Molecular diagnosis in HAP/VAP

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Purpose of review
This review describes recent findings related to molecular-based methods of potential application in the diagnosis of bacterial hospital-acquired pneumonia (HAP)/ventilator-associated pneumonia (VAP). It focuses on methods capable of providing organism identification and keys of bacterial resistance necessary in clinical and epidemiological management of patients and on their ability to provide quantitative results.

Recent findings
Significant advances have been made in recent years in the field of molecular diagnosis of bacterial pathogens. Real-time PCR, hybridization and mass spectrometry-based platforms dominate the scene. Some of the new technologies provide high sensitivity and specificity in the identification of single or multiple pathogens or a combination of etiological identification and antimicrobial resistance determinants in Staphylococcus aureus, nonfermenter Gram-negative bacilli and Enterobacteriaceae that are often associated with the cause of bacterial HAP/VAP in the late onset of the disease. In diagnosis made directly from clinical specimens and quantification of targets for bacterial load, some of them are promising.

Summary
Despite some limitations, current molecular diagnostic methods have a great potential to include bacterial targets useful in the identification of microorganisms and antimicrobial resistance, to analyze directly unprocessed samples and to obtain quantitative results in bacterial HAP/VAP, an entity of complex microbiological diagnosis due to the features of the pathogens commonly implicated.

Keywords
bacterial pneumonia, diagnosis, hospital-acquired pneumonia, methods, molecular, ventilator-associated pneumonia

INTRODUCTION
Microbiological diagnosis is crucial in the management of hospital-acquired pneumonia (HAP) and ventilator-associated pneumonia (VAP) [1,2]. Many studies argue that an early appropriate pathogen-directed antimicrobial therapy improves outcomes [3–5]. The episodes of bacterial cause in HAP/VAP due to the characteristics of prevailing bacteria and emerging drug resistance require a rapid and reliable diagnosis to help establish the most suitable treatment [6**].

Validated methods for microbiological diagnosis in HAP/VAP are culture-based (quantitative or semi-quantitative cultures) [7]. Recently, new molecular-based methods for identification of respiratory bacterial pathogens have been used (and commercialized). Their objective is to offer an accurate, fast and reliable diagnosis, if possible, of multiple pathogens, without compromising the sensitivity and specificity [8]. The molecular-based methods of diagnosis could offer speed in obtaining results, detection of a very low quantity of target sequences, independency of the viability of target organisms, less effect on results due to the previous use of antibiotics, identification of sequences related to antimicrobial resistance [9] and the possibility of detecting microorganisms difficult to diagnose through conventional methods [2]. In light of these advantages, nucleic acid-based methods in bacterial identification offer a higher sensitivity and specificity over traditional microbiological techniques [8]. More recently, mass spectrometry (MS)-based techniques have begun to appear on the scene. However, these methods are not flawless. Contamination, lack of validations of some assays, complex interpretation of results and increased cost are possible limitations [8].
KEY POINTS

- The molecular diagnosis in hospital-acquired pneumonia (HAP)/ventilator-associated pneumonia (VAP) must provide accuracy and rapidity in the detection of pathogens in order to be considered as a valid tool to lead the antibiotic therapy.

- Significant advances have been made in the past years in the design of molecular diagnosis platforms based on respiratory infections. These platforms have become an additional option of diagnosis and sometimes have surpassed conventional methods.

- Potential applications have multiplex molecular methods in the detection of *Staphylococcus aureus*, nonfermenter Gram-negative bacilli, *Enterobacteriaceae* and antimicrobial resistance genes and/or virulence factors.

- It is still to be determined, on the basis of their performance characteristics, the most appropriate molecular methods for the microbiological diagnosis regarding the identification of potential pathogens in HAP/VAP.

Topics of constant concern in the microbiological diagnosis of bacterial HAP/VAP are the need for rapid and accurate pathogen identification to guide the treatment, the involvement of multidrug-resistant organisms that influence the choice of antimicrobials [7,10,11] and the colonization of the lower respiratory tract by nosocomial pathogens, when distinguishing colonization of the airway from pulmonary infection proves difficult [2]. The role of molecular-based diagnostic methods in this respect is in continuous development.

The objective of this article is to review a selection of recent scientific literature that describes the use of molecular-based methods potentially useful in bacterial HAP/VAP in the late onset of the disease, with particular focus on novel technologies currently available.

CAUSE AND MOLECULAR TARGETS

The microbiology of bacterial HAP/VAP clearly differs from that of community-acquired pneumonia [7,12]. In recent years, no important variations have been registered in the bacterial cause of the nosocomial pneumonia typically caused by *Staphylococcus aureus* or methicillin-resistant *S. aureus* (MRSA), nonfermenter Gram-negative bacilli (such as *Pseudomonas aeruginosa* and *Acinetobacter* species) and *Enterobacteriaceae* (such as *Escherichia coli*, *Klebsiella* species and *Enterobacter* species) [6*,7,13,14]. *Streptococcus pneumoniae* and *Haemophilus influenza* have a low prevalence as cause of HAP (<5%) [6**,7]. In the SENTRY Antimicrobial Surveillance Program 80% of hospital pneumonia is caused by these microorganisms, the rate of drug resistance among bacterial pathogens increased by 1% per year between 2004 and 2008, and isolates of the same species in VAP had a mean of 5–10% less susceptibility to frequently used extended-spectrum antimicrobials [6**]. The emergence of resistance previously unrecorded [i.e. *Klebsiella pneumoniae* carbapenemase (KPC) produced by *K. pneumoniae*] [15] and the detection of virulence factors [i.e. Panton-Valentine leukocidin produced by *S. aureus*] [16*] that could determine the seriousness of the clinical picture have significantly increased.

On the basis of the above facts, potential targets in multiplex molecular assay in HAP/VAP are mecA gene in *S. aureus*; blaVIM, blaIMP genes in *P. aeruginosa*; blaOXA genes in *Acinetobacter* species; and blaKPC gene in *Enterobacteriaceae* and *Stenotrophomonas maltophilia* [17**]. These are aspects under consideration in the design of molecular methods for diagnosis of HAP/VAP.

MOLECULAR DIAGNOSTIC METHODS

Nucleic acid-based amplification methods have been, and continue to be, the most commonly used in the molecular diagnosis of infectious diseases [17**]. They have become the reference method for the microbiological diagnosis of some respiratory pathology surpassing other conventional procedures [18,19].

The molecular technologies in the diagnosis of respiratory infections are focused on multiple target detection systems (or multiplex assays), in general using the nucleic acids amplification by PCR, most frequently multiplex real-time PCR and detection through arrays, such as the two-dimensional microchips or three-dimensional beads [20*,21,22], dye-labeled probes [23*] and more recently by MS, such as matrix-assisted laser desorption ionization time-of-fly (MALDI-TOF MS) and electrospray ionization (ESI MS) [24].

NEW PLATFORMS

A number of recent publications have offered a summary of novel multiplex platforms directed at respiratory infections [25*,26**]. In most of them, the microorganisms targeted are only viruses or the combination of viruses and bacteria related to the community-acquired respiratory tract infection involving adults, pediatric or immunocompromised patients [27]. Some features, such as the possibility to provide results within a few hours, starting from direct respiratory samples, the ability to detect
multiple targets leading to the identification and antimicrobial resistance simultaneously, and the option to obtain quantitative results, may make them useful or potentially promising in bacterial HAP/VAP [26**].

**BACTERIAL IDENTIFICATION**

GenXpert (Cepheid) and BD GeneOhm (Becton-Dickinson) systems are examples of commercialized platforms with assays of rapid detection of *S. aureus* or MRSA identification. As they combine bacterial identification and detection of antimicrobial resistance simultaneously we will consider them in this section. GenXpert for *S. aureus* or MRSA is based on an automated microfluidic procedure that depends on real-time PCR, and BD GeneOhm MRSA uses a real-time PCR with a fluorogenic target hybridization probe [26**]. Their DNA targets are specific genes of *S. aureus* in addition to the staphylococcal cassette chromosome mec (SCCmec) and sequences in the orfX region (where SCCmec joins the *S. aureus* chromosome). These systems can detect *S. aureus* or MRSA from colonies or directly from blood cultures [28,29], or nasal [30*,31,32], skin and soft tissue swabs [31] in approximately 1–2 h. In MRSA detection the GeneXpert and BD GeneOhm systems have reported percentages of sensitivity, specificity and negative predictive value of up to 75, 94.5, and 99.1%, and 85.2, 96.5, and 99.5%, respectively, in reference to cultures and depending on anatomical sites sampled [31]. Other studies have performed better results in sensitivity, up to 92% for GeneXpert assay [33] and 97.2% for BD GeneOhm test [34**], and similar results in specificities and negative predictive values [33,34**]. In two studies, both rapid MRSA detection assays, validated for nasal colonization, were used in sputum samples [35,36]. In one of them, BD GeneOhm showed a high sensitivity (97%) and specificity (92%) in MRSA identification in relation to 32 positive cultures [36] (cited by [37*]). In the other one, GeneXpert assay in lower respiratory tract samples from patients with suspicion of VAP registered sensitivity and specificity of 98.4 and 79.4%, respectively, in relation to 71 positive quantitative cultures (cut-off value 10⁴). When qualitative cultures were under consideration the specificity increased to 95.2% [35].

In addition to MRSA detection, the presence of vanA and vanB genes could also be detected (BD GeneOhm VanR Assay) [34**]. Although the use of these tests in pneumonia diagnosis has been little reported, one of the main contributions of these real-time PCR assays in the context of this clinical entity is to determine the *S. aureus* colonization status [31]. The screening and decolonization in epidemiological control measures can eliminate the possibility of MRSA infection and lower the risk of MRSA pneumonia [37*,38]. The development of a reliable test for detection of *S. aureus* and MRSA in diagnosis of pneumonia remains a challenge.

Another emerging system, based on multiplex PCR, designed to detect a large panel of respiratory pathogens in an integrated diagnostic platform, is FilmArray (Idaho Technologies). It is simple to use with minimal hands-on time and has a total run time of about 1 h. Starting from an unprocessed clinical sample, all steps, such as nucleic acid purification, reverse transcription, PCR amplification and melting curve analysis, occur in a single assay, minimizing the risk of carryover contamination [39*]. The panel includes 17 respiratory viruses (also influenza A virus H1–2009) and three bacteria (*Bordetella pertussis, Chlamydia pneumoniae* and *Mycoplasma pneumoniae*) [39*]. By using nested multiplex PCR, the targeting of conserved housekeeping genes can accurately detect bacteria. It is a technology with broad possibilities in the detection of other Gram-negative bacilli [26**]. There could be applications under development. So far, comparative studies on FilmArray diagnostic performance in relation to other validated platforms for respiratory viruses have been described [27,40*,41*], without benchmark results relating to bacterial detection.

In the past years one of the most promising technologies applied in microbial identification is the MS [42,43**]. ESI and MALDI ionization techniques have introduced the MS in this field. MALDI-TOF MS and PCR/ESI MS offer results on bacterial identification (species, subspecies and lineage levels), bacterial virulence factors (as toxins) [44,45] and antibiotic resistance [46,47**]. The first obtains results in 1–2 min per sample starting from crude bacteria [48] or even directly from some types of specimens [49], and the second is currently the fastest diagnostic MS-based method, with results in 4–6 h and directly from a clinical sample bypassing bacterial cultures, respectively [24].

Bacterial identification through the analysis of whole cells by MALDI-TOF MS has been demonstrated in the past, but its use in the clinical laboratory is recent. The basis of this approach is the generation of a spectral profile of bacterial proteins. The analysis of these proteins does not require biochemical reactions, making it a more rapid and lower-cost identification tool than traditional methods [50]. In several studies it has been tested in large batches of bacterial isolates. The accuracy of the identification is higher at the genus level and lower, but acceptable, at the species level [48,51*]. In this platform the integrity and comprehensiveness of the databases used for the spectrum analysis are
fundamental for the accuracy of identification results [52]. In a study of more than 1000 bacterial isolates, MALDI-TOF MS showed a sensitivity of 95% and a specificity of 84.1% for the identification of samples compared with conventional systems [43**]. More recently, a work on 767 strains reported a correct identification in 96.2% of the isolates comparing the results with reference to the results obtained with a phenotypic automated system and 16S rRNA gene sequencing. Bearing in mind the bacterial groups, a good identification was observed, overall to the genus level, with 98.2% of Enterobacteriaceae, 94.7% of the nonfermenter Gram-negative bacilli, 94.5% of the staphylococci and 97.2% of streptococci [48]. Specifically on nonfermenter Gram-negative bacilli identification, an evaluation study of two known MALDI-TOF MS databases by comparison with conventional biochemical or molecular methods described, in 200 clinical isolates of cystic fibrosis, the identification agreement to the species, complex and genus level, as a whole reaching up to 97%, and the agreement to the species level with the reference method was similar for both systems (72.5%) [51*]. Moreover, in respiratory infections MALDI-TOF MS has been used on isolated strains of throat swab samples of community-acquired pneumonia patients, and 99.1% were successfully identified [52].

One of the limitations of this MS platform in the diagnosis of HAP/VAP could be related to the correct identification of various bacterial species when a mixture of bacteria is used. This implies that its use in direct sputum samples must be carefully measured in view of how frequently a polymicrobial flora is present. This has not been tested yet in the detection of pathogens in unprocessed respiratory samples [26**].

The latest option in MS-based molecular diagnostics is PCR/ESI MS (PLEX-ID Technology, Abbot Molecular) based on the combination of PCR and ESI MS. The premise of bacterial identification by this approach is the measurement of the mass of each amplicon and the determination of its nucleotide base. The results of the base sequences are compared with a database comprising known sequence data of a broad range of pathogens [53]. This approach allows the detection of bacteria, viruses, fungi and parasites, and even the detection of unforeseen pathogens or the discovery of new ones [53]. This technology has been used in viral upper respiratory tract infections [23*] and for the detection of biological markers of inflammation in pneumonia [54,55]. Recently, it has been described in the identification of respiratory pathogens and mechanisms of antibiotic resistance [54–58]. It is an adequate technology for the identification of bacterial species and, as it is semi-quantitative, can determine a relative intensity of microorganisms [59**]. It stands as an option for the future diagnosis of HAP/VAP. Also, it gives information on the clonal relatedness of strains, which is of epidemiological value for surveillance and infection-control practices [58]. Among its disadvantages are the potential contamination of the workspace and samples (due to the fact that it is an open platform), the difficulty in the assessment of results of polymicrobial specimens and the high cost of instrumentation [59**].

The ability of PCR/ESI MS to identify P. aeruginosa and S. aureus has been described by Eshoo et al. [60], who incidentally detected these organisms directly in whole-blood specimens. They used primers targeting 16S and 23S rRNA genes, designed for the amplification of these regions from all bacteria. These ribosomal primers detected these pathogens. The results were confirmed by culture.

**DETECTION OF ANTIMICROBIAL RESISTANCE**

In this field, recent publications have reported molecular-based detection of antimicrobial-resistance determinants, mainly about the identification of β-lactamase genes. Microarray-based assays such as Check KPC/extended-spectrum β-lactamase (ESBL) (Check-Points) have been used to identify β-lactamase (bla) genes present in microbial isolates. They do not give information about bacterial identification but provide it on antimicrobial resistance mechanisms. This assay uses a PCR amplification and hybridization to a low-density DNA microarray [26**]. Several works highlighted the ability of this assay to detect bla genes corresponding to TEM, SHV, CTX-M and KPC β-lactamases, and reported similar sensitivities and specificities up to 100% [56,61*]. Recently, a new one has been evaluated (also a Check-Points product) that employs a greater number of specific markers to identify bla genes of ESBL (TEM, SHV and CTX-M), plasmid-mediated cephalosporinase (pAmpC) and carbapenemases (KPC, OXA-48, VIM, IMP and NDM) in 187 well-characterized Gram-negative bacilli carriers of different bla genes, and sensitivities and specificities of 100% for the most tested genes have been observed [56,61*]. Their use on a daily routine is limited by the low prevalence of carbapenemase-producing isolates and their high price [61*].

Other studies on antimicrobial-resistance sequence detection have used multiplex real-time PCR and intercalating fluorophores (i.e. SYBR Green) [62] or hybridization probes (i.e. molecular beacons) [23*] to identify multiple K. pneumoniae
carbapenemase gene variants (blaKPC) [23*,62]. Chen et al. [23*] describe a novel assay to detect and classify all variants currently known (blaKPC2 to blaKPC11). This approach allowed them to get results easily within 3 h. It proved to be a robust method that showed high sensitivity (reporting a detection limit of 5–40 DNA copies per reaction and four colony-forming units per reaction laboratory-prepared sample) and specificity (with no cross-reactivity observed using bacterial DNA from other species). It also offers the possibility to be modified depending on whether novel variants of blaKPC emerge.

Mass spectrometry-based methods have been tested in the detection of drug resistance. Recently MALDI-TOF MS-based β-lactamase assay was described as a novel approach that can be helpful to detect resistance against different β-lactam antibiotics, including carbapenems. It requires, as a prior step to the spectrometry analysis, the incubation of the microorganism with an antibiotic β-lactam and detects the mass shift resulting from the hydrolysis of the central β-lactam ring. Other mechanisms, such as porins or up-regulation of the efflux pumps, were not detected. Results in relation to the β-lactamase activity can be available in a few hours; however, further validation of this assay will be necessary before it is applied for diagnostic use. The information given can also be helpful in the development of new β-lactam antibiotics and β-lactamase inhibitors [47**].

The use of PCR/ESI MS has also been described in the analysis of specific genetic elements associated with antibiotic resistance and virulence. In S. aureus it has detected the presence of mecA gene, describing a good correlation with MRSA phenotype, and identified genes that encoded toxins such as Panton-Valentine leukocidin, correlating the results with those obtained by PCR of this toxin [58]. In Enterobacteriaceae, Endimiani et al. [56] used this approach for the detection and identification of blaKPC genes. They used 74 strains of K. pneumoniae, E. coli, Enterobacter cloacae of clinical isolates and E. coli DH10B; the latter was control strain constructed, possessing a blaKPC gene. All tested strains were correctly identified at the species level and had a sensitivity and specificity of 100% in the detection of the antimicrobial-resistant Gram-negative bacilli determinants. Also, in nonfermenter Gram-negative bacilli, Hujer et al. [57], using a strain collection of well-characterized multidrug-resistant Acinetobacter species, describe that PCR/ESI MS identifies accurately the specific mutations which determine the resistance to quinolones contained in gyrA and parC genes, remarking the exact relation between phenotypic in-vitro resistance patterns and target sequences identifying the correct phenotype in 98.7% of the isolates. They mention quantitative results by comparison of peak heights with internal calibration PCR standards. The usefulness of this platform has also been proven in the genotyping of Acinetobacter species and comparison with pulsed field gel electrophoresis to establish clonal relations [57,63].

When assessing outcomes related to antimicrobial resistance, it is important to bear in mind that the molecular methods offer specific antimicrobial sensitivities when including these resistance genes or related sequences. However, the antimicrobial susceptibility test standard has to continue being performed from the strain of the microorganism isolated from culture [17**].

BACTERIAL QUANTIFICATION

Among available technologies, the real-time PCR is well known for its ability to quantify targets [64] and probably PCR/ESI MS may provide experiences in microbial load. Technically, quantitative real-time PCR is performed by the addition of standards which have known or calibrated levels of target nucleic acid [65], whereas PCR/ESI MS does it by comparing height of spectral peaks with the calibration standard, and the amplified nucleic acids of the microorganism enables determination of the concentration [53]. So far, publications about bacterial quantification agree to use real-time PCR, but probably in the next years PCR/ESI MS will become a common tool for microbial quantification.

Specific bacterial pathogens related to HAP/VAP have been subjected to quantitative studies by molecular techniques. Detection and quantification of S. aureus, P. aeruginosa and S. pneumoniae along with other respiratory pathogens have been tested by real-time PCR assay in sputum samples from patients with chronic obstructive pulmonary disease during stable periods and acute exacerbations of the disease [66*]. Quantitative PCR results obtained by applying several thresholds in the PCR data were compared with the culture, showing a high sensitivity and without observed cross-reactivity between species [67].

A recent and interesting work has studied the bacterial load of pathogens found in the condenser humidifier filter of the ventilator circuit to observe the degree of correlation with bronchoalveolar lavage quantitative procedures and clinical suspicion of VAP. Bacterial DNA present in patient samples was identified by amplification and sequencing of the 16S ribosomal DNA gene and they elaborated standard curves for real-time PCR determination of bacterial load from 16S ribosomal DNA samples. A good
correspondence was found between the cultures and PCR findings both in pathogen identification and when providing quantification greater than 104 bacteria. Variations in the quantification of pulmonary pathogens assessed by culture and a rapid molecular-based method and the clinical course of the respiratory process may alert about an increased risk of pneumonia before the development of clinical signs [68-**].

In bacterial pneumonia results of pneumococcal load in clinical specimens (including sputum samples) have been reported to help establish the cause [69] or in association with disease severity [70,71*]. In a study of 304 patients with community-acquired pneumonia a duplex real-time PCR was used to determine pneumococcal load in serum, urine and sputum specimens. The authors found association between the increase of pneumococcal load in serum and probably in urine with disease severity but not with sputum. They suggest the colonization or coincidental carriage status could be clarified by future studies on bacterial load in respiratory samples [71*].

Molecular-based diagnostic methods currently available are helpful in detecting multiple bacterial pathogens simultaneously, including the most frequent cause of HAP/VAP, but the possibility of quantitative results defining a threshold for classification, such as a colonizer or as an invasive pathogen, could be an arduous task considering the pathogenesis of this clinical entity.

**CONCLUSION**

Significant progress exists on the development and improvement of molecular-based methods feasible to be applied to the diagnosis of lower respiratory tract infection. Multiplex assays, user-friendly formats, results in a few hours, high sensitivity and specificity in pathogen identification, detection of antibiotic resistance genes and target quantification, among others, are some of the contributions of novel molecular-based diagnosis approaches. Some of the available rapid diagnosis platforms are directed to detect viruses and bacteria in the community-acquired respiratory tract infection, and could be potentially useful in HAP/VAP. Detection of pathogens in unprocessed samples and determination of the microbial load by quantitative tests are some of the challenges of molecular diagnosis in HAP/VAP.

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**Conflicts of interest**
There are no conflicts of interest.

**REFERENCES AND RECOMMENDED READING**

Papers of particular interest, published within the annual period of review, have been highlighted as:

* of special interest
** of outstanding interest

Additional references related to this topic can also be found in the Current World Literature section in this issue (pp. 570–571).

7. This study describes the pathogens most likely to cause bacterial HAP/VAP based on SENTRY Antimicrobial Surveillance Program (1997–2008) and reviews recent medical and microbiology literature.
18. Recent guidelines for the management of pneumonia in adult patients.


In this recent and comprehensive review the author discusses key issues to be considered in the development of new molecular amplification methods for the rapid diagnosis of bacterial respiratory infection. It describes potential targets for molecular assays by respiratory syndromes.


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This study describes the advantages and limitations of a microarray-based assay for the detection of extended-spectrum β-lactamases and carbapenemases from bacterial cultures.


A recent review on the use of molecular methods to detect viral and bacterial pathogens in exacerbations of chronic obstructive pulmonary diseases.


In this recent and interesting study the authors provide preliminary data regarding the possible use of quantitative PCR in samples of the hygroscopic condenser humidifier filter of the ventilator circuit as detection method for pulmonary pathogens.


A prospective study in which the association of the bacterial load in different types of clinical specimens with severity of community-acquired pneumonia in adult patients was investigated. Bacterial load in serum, but not in sputum, was associated with severity.