting a diagnosis of HAP/VAP.

3. Emerging strategies for diagnosis of HAP/VAP

3.1. Imaging

Most clinicians diagnose pneumonia based on the finding of a new or progressive radiological infiltrate and at least one clinical feature (e.g. fever, leukocytosis, worsening oxygenation, or purulent tracheal secretions) [6]. This approach has high sensitivity but low specificity for diagnosing VAP, and a normal chest radiograph may not be completely excluding pneumonia [17]. Although chest radiography is widely used despite this limitation, computed tomography (CT) remains the gold standard. CT chest has the advantage of detecting subtle lung infiltrates, particularly in patients with chronic obstructive pulmonary disease, which are often missed on chest radiography [18]. Another noninvasive option is point-of-care lung ultrasound, which has gained popularity in ICUs over recent years [19]. Compared with other methods, lung ultrasound is free of radiation, can be used at the bedside and on pregnant woman, allows for dynamic evaluations, is more accurate than chest radiography for detecting pleural effusions, and takes less time [20]. Although visualizing a consolidated region with a linear/arborescent dynamic air-bronchogram had good specificity (81%), visualization of subpleural consolidation has good sensitivity (81%) and limited specificity (41%) [21]. In experienced hands, a normal lung ultrasound excludes VAP [16]. Lung ultrasound can also be used to monitor infiltrates during active treatment [22].

3.2. Non-cultured based methods

In general, rapid methods for detecting respiratory pathogens are based on immunological or molecular techniques. Most immunological methods use antigen detection by immunofluorescence, enzyme immunoassay, or immunochromatography. Direct and indirect immunofluorescence are widely used to detect respiratory viruses, but are limited by their qualitative nature, limited sensitivity, and inability to distinguish infective viruses.

The immunochromatographic urinary antigen test for *S. pneumoniae* has a sensitivity of 65%–100% and a specificity of 94% [23]. The urinary antigen test may also be applied to pleural fluid and serum samples, albeit with lower sensitivity (40%–50%) [24]. The most commonly available *Legionella* urinary antigen assays have a sensitivity of around 80% for serogroup 1 infection (approximately 85% of infections in the US and Europe), but do not detect other serotypes. Advances in urine antigen detection tests for patients with pneumonia mean that new kits show greater sensitivity, can detect *S. pneumonia*e and L. *pneumophila* in a single test, and can detect pneumococcal serotypes [23,25]. We have to mention that urine antigen testing has only really been validated in adult (not paediatric) patients.

3.2.1. Molecular techniques

The use of molecular techniques, especially nucleic-acid-based amplification methods, have many advantages over conventional methods [26]. They provide high sensitivity and specificity when identifying respiratory pathogens, the possibility of detecting multiple pathogens and the potential for establishing antimicrobial resistance patterns [27]. The ideal rapid molecular diagnostic platform would also provide these by point-of-care testing. The possibility of direct identification and quantification of a pathogen and its load from a single clinical sample are the main advantages of these methods over conventional techniques [28,29].

3.2.1.1. Bacterial and respiratory viral identification. The basis for most molecular assays includes PCR (DNA amplification) or reverse-transcription PCR and nucleic acid-sequence-based amplification. Nucleic acid-based technologies (NATs) have several advantages over other diagnostic tools. They potentially detect low levels of all known pathogens in clinical specimens, do not depend on the viability of the target microbe, and can provide results within a clinically useful time frame [30]. NATs may also quantify target organism density, and (in the case of respiratory viruses) strain typing with high sensitivity and specificity,, making them the method of choice for testing respiratory viruses [31].

Respiratory viruses commonly detected by NATs in large multiplex panels include influenza A and B viruses, respiratory syncytial virus, parainfluenza viruses, human metapneumovirus, human rhinoviruses, enteroviruses, adenoviruses, human bocavirus, and several coronaviruses (e.g. OC43, 229E, NL63, and HKU1) [26]. Bacterial targets such as *Bordetella pertussis, Mycoplasma pneumoniae*, and *Chlamydophila pneumoniae* have also been included in some commercial multiplex respiratory virus PCRs [27,32]. A similar approach can be used to detect bacterial pathogens that complicate respiratory viral infections [29]. Given that co-infection in critically ill patients with influenza has increased from 11.4% (110/968) in 2009 to 23.4% (80/342) in 2015 ($p < 0.001$), NATs are useful in patients with HAP/VAP [33].

More recent assays have allowed multiplexing of over 20 viral targets with good sensitivity. These include those using PCR with melting curve analysis (FilmArray, AusDiagnostics), microcapillary electrophoresis (Seeplex, RespiFinder, ICEPlex), microsphere hybridization associated with flow cytometer detection, solid phase hybridization (Infiniti, NGEN, Verigene, iCubate, eSensor), and PCR coupled with electrospray ionization-MS (ESI-MS; PLEX-ID).

A variety of new platforms have been developed to speed up pathogen identification, including sensitivity testing. Assays which have been designed for organism identification include:

- GeneXpert. This is an all-in-one platform designed as a point-of-care testing assay with a turnaround time of under two hours. Thus, results can be available fast enough to affect empiric treatment choices. GeneXpert systems can detect *S. aureus* or MRSA from colonies or directly from blood cultures, or from nasal, skin, and soft tissue swabs [2,34]. GenXpert uses an automated microfluidic procedure that depends on real-time PCR for *S. aureus* or MRSA, and the BD GeneOhm MRSA uses real-time PCR with a fluorogenic target hybridization probe [35]. A GeneXpert assay for influenza A and B was compared with two commercially available rapid antigen tests and was shown to have excellent sensitivity (97.3% and 100% for A and B, respectively) and specificity (100%, both) [36]. The use of GeneXpert on lower respiratory tract samples from patients with suspected VAP had high sensitivity (98.4%) and moderate specificity (72%), compared with bacterial culture in the detection of *S.aureus* [37].
- BD GeneOhm. This assay uses real-time PCR with a fluorogenic target hybradization probe, designed to detect *S. aureus* or MRSA in respiratory specimens [35].
- Rapid molecular assays for the detection of *S. aureus* have major clinical benefit in that they can determine *S. aureus* colonization status [38].
- FilmArray Respiratory Panel. Produced by bioMérieux BioFire (Salt Lake City, UT), this array integrates nucleic acid extraction, nested PCR, detection, and data analysis in a single-use pouch. It is the first assay to be approved by the Food and Drug Administration (FDA) for the qualitative detection of viral and bacterial nucleic acid targets in nasopharyngeal swab specimens [39]. The FilmArray Respiratory Panel 2 can detect 17 viral targets and the three bacterial species (*B. pertussis, C. pneumoniae, and M. pneumoniae*) typically associated with pneumonia (Table 1.) This assay has a turnaround time of approximately 1 hour and has been applied to direct respiratory specimens, including BAL specimens from mechanically ventilated patients [40]. Also, given the multifocal nature of VAP, even mini-BAL samples obtained blindly without the use of bronchoscopy can be effective [41]. More recently, the FilmArray Respiratory Panel has been employed to demonstrate that more

Table 1. Diagnostic tests for VAP/HAP.

Diagnostic Method	Sample	Technology	Target profiles	Time
FilmArray Respiratory panel	Blood.NP swabs	Multiplex-PCR and DNA melting curve analysis	Gram(+), gram(-), atypicals, fungi, resistance genes: *mecA, vanA, vanB, KPC*	1–1,5 h
GeneXpert system	Blood, nasal swabs	Multiplex -rt-PCR	MRSA, MSSA	1 h
GeneOhm	blood	Multiplex -rt-PCR	MRSA, MSSA	1-2h
Light Cycler Septifast MecA	Nasal swabs blood	PCR and amplification of MRDA DNA	MRSA	4-6h
Nanosphere Verigene	blood	Multiplex rt-PCR	Gram(+), gram(-) Gram(+)resistance genes: mecA,vanA,vanB Gram(-) res.genes: ^{bla}CTX-M, IMP,KPC, NDM, OXA, VIM	2,5h for gram(+) and 2h for gram(-)
PCR/ESI-MS	Blood, sputum	PCR/electrospray mass spectrometry	Bacteria, viruses, fungi and resistance genes	6h
Check KPC/ESBL microarray	blood	DNA microarrays	bla_{CTX-M} genes	7–8h
PNA-FISH	blood	Fluorescent in-situ hybridization	Gram(-) and gram(+) bacteria	2,5h
VOC fingerprints	Exhaled breath	Solid microextraction &gas chromatography -MS	VOCs biomarkers	2-4h

than 24% of HAP episodes were associated with respiratory virus infection alone or concomitant viral and bacterial infection [42]. The FilmArray Respiratory Panel has recently been extended to include 24 common respiratory pathogens (including 8 gram-positive bacteria, 11 gram-negative bacteria, and 5 Candida species) with global sensitivity of 78.6% and specificity 98.1% compared to conventional microbiologic techniques [43].

- The LightCycler SeptiFast. This real-time multiplex PCR assay can identify 20 bacterial and fungal species that account for up to 95% of cases of bacteremia/fungaemia. The assay requires lengthy DNA extraction times of between 4 and 6 hours, so it is not optimized for point -of -care testing as yet. In a pilot study, Baudel et al. [44] suggest that multiplex PCR performed on BAL fluid in critically ill patients could improve the rates of pathogen detection in pneumonia (66%) compared with direct examination (23%) and culture (44%) ($p < 0{,}001$). The pathogen identification rate provided by multiplex PCR was not modified by prior antibiotic treatment (66% vs. 64%, non-significant) and remained superior to culture (23%, $p < 0.001$).
- Matrix-assisted laser desorption ionization (MALDI) time-of-flight (TOF) Mass Spectrometry (MS). This is a very powerful microbiological tool that is being gradually implemented for pathogen identification in conjunction with conventional methods [27,28]. MALDI-TOF MS is a protein/peptide-based diagnostic MS method that relies on the measurement of the molecular mass of all cellular proteins to determine the unique global protein profile that is characteristic of the pathogen. In a study of more than 1000 bacterial isolates, MALDI-TOF MS showed a sensitivity of 95% and a specificity of 84.1% for sample identification compared with conventional systems, taking 6 min/isolate for identification at a cost of 22%–32% less than that of current methods [45] It requires pre-treatment for approximately 1 h [46].
- Huang et al. [47] from the University of Michigan performed a quasi-experimental study to analyze the impact of MALDI-TOF MS in conjunction with intervention by an antimicrobial stewardship team in patients with bloodstream infections. Use of MALDI-TOF MS significantly decreased time to organism identification and improved time to effective antibiotic therapy. It also optimized antibiotic therapy. In a recent prospective large-scale clinical evaluation, a combined MALDI BioTyper and FilmArray BCID-based algorithm significantly shortened the median time to identification (19.5 vs. 41.7 h) with a 5% rate of incorrect or invalid results [48].
- PLEX-ID Technology. This unique system combines PCR with ES-MS to detect a wide range of pathogens, including bacteria, viruses, fungi, and parasites. The technology has been used in viral upper respiratory tract infections [49] and for the detection of biological markers of inflammation in pneumonia [50]. Two studies by the same group evaluated the diagnostic performance of the IRIDICA PCR/ESI-MS assay with BAL samples from ventilated patients with suspected pneumonia [51,52] and showed promising results. By contrast, Huttner et al. [53] documented a concordance of 45% between PLEX-ID and standard diagnostic methods for bacterial and fungal detection in BAL specimens. Among patients with confirmed pneumonia after bronchoscopy, overall specimen concordance was only 30%. Unfortunately, PLEX-ID cannot currently be recommended as a standard diagnostic tool for the detection of bacteria and fungi in BAL specimens. Among its disadvantages are the potential contamination of the workspace and samples (i.e. it uses an open platform), the difficulty in assessing polymicrobial specimens, and the prohibitive costs [54].

3.2.1.2. Detection of antimicrobial resistance. Molecular techniques might also be helpful in examining drug resistance, determining if resistance has been transferred from another organism, supporting for optimal timely antibiotic prescribing, de-escalation and stewardship. Importantly, NAATs, mass spectrometry are capable of identifying presence of selected antibiotic resistance genes and FISH based microscopy can evaluate antibiotic sensitivity against HAP causative pathogens as describing below:

3.2.1.2.1. DNA microarrays. Microarray technology, based on the principle of hybridization, allows the mass screening of

genetic sequences and has been applied to identifying pathogens, monitoring antimicrobial resistance, and typing strains. Simply defined, a microarray is a collection of microscopic features (e.g. DNA) that can be probed with target molecules to produce quantitative (gene expression) or qualitative (diagnostic) data.

Recently developed assays which have been developed to expedite antimicrobial resistance testing include:

- The Verigene Nanosphere system. This is a multiplex nucleic acid detection assay that is used in clinical laboratories for identifying pathogens and for detecting resistance genes in positive blood cultures [55]. Antimicrobial resistance markers can be identified within 2.5 h of a positive blood culture. The use of the Verigene assay in bacteremia has been associated with reduced length of stay, reduced mortality, and improved time to optimal antimicrobial therapy [56].
- SeptiCyte is the only diagnostic tool for infectious disease host gene expression to be approved by FDA [57]. Although it has a short assay time of 4–6.5 h, it is generally considered too complex and lengthy for routine clinical use [58]. SeptiCyte measures the differential expression of four genes (*CEACAM4, LAMP1, PLA2G7*, and *PLAC8*) and has been validated in observational studies among adults and children with suspected sepsis [59].
- The VAPChip assay. This assay is based on BRapid-Array-PCR technology that directly targets 13 respiratory pathogens and 24 β-lactam resistance genes in clinical respiratory specimens. Roisin et al [60] evaluated the diagnostic performance of the assay for detecting and characterizing the etiologic agents in nosocomial pneumonia, using the respiratory samples of patients admitted to ICU. For bacterial identification, the VAPchip had a sensitivity of 72.9% and a specificity of 99.1%.

3.2.1.2.2. Microarrays for detecting β-lactamases. Microarrays possess considerable multiplexing capacity and can be used to detect an unlimited number of genes within a reaction mixture [35]. Gram-negative MDR pathogens are typically causative in VAP, with more than 2,000 β-lactamase genes responsible for resistance to β-lactam antibiotics in these cases Microarray-based assays, such as Check KPC/ESBL (Check-Points), have been used to identify β-lactamase (*bla*) genes present in microbial isolates. They do not identify bacteria, but they do provide information about the mechanism of antimicrobial resistance [61]. The assay takes 7-8 hours. Several studies have highlighted the ability of this assay to detect *bla* genes corresponding to TEM, SHV, CTX-M, and KPC β-lactamases, and have reported high sensitivity and specificity (100%). In a study by Endimiani et al, for example, sensitivity and specificity of the Check KPC/ESBL assay compared to conventional microbiologic susceptibility testing among 106 gram-negative strains ranged from 96% to 100% [62]. In a follow-up study by Fishbain et al. [63], when used with a water lysis step, this platform had a 94.4% sensitivity and a 100% specificity in blood culture samples containing ESBL- and KPC-producing pathogens.

3.2.1.2.3. Loop-mediated isothermal amplification. Advances in traditional and real-time PCR have led to the development of loop-mediated amplification (LAMP), transcription-mediated amplification, and helicase-dependent amplification. LAMP and helicase-dependent amplification are particularly cost-effective because they do not require the use of expensive thermocyclers [27]. LAMP relies on auto-cycling strand displacement to generate DNA or RNA (using reverse transcriptase). Isothermal amplification has been used to detect several respiratory viruses, including human and avian influenza, RSV, human metapneumovirus, human coronavirus-NL63, and Middle East respiratory syndrome coronavirus [27,64,65]. Zhang et al [66]. applied this technique to the rapid diagnosis of major pathogenic bacteria (i.e. *S. pneumoniae, S. aureus, Escherichia coli, K. pneumoniae, P. aeruginosa, Acinetobacter baumannii, Stenotrophomonas maltophilia*, and *H. influenzae)* in 120 sputum samples from patients with suspected exacerbations of chronic obstructive pulmonary disease. Compared with conventional cultures, LAMP obtained a greater number of positive results that had a higher correlation with clinical symptoms. In a recent study, Yamamoto et al. [67] described a LAMP method for detecting blaOXA-23-positive carbapenem-resistant *A. baumannii* that had a sensitivity of 88.6% (39/44) and a specificity of 92.1% (70/76), compared with culture as the gold standard.

3.2.1.2.4. Mass spectrometry. MS-based diagnostic platforms are in common use, including MALDI-TOF MS, ESI-MS, and ion trap-based identification. These methods have been tested for detecting drug resistance with the most common MDR bacteria in VAP, such as MRSA, *P. aeruginosa, A. baumannii*, and *Enterobacteriaceae*.

PCR-ESI/MS has emerged as a technology that is capable of identifying nearly all known human pathogens either from microbial isolates or directly from clinical specimens. With broad-range amplification followed by detection of mixed amplicons, the method can identify genetic evidence of known and unknown pathogens. Starting, when Wolk et al [54] identified the presence of *mecA* gene and showed very good correlation with MRSA phenotype. Also, the identification of toxin genes(PVL,TSST-1) by PCR/ESI-MS correlated with independent PCR analyses of these genes, Recently, a MALDI-TOF MS-based β-lactamase assay was shown to be of use in detecting resistance against different β-lactam antibiotics, including carbapenems [68,69]. Also, PCR/ESI-MS is currently the fastest MS-based method for diagnosis, producing results within 4–6 hours directly from clinical specimens, thereby bypassing the need for bacterial culture (Table 1). In a multicenter observational study, a novel and culture-independent PCR/ESI-MS method was tested in 529 patients [70]. Among the 185 patients with pneumonia, cultures were positive and PCR/ESI-MS was negative in 13 cases; in addition, both were negative in 384 cases. Thus, PCR/ESI-MS was shown to have a sensitivity of 81%, a specificity of 69%, and a negative predictive value of 97% at 6 h from sample acquisition. Independent clinical analysis of the results suggested that PCR/ESI-MS could have altered treatment in as many as 57% of cases [70].

3.2.1.2.5. Rapid automated microscopy. Additionally, automated microscopy methods such as the identification/antibiotic susceptibility testing system (Accelerate Diagnostics Inc) are in development using both genomic and phenotypic technologies

to provide pathogen identification and antimicrobial susceptibilities in a rapid manner [71]. Other researchers performed a pilot clinical trial using a prototype of the Accelerate Pheno (Tucson, AZ) rapid automated microscopy system for detecting potential MDR pathogens in mini-BAL surveillance samples from mechanically ventilated adults at risk for VAP [72]. Microscopy revealed all microbiologically positive samples and organisms (n = 7) and most negative samples (64 of 66) compared with culture (100% sensitivity and 97% specificity). Specifically, it can rapidly detect carbapenem resistance in *K. pneumoniae*, and, if present, predict if the resistance can be attributed to KPC carbapenemase.

Fluorescence in-situ hybridization

Automated microscopy tools based on fluorescent in-situ hybridization (FISH) also offer an interesting methodology for both rapid identification and antibiotic susceptibility testing, from blood or respiratory samples. The FISH technique is based on fluorescently labeled oligonucleotide probes that bind complementarily to specific target ribosomal RNA sequences of bacteria, yeasts, or other microorganisms. FISH can be used to detect pathogens that are difficult or time consuming to identify by traditional methods, especially when more than one species is present, as occurs with polymicrobial infection typical of VAP. For instance, Metzger et al. [73] observed high rates of concordance (81%–95%) between FISH microscopy and conventional microbiologic techniques in detecting organisms in respiratory specimens (BAL) containing target species above and below the diagnostic threshold. Similarly, this technique has been used as a microbiological surveillance method with a sensitivity of 100% and a specificity of 97% compared to standard respiratory cultures [74].

3.2.1.2.6. β-lacta test (bio-rad). β-LACTA test (Bio-Rad) is a new chromogenic test that allows rapid detection of 3GC resistance among *Enterobacteriaceae*. The test may be performed either directly with *Enterobacteriaceae* isolated colonies or with bacterial pellets from positive blood cultures or urines. From a Greek study, the test exhibited excellent sensitivity (96.1%) and specificity (98.5%) for the detection of ESC non-susceptibility in the 235 clinical isolates, which harboured mainly extended-spectrum β-lactamase (ESBL) and KPC- and NDM-type enzymes [75]. In a recent study, Garnier et al. [76], evaluated this as a tool for early escalation or de-escalation of antimicrobial therapy in gram-negative bacterial infections (54% were HAP). Their results showed that the rate of appropriate antimicrobial therapy was about 100% and that there was a significant reduction in the time to antimicrobial change (27 vs 51 h).

In another study β-LACTA test detected with 100% sensitivity the presence of CTX-M-15-producing *K. pneumoniae* spiked in sterile bronchial aspirate sample (BAS) for upon two or more GNB per field upon microscopic Gram staining examination [77]. So, the authors concluded that β-LACTA is an accurate tool for ESBL-PE-GNB detection directly on BAS.

3.2.2. Exhaled breath condensate fluid

Exhaled breath analysis is a promising noninvasive method that can rapidly differentiate healthy individuals from those with diseases that produce volatile organic compounds (VOCs). VOC-guided microbiological identification has been reported using conventional gas chromatography-MS and novel nanocomposite sensor arrays (E-nose) among ventilated patients in ICU [78]. Schnabel et al. [79] found a subset of 12 VOCs that correctly discriminated between VAP-positive and VAP-negative patients with a sensitivity and specificity of 75.8% and 73.0%, respectively. May et al. [80] also described a novel strategy for the rapid diagnosis of VAP, using exhaled breath condensate fluid from heat-moisture exchangers to provide a testing substrate to identify bacterial DNA by PCR. They showed excellent concordance in pathogen identification by PCR of exhaled breath condensate fluid and pathogens isolated from BAL fluid. Additionally, they found that increasing DNA load among serial exhaled breath condensate fluid samples predicted subsequent VAP. The utility of VOCs produced by respiratory pathogens is therefore of great interest, particularly as a noninvasive early diagnostic tool for HAP/VAP. Serial VOC samples are simple to acquire and may allow for preemptive or targeted preventative treatment of early VAP or tracheobronchitis. A large multicenter European study is in progress to evaluate the utility and performance of VOC-guided diagnosis for VAP (BreathDx NTR 6114) [81].

3.3. A need for new studies

Adoption of the novel diagnostic techniques remains relatively low, with molecular techniques and direct susceptibility testing used in only 12% of eligible respiratory samples among patients with potential VAP in 80 Spanish hospitals in 2016 [82]. Thus, we urgently need studies to be conducted with these new molecular technologies, especially in patients with VAP. The gold standard method for diagnosing VAP must remain BAL samples, but comparison should be made to endotracheal aspirates and to conventional methods. However, this should be done in the knowledge that BAL samples are particularly challenging to work with and that contamination with purulent debris from respiratory epithelial and inflammatory cells may render samples difficult to assay by nucleic acid amplification or proteomic techniques [83]. Additionally, studies are needed that evaluate the clinical utility of rapid microorganism identification via molecular methods with BAL fluid in VAP. In a pre-/post-implementation study, Mok et al. [84] reported that rapid microorganism identification via MALDI-TOF MS decreased the length of ICU stay after analyzing BAL fluid ($p = 0.027$).

Additionally, there is a need for further research into the optimization of assays ideally for point-of-care testing to achieve rapid, reliable aetiologic diagnoses in HAP/VAP as being an important field of ongoing research.

4. Conclusion

The development and marketing of technically sophisticated methods in the last decade have started to transform the process of microbiological identification from relying on blood cultures to a much more rapid process that can be performed directly from specimens. These rely on nucleic acid amplification and include MS, real-time automated microscopy, and other technologies. Their ability not only to identify the organism but also to detect any resistance mechanisms before starting empirical antimicrobial therapy could be essential for the control of the spread of MDRs, especially in the context of epidemics. However, as with

any new medical technology, these need to be properly validated in clinical practice.

5. Expert opinion

A major reason for the slow uptake and acceptance of recently developed novel assays for the diagnosis of respiratory infections has been the lack of commercial assays. NATs are more sensitive than gold standard cell culture methods for the diagnosis of respiratory viruses and are more specific than antigen detection tests, including immunochromatographic and immunofluorescence assays [29]. However, these new technologies also have important limitations. They are unable to differentiate colonization from infection, which could be highly problematic in mechanically ventilated patients. They are also unable to give the true susceptibility patterns of the responsible pathogens. For example, although Peptide Nucleic Acid -FISH (PNA-FISH) can provide rapid identification and susceptibility information, the method is limited by its ability to identify relatively few organisms and by being labor intensive in its current formulation [85]. By comparison, MALDI-TOF can identify many more organisms with a comparatively straightforward method. Although nucleic acid extraction-independent assays such as MALDI-TOF MS, microarrays and LAMP are promising, these are currently limited by the limited range of organisms that can be detected. Among the main drawbacks of most of these new techniques are that they rely on qualitative evaluations, they are subject to contamination risk, their costs are often prohibitive, and they often lack validation.

One approach to help distinguish infection from contamination or colonization is to quantify microbial load by molecular methods. Those involved in molecular diagnostic test development for pneumonia need to look beyond the simple detection of specific known pathogens and provide a means to interpret the clinical significance of a positive result accurately. To distinguish between colonization and infection, the use of a quantitative cut-off for the number of CFUs is recommended (e.g. $\geq 10^5$ CFU/mL) [69]. However, studies are still needed to confirm the efficacy, safety, and cost-effectiveness of using these tools for initial antibiotic selection in the ICU.

A promising technology is the 'electronic nose,' which can use gas chromatography to identify the presence of VAP and specific pathogens by real-time exhaled gas analysis from the ventilator, potentially leading to earlier and more accurate initial therapy. VOC signatures were effective in differentiating patients with colonization from those with VAP and also predicted the disease course. This may supersede currently available methods that are less sensitive and specific for the early detection of VAP (e.g. chest radiograph and CT imaging, sputum cytology, inflammatory markers, and gas exchange analysis) [74]. It is anticipated that, as the body of evidence expands through findings of well-designed clinical research undertakings focused on evaluating the performance of these novel assays, and the assays themselves are optimized for routine clinical use, significant advances will be made in expedited diagnosis and goal-directed treatment of HAP/VAP."

Two main technological advances are ongoing. First, the development of point-of-care testing with miniaturized and portable machines that allows rapid testing at the bedside. Second, the development of new methods of analysis for gene expression (genomics), RNA activation (transcriptome), protein production (proteomics), lipids (lipidomics), or metabolites (metabolomics). In addition to accelerating the time to diagnosis of culturable pathogens, real-time metagenomics holds the potential to identify pathogens that cannot be grown in culture [86].

Next-generation sequencing allows for quicker and cheaper DNA and RNA sequencing than is permitted by Sanger sequencing [87].

With PCR and 16S ribosomal RNA gene-based sequencing approaches, a metagenomic approach can identify bacteria, viruses, and fungi alike [88]. 16S rRNA gene sequence analysis is a standard method in bacterial taxonomy and identification, and is based on the detection of sequence differences (polymorphisms) in the hypervariable regions of the 16S rRNA gene which is present in all bacteria. Amplification of the 16SrRNA gene in a PCR assay results in amplification of all bacteria in a sample. Although the 16SrRNA gene sequencing has been used to explore the microbiome in ventilated patients., Morris et al, using BAL samples, assessed the diagnostic accuracy of two 16S ribosomal RNA gene PCR assays for the rapid exclusion of VAP (within 6 h) [89].

Nguyen et al. [90], applying a modern proteomic approach, identified that the presence of S100A8, lactotransferrin, and actinin-1a in BAL samples discriminated patients with VAP and acute lung injury. In addition, Kothari et al. [91] reported that overexpression of the tumor necrosis factor alpha gene was associated with an increased incidence of severe sepsis and septic shock from all causes, including pneumonia. This sequencing technique can effectively narrow the microbiome diversity in the setting of longer mechanical ventilation durations and can be used to predict future VAP onset when dominant bacterial taxa emerge [92]. Further work is needed to streamline the bioinformatic analysis of metagenomic sequencing data before this approach can be scaled for testing in a clinical context.

Novel, rapid techniques of molecular DNA quantification (e.g. droplet digital or nanorod PCR) may prove useful adjuncts in helping clinicians discriminate between health, contamination, colonization, and infection.

The discovery of CRISPR–Cas9 technology as a bacterial immune system against pathogens and its usage as an efficient tool for making targeted changes in the genome has led to a huge revolution in basic biology research [93].The development of the clustered regularly interspaced short palindromic repeats (CRISPR)-Cas9 system has revolutionized the field of gene editing, thus facilitating efficient genome editing through the creation of targeted double-strand breaks of almost any organism and cell type. The implementation of the CRISPR-Cas 9 system has increased the number of available technological alternatives for studying gene function, thus enabling generation of CRISPR-based disease models [94]. CRISPR is a tool that allows researchers to edit genes very precisely, easily and quickly. It does this by harnessing a mechanism that already existed in bacteria. Improvements are urgently needed for various aspects of the CRISPR/Cas9system, including the system's precision, delivery and control over the outcome of the repair process.

The recent discovery of the lung microbiome has provided new insights into the pathogenesis of pneumonia based on interactions between multiple microorganisms [95]. Although it will be some time before next-generation sequencing becomes a standard tool in diagnostic laboratories, this technology may provide valuable insights into the microbial ecology of the lung in health and disease, thereby improving our understanding of the pathogenesis of pneumonia.

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Declaration of interest

The authors have no relevant affiliations or financial involvement with any organization or entity with a financial interest in or financial conflict with the subject matter or materials discussed in the manuscript. This includes employment, consultancies, honoraria, stock ownership or options, expert testimony, grants or patents received or pending, or royalties.

Reviewer disclosures

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