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Molecular diagnostic techniques in respiratory infections

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ABSTRACT

The diagnosis of community-acquired pneumonia and hospital-acquired pneumonia is based on clinical, radiological, and microbiological findings. Conventional methods are based on the culture of respiratory samples, including sputum, endotracheal aspirate, bronchoaspirate, and bronchoalveolar lavage, followed by strain identification and antibiotic susceptibility testing. The gold standard for microbiological diagnosis of pneumonia remains the culture-based methods. These take > 24 h to identify the bacteria and 48 h to provide antibacterial susceptibility. Culture is insensitive, only detecting a pathogen in 23-40% of patients with clinically diagnosed pneumonia and an even smaller proportion after the administration of antibiotics. The utilization of multiplex panels for the simultaneous detection and identification of respiratory pathogens, including the detection of resistant determinants, can streamline testing procedures and enhance both the sensitivity and speed of diagnosis compared to traditional. In this review, molecular testing is currently available, and the potential future applications of next-generation sequencing are developed.

Keywords: Diagnostics. Respiratory tract infections. Molecular diagnosis. Conventional diagnosis.

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INTRODUCTION

Respiratory tract infections (RTIs) are infections that affect the respiratory system, which includes the airways, lungs, and related structures. They are generally classified into upper respiratory tract infections and lower respiratory tract infections. The most important respiratory infection is pneumonia[1]. Overall, two types of pneumonia are considered: (i) Community-acquired pneumonia (CAP), which is acquired outside of the hospital, and (ii) hospital-acquired pneumonia (HAP), which is the pneumonia that occurs 48 h or more after hospital admission and is not present at the admission time. This can be classified into three entities: 1) Hospital pneumonia; 2) Hospital pneumonia that subsequently requires ventilation, and 3) Pneumonia-associated with mechanical ventilation (VAP). When the etiology of these two types of pneumonia (CAP and HAP) is analyzed (Table 1), some differences can be found: *a*) Despite the use of vaccines against *Streptococcus pneumoniae* and *Haemophilus influenza,* they remain together with *Mycoplasma pneumoniae,* the most frequent bacteria causing CAP. Although *S. pneumoniae* can also be found as a causative agent of HAP, the main bacteria causing this type of pneumonia are mainly Gram-negative bacilli (GNB) and *Staphylococcus aureus*[1,2]. There are no significant differences in the type of microorganisms that cause the three types of HAPs, with the majority of these infections caused by GNB often with high resistance to antibiotics, which makes it difficult to select the initial antimicrobial treatment; *b*) Viruses such as influenza virus, respiratory syncytial virus (RSV), and SARS-CoV-2 are common as a cause of CAP but less prevalent in HAP; and *c*) Although the overall prevalence of pneumonia caused by fungi is low, many fungi causing pneumonia, mainly in immunocompromised patients, are endemic to specific geographic areas, whereas others such as *Aspergillus, Candida,* and *Pneumocystis* are ubiquitous (Table 1)[1,2]. It is important to highlight that 10% of the patients in an ICU present pneumonia, and 66% of them have VAP and *Pseudomonas aeruginosa*, the most frequently isolated microorganism causing it[2].

An adequate microbiological diagnosis of RTIs, both CAP and HAP, is important not only for patient management because it helps to define the presence of the infection and the etiological agent to provide the appropriate treatment but also, in the case of bacterial pneumonia, to reduce the selective pressure of antibiotic use, and the unnecessary use of broad-spectrum antibiotics, as well as the costs and side effects. The impact of delayed appropriate antibacterial therapy on clinical outcomes, increased healthcare cost, and mortality in patients with HAP has been reported in several studies[3-7].

It is worth mentioning that in 2024, the World Health Organization (WHO) updated a list of bacterial pathogens of public health importance to guide research, development, and strategies to prevent and control antimicrobial resistance[8]. The study followed a succession of prioritization criteria, including mortality, incidence, non-fatal burden, transmissibility, trend of resistance, preventability, treatability, and current antibacterial pipeline. The report ends up with a three-group classification based on the priority level, with a total of 14 pathogens heading the top positions (Fig. 1). Over the past decade, the

Table 1. Main microorganisms causing CAP and HAP

Feature	CAP	HAP
Setting	Acquired outside hospitals	Acquired ≥ 48 h after admission
Common bacteria	*S. pneumoniae*, *H. influenzae*, *M. pneumoniae*	*P. aeruginosa*, *K. pneumoniae*, *S. aureus* (MRSA)
Viral causes	Influenza, RSV, SARS-CoV-2	Less common
Fungal causes	*P. jirovecii* (HIV/AIDS)	*Candida*, *Aspergillus* (ICU, immunocompromised)
Antibiotic resistance	Less common	More common (MDR pathogens)

CAP: community-acquired pneumonia; HAP: hospital-acquired pneumonia; MRSA: methicillin-resistant *Staphylococcus aureus*; RSV: respiratory syncytial virus; HIV: human immunodeficiency virus; AIDS: acquired immunodeficiency syndrome; ICU: intensive care unit; MDR: multidrug-resistant.

percentage of antibiotic resistance in GNB has shown a concerning upward trend. Special concern is for carbapenem-resistant *Klebsiella pneumoniae* bloodstream infections, which showed an increase of over 50% in 2023 compared to 2019 among clinical isolates causing bacteremia from European countries[9]. Moreover, a global burden of severe infections due to carbapenem-resistant pathogens focusing on ICU has been reported[10]. In addition, the increase in the resistance to a "new" antibiotic such as ceftazidime-avibactam should be highlighted. In a recent metanalysis, it has been reported that from 2015 to 2020, the resistance proportion was around 5.6% (95% CI 4.1-7.6) of 221,278 GNB isolates and increased to 13.2% (95% CI 11.4-15.2) of 285,978 GNB isolates from 2021 to 2024[11].

Although *S. pneumoniae* and some viruses remain the main cause of CAP, some cases of CAP caused by GNB, such as *P. aeruginosa, K. pneumoniae,* and *Acinetobacter baumannii,* have been reported[12-14]. In a worldwide study including 54 countries, *P. aeruginosa* was found as a cause of CAP in 4.2% of the cases (range 3.1-5.5%), and multi-drug resistance was associated with 1.38% of the cases (range 0.9-2.3%)[15]. Approximately 1-7% of cases of CAP are caused by *K. pneumoniae*, with 5-36% of these being multidrug-resistant (MDR) strains[16]. Although *A. baumannii* is mainly considered a nosocomial pathogen, CAP produced by this microorganism has also been shown, and despite the fact that it does not present a level of resistance that its nosocomial counterpart, it causes severe pneumonia[17].

In HAP patients, the prevalence of GNB ranges between 76.13% and 95.3%[18]. These GNB-causing HAPs include: *P. aeruginosa, Escherichia coli, K. pneumoniae, Enterobacter spp. A. baumannii.* Multi-drug resistance, extended-drug resistance, or even pan-drug resistance is the main concern when it comes to treating these above-mentioned GNB-caused infections[19]. In addition, *S. aureus,* including methicillin-resistant *S. aureus,* is also a frequent cause of HAP. The frequency of each one of these HAP etiological agents can vary according to geographical regions. In a Spanish survey, the distribution of these pathogens was: *P. aeruginosa* (18.2%), *S. aureus* (12.2%), *K. pneumoniae* (6.9%), *and E. coli* (6.7%)[20]. Although the prevalence of *A. baumannii* in this survey was below 1%, it is worth mentioning that this microorganism can reach more than 20% in some Eastern European countries[21].

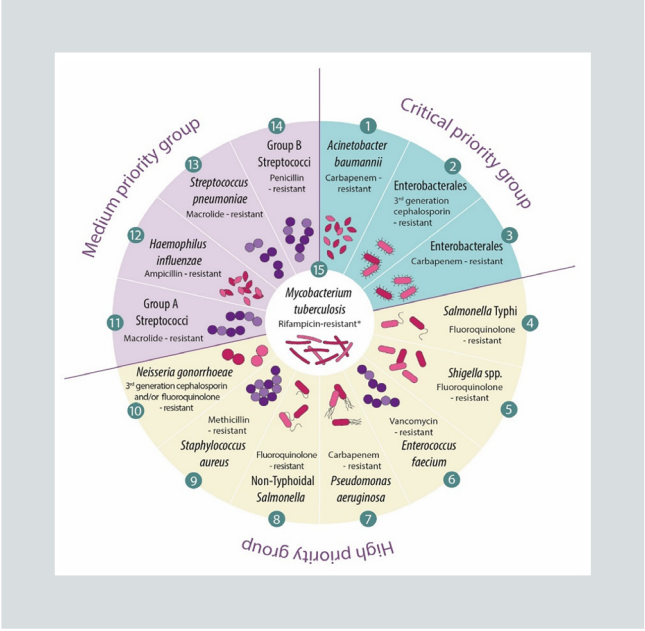

Figure 1. World Health Organization priority pathogen list to guide research, development, and strategies to prevent and control AMR and classification criteria into medium, high, and critical groups *(figure kindly provided by Dr. Natalia Roson from her PhD thesis).*

In a study investigating the clinical outcomes associated with initial antibiotic therapy to treat patients with pneumonia, it was shown that initial antibiotic therapy failure was recorded in 72.5% of patients and was significantly associated with the isolation of a MDR pathogen[22]. In other studies, multidrug-resistant Gram-negative bacilli (MDR-GNB) infection was among the factors associated with mortality in patients with HAP[23,24].

All the above-mentioned highlights the significant challenge posed by antibiotic-resistant GNB in pneumonia, emphasizing the need for effective treatment strategies. To achieve this goal, a rapid diagnosis is crucial to provide the most adequate antimicrobial treatment.

The diagnosis of CAP and HAP is based on clinical, radiological, and microbiological findings. Conventional methods are based on the culture of respiratory samples, including sputum, endotracheal aspirate, bronchoaspirate, and bronchoalveolar lavage, followed by strain identification and antibiotic susceptibility testing. The gold standard for microbiological diagnosis of pneumonia remains the culture-based methods. These take > 24 h to identify the bacteria and 48 h to provide antibacterial susceptibility. Culture is insensitive, only detecting a pathogen in 23-40% of patients with clinically diagnosed pneumonia and an even smaller proportion after the administration of antibiotics. In addition, antigen detection in urine is useful for *Legionella pneumophila* and *S. pneumoniae* detection. For the microbiological diagnosis of HAP, it is recommended to perform a qualitative or quantitative culture (preferable) of respiratory secretions or to use techniques based on the detection of nucleic acids.

The utilization of multiplex panels for the simultaneous detection and identification of respiratory pathogens, including in some of them the detection of resistant determinants, can streamline testing procedures and enhance both the sensitivity and speed of diagnosis compared to traditional methods such as culture[25,26]. Polymerase chain reaction (PCR) for a large number of viruses and a few bacteria, along with atypical bacteria, are normally included in syndromic panels to diagnose CAP, whereas, for HAP, they mainly include a plethora of bacteria, few viruses, and detection of genes encoding resistant determinants. In the following sections, the molecular testing currently available, including the potential future application of next-generation sequencing (NGS), is developed.

Table 2. Commercial multiplex polymerase chain reaction panels for HAP

Name	Methods	Detected pathogens	Viruses	Bacteria	Resistant genes	Time to results (hours)
Filmarray Pneumonia Plus**	Nested multiplex PCR + analysis melting	18 bacteria; 9 viruses	Inf A/B; Adv; CoV; PiV; RSV; hMPV; RV/EV	*Ac/b; Ecl; Ec; Hi; Ka; Ko; KpC; Mc; P; Pa; Sm; Sa; Sag; Sp; Spy; Lp; Mp; Cp*	(8) CTX-M; KPC; NDM; Oxa48-like; VIM; IMP; mecA/mecC and MREJ	1
Unyvero Pneumonia HPN*	RT-PCR + detection by array hybridization	20 bacteria; 1 fungus		*Sa; Sp; Cf; Ec; Ecl; Ka; P; KpC; Ko; Kv; Sm; Mm; Mc; Pa; AbC; Sma; Lp; Pj; Hi; Cp; Mp*	(17) ermB; mecA; mecC; mutations at gyrA83 and gyrA87; CTX-M; IMP; KPC; NDM; Oxa-23; Oxa-24/40; Oxa-48; Oxa-58; sul1; SHV; TEM; VIM	4

*Integrated system.
**Non-integrated system.
AdV: adenovirus; CoV: SARS-CoV; EV: enterovirus; hMPV: human metapneumovirus; Inf: influenza virus; PiV: parainfluenza virus; RSV: respiratory syncytial virus; RV: rhinovirus; Ac/b: *Acinetobacter calcoaceticus-baumannii* complex; Cp: *Chlamydia pneumoniae*; Cf: *Citrobacter freundii*; Ecl: *Enterobacter cloacae*; Ec: *Escherichia coli*; Hi: *Haemophilus influenzae*; Ka: *Klebsiella aerogenes*; Ko: *Klebsiella oxytoca*; KpC: *Klebsiella pneumoniae* complex; Kv: *Klebsiella variicola*; Lp: *Legionella pneumophila*; Mc: *Moraxella catarrhalis*; Mm: *Morganella morganii*; Mp: *Mycoplasma pneumoniae*; P: *Proteus spp.*; Pj: *Pneumocystis jirovecii*; Sm: *Serratia marcescens*; Sa: *Staphylococcus aureus*; Sma: *Stenotrophomonas maltophilia*; Sag: *Streptococcus agalactiae*; Spy: *Streptococcus pyogenes*; Sp: *Streptococcus pneumonia*.

MOLECULAR TESTING BASED ON PCR

Notwithstanding that a molecular technique of choice has not been defined yet, rapid PCR-based methods seem to be the most advantageous in diagnosing common CAP and HAP. Due to their accuracy in targeting pathogens and the robustness of results, the Infectious Diseases Society of America and the American Thoracic Society emphasize the need for further research into new PCR-based diagnostic tools for pneumonia[27]. Similarly, ERS/ESICM/ESCMID/ALAT guidelines state that the introduction of PCR in diagnostic routine represents a useful implementation to detect multiple viral and bacterial pathogens, as well as antimicrobial resistance genes (ARG), in < 4 h[28,29]. Commercial syndromic panels are available for both HAP and CAP. Given the fact that HAP origin is linked to nosocomial settings, panels targeting a broader spectrum of ARGs are recommended[30]. Conversely, CAP's diagnostic mainly focuses on viruses and bacterial agents such as *S. pneumoniae* and atypical bacteria[28]. In pediatric patients, real-time multiplex PCR (rt-mPCR) panels could support and confirm a clinical suspicion of VAP. Considering the variety of causative agents and the fact that pneumonia causes 15.6% of mortality in children under 5 years, the introduction of syndromic panels could decrease the mortality and enable timely antibiotic treatment initiation or discontinuation[31]. In this context, the rt-mPCR FilmArray Pneumonia panel (BioFire Diagnostics, Salt Lake City, UT) has shown high sensitivity (> 94.0%) and negative predictive value (> 75.0%) compared to the microbial culture. This could suggest a potential change in antibiotic treatment in 48-84% of cases (Table 2)[31,32]. Likewise, to reduce CAP morbidity and mortality in infants and children, the Pediatric Infectious Diseases Society and the Infectious Diseases Society of America recommend PCR for suspected viral respiratory infections[33]. An interesting study applied the FilmArray Pneumonia panel and the Respiratory Panel 2.1 (BioFire Diagnostics, Salt Lake City, UT) to upper respiratory samples from pediatric CAP cases achieving sensitivities of 87% and 78%,

Table 3. Commercial multiplex polymerase chain reaction panels for CAP

Name	Methods	Detected pathogens	Viruses	Bacteria	Time to results (hours)
Allplex™ Respiratory Panel 3**	Real-time RT-mPCR	5 viruses	BoV 1/2/3/4; CoV 229E/NL63/ OC43; RV		4
Allplex™ Respiratory Panel 1**	Real-time RT-mPCR	7 viruses	Inf A/A-H1/A-H1pdm09/A-H3/B; RSV A/B		4
Allplex™ Respiratory Panel 2**	Real-time RT-mPCR	7 viruses	AdV; EV; hMPV; PiV 1/2/3/4		4
Allplex™ Respiratory Panel 4**	Real-time RT-mPCR	7 bacteria		*Bpp; Bp; Cp; Hi; Lp; Mp; Sp*	4
LIAISON PLEX® Respiratory Flex Assay**	Automated PCR	5 bacteria; 14 viruses	AdV; CoV; hMPV; inf A/H1/ H3/B; PiV 1/2/3/4; RV/EV; RSV A/B; CoV2	*Bh; Bpp; Bp; Cp; Mp*	2
TrueMark™ Respiratory Panel 2.0; TaqMan™ Array card**	Simultaneous real-time PCR by microfluidic wells system	10 bacteria; 1 fungus; 24 viruses	AdV; BoV; CoV 229E/HKU1/ NL63/OC43; EV pan/D68; hMPV; PiV 1/2/3/4; PeV; RSV A/B; RV 1/2; HHV3; HHV4; HVV5; HVV6; InfA/B; Inf A/ H3; Inf A/H1-2009; MV; MERS-CoV; MuV; CoV; CoV2 S/N	*Bb; Bpp; Bp; Cp; Hi; KpC; Lp; Mc; Mp; Sa; Sp; Pj*	5
Respiratory Finder SMART22**	MLPA Capillary electroph.	18 viruses; 4 bacteria	Inf A/H1pdm09/B; RSV A/B; AdV; CoV 229E/OC43/NL63/ HKU1; hMPV; PiV 1/2/3/4; RV/EV; EV; BoV; MERS; CoV2	*Mp; Lp; Cp; Bp*	4
BioFire® Respiratory 2.1 panel *plus**	Nested multiplex PCR + analysis melting	19 viruses; 4 bacteria	Inf A/H1/H3/H1-2009/B; SRV; AdV; CoV 229E/OC43/NL63/ HKU1; hMPV; PiV 1/2/3/4; RV/EV; MERS-CoV; CoV2	*Mp; Cp; Bp; Bpp*	1
QIASTAT-DX Resp. Panel*	Multiplex RT-PCR	19 viruses; 3 bacteria	Inf A/H1/H3/H1-2009/B; SRV; AdV; CoV 229E/OC43/NL63/ HKU1; hMPV; PiV 1/2/3/4; RV/EV; BoV; CoV2	*Mp; Lp; Bp*	1
Anyplex™ II RV16 Detection	Multiplex RT-PCR	(2 panels) 16 viruses	AdV; inf A/B; PiV1/2/3/4; RV; BoV 1/2/3/4; CoV 229E/NL63/ OC43; EV; hMPV; RSV A/B		6-7
xTAG Resp.Vir. Panel**	Microarrays in suspension Flow cytomet. Detection	19 viruses	Inf A/H1/H3/H5/B; SRV A/B; AdV; CoV 229E/OC43/NL63/ HKU1; hMPV; PiV 1/2/3/4; RV/EV; BoV		6.5-8
NxTAG® Respiratory Pathogen Panel (RPP) v2*	Integrated multiplex PCR and bead hybridization.	19 viruses; 2 bacteria	AdV; CoV 229E/HUK1/NL63/ OC43; hMPV; Inf A/H1/ H1pdm09/H3/B; PiV 1/2/3/4; RSV A/B; RV/EV; CoV2	*Cp; Mp*	3
ePLEX Resp.Vir. Panel RP2**	Microarray + electrochemical detection	21 viruses; 2 bacteria	Inf A/H1/H3/H1-2009/B; SRV A/B; AdV B/E/C; CoV 229E/ OC43/NL63/HKU1; hMPV; PiV 1/2/3/4; RV/EV; CoV2	*Mp; Cp*	6-8

(Continues)

Table 3. Commercial multiplex polymerase chain reaction panels for CAP *(continued)*

Name	Methods	Detected pathogens	Viruses	Bacteria	Time to results (hours)
Fast Track RP33**	RT-PCR with labeled probes	21 viruses; 11 bacteria; 1 fungus	Inf A/B/C; SRV; AdV; CoV 229E/OC43/NL63/HKU1; hMPV; PiV 1/2/3/4; RV; EV; BoV; PcV; CMV;	*Bp; Mp; Cp; L; Pj; Sp; HiA; HiB; Sa; Mc; Kp; S*	3
CLART Pneumovir 2**	Low-density microarrays CLART® detection	22 viruses	Inf A/H1/H3/H1-2009/B/C; SRV A/B; AdV; CoV OC43/ NL6/229E; hMPV A/B; PiV 1/2/3/4a/4b; RV; BoV; EV		9

*Integrated system.
**Non-integrated system.
AdV: adenovirus; BoV: human bocavirus; CMV; cytomegalovirus; CoV: SARS-CoV; CoV2: SARS-CoV-2; EV: enterovirus; hMPV: human metapneumovirus; HHV: human herpes virus; HVV: hidden valley virus; Inf: influenza virus; MuV: mumps virus; MV: measles virus; PcV: Parechovirus; PiV: parainfluenza virus; RSV: respiratory syncytial virus; RV: Rhinovirus; Bb: *Bordetella bronchiseptica*; Bpp: *Bordetella parapertussis*; Bp: *Bordetella pertussis*; Cp: *Chlamydia pneumoniae*; Hi: *Haemophilus influenzae*; *Kp: Klebsiella pneumoniae*; *KpC: Klebsiella pneumoniae complex*; *L: Legionella*; Lp: *Legionella pneumophila*; Mc: *Moraxella catarrhalis*; Mp: *Mycoplasma pneumoniae*; Pj: *Pneumocystis jirovecii*; S: *Salmonella*; Sa: *Staphylococcus aureus*; Sp: *Streptococcus pneumoniae.*

respectively. This led to a robust diagnosis, targeted therapy, and improved infection control (Table 3)[34].

More broadly, the extended time-to-result and the low sensitivity of conventional microbiological testing do not grant a prompt de-escalation, even in adult patients. In contrast, rt-mPCR panels offer a long-term solution by reducing the turn-around time, increasing the reliability of results, and overcoming the limitation of an absent or poor bacterial growth, particularly in patients receiving antibiotic treatment[35]. A randomized control trial to investigate the BioFire FilmArray Pneumonia Panel compared to the standard of care testing on the antibiotic in 1,152 hospitalized patients with suspected pneumonia has recently been carried out. The authors found that the incorporation of the BioFire FilmArray pneumonia panel in the diagnostic algorithm might lead to faster antibiotic escalations, including for Gram-negative or Gram-positive bacilli, and faster antibiotic de-escalations directed at Gram-positive bacteria[36].

A critical clinical setting is represented by intensive care unit patients suffering from HAP or VAP (5-40% of cases), where the estimated mortality rate ranges from 10% to 50%[37]. Patients receive empirical broad-spectrum antibiotic treatment while awaiting microbial culture results. In the INHALE WP3 trial, lower respiratory tract samples were analyzed with the FilmArray Pneumonia *Plus* panel to improve antibiotic stewardship. An absolute tailored therapy was achieved in 21% of patients[37]. However, the impact of syndromic panels on antibiotic treatment modification requires further investigation. Negative PCR results alone cannot be considered sufficient for changing treatment, as the absence of common ARG does not necessarily indicate antibiotic susceptibility[31,37]. In another study by Poole et al.[38], investigating the impact of molecular point of care (Filmarray Pneumonia Plus Panel), it was found that more pathogens were identified using this approach than conventional methodology 71 versus 51%, respectively. In addition, HAP causative agents such as *Burkholderia cepacia* complex and *Haemophilus parainfluenzae* are not typically

included, posing a risk of undiagnosis or underdiagnosis. Similarly, non-tuberculous mycobacteria cause respiratory infections in immunocompromised patients and require additional PCR-based testing for accurate detection[39]. The study of Abelenda-Alonso et al.[40] investigated the use of the FilmArray Pneumonia *Plus* panel for CAP diagnosis to improve antibiotic stewardship in hospitalized patients. The authors did not obtain significant benefits from introducing the panel into the routine diagnostic, as they observed no reduction in treatment duration and mortality when CAP was diagnosed by PCR.

A significant step forward is the detection of ARGs directly from respiratory samples using commercialized kits designed for other biological matrices. The Xpert Carba-R kit (Cepheid, Sunnyvale, California, USA), a multiplex PCR to detect carbapenemase-producing bacteria, was successfully applied to bronchoalveolar lavage (BAL) fluids. The PCR test achieved 100% agreement to detect bla_{KPC}, bla_{NDM}, bla_{VIM}, bla_{OXA-48}, and bla_{IMP-1} genes in a concentration range of 10^4-10^2 CFU/ml of *K. pneumoniae, E. coli,* and *Enterobacter cloacae*[41]. Further research is needed to implement rt-mPCR panels to help withdraw the administration of inappropriate antibiotics in the future and organize new treatment algorithms[27]. PCR's reliability for diagnostics use was confirmed during the COVID-19 era, and after its end, the detection of winter pneumonia respiratory viruses' detection such as Flu A, Flu B, and RSV continued to benefit from the technological implementations introduced during that period[42]. Integrated systems, such as the Xpert Xpress CoV-2/Flu/RSV *plus* (Cepheid, Sunnyvale, California, USA), a triplex PCR, were introduced as point-of-care testing, demonstrating a sensitivity of around 100%. These test's high-efficiency results may be attributed to the detection of ten viral genetic targets in one single channel (Table 4)[43,44]. In addition, the triplex RT-PCR integrated system STANDARD M10 Flu/RSV/SARS-CoV-2 (SD BioSensor, Suwon, Korea) was launched on the market, and its performance was compared with other available tests. However, studies by Jensen et al.[43] and Hong et al.[45] highlighted a lower sensitivity (47–67%) and an overall higher Ct range compared to the Xpert Xpress system. Nevertheless, the M10 system was found to be as sensitive as the Allplex SARS-CoV-2 and Allplex Respiratory Panel 1 (RP1) (targeting Flu A/H1/H1pdm09/H3, Flu B, RSV A/B) (Seegene Inc., Seoul, Korea) with an overall agreement of 94.6%, a positive predictive agreement of > 99.7 and negative predictive value agreement of > 98.4 for the three targets[46]. Further efforts should focus on detecting atypical and difficult-to-identify pathogens, such as *M. pneumoniae* and *Chlamydophila pneumoniae* causing respiratory infections, especially in children. In these cases, an in-house PCR setup provides an alternative diagnostic tool with high sensitivity and specificity[47]. In past years, the relevance of *M. pneumoniae* and *C. pneumoniae* infections in CAP has prompted the evaluation of PCR strategies aimed at simultaneously targeting the 16S rRNA gene to differentiate between the two pathogens and 23S rRNA to detect putative resistance mutations related to macrolides in *M. pneumoniae*[48].

In 2023, Sugimoto et al.[49] successfully standardized a duplex real-time RT-PCR to diagnose two major human metapneumovirus subgroups (A and B) in a single reaction.

Table 4. Commercial singleplex, duplex, and triplex polymerase chain reaction panels for RTIs

Name	Methods	Detected pathogens	Microorganism	Time to results (hours)
TaqPath COVID-19 CE-IVD RT-PCR kit**	Real-time RT-PCR	1 virus	CoV2	2
cobas SARS-CoV-2 Test**	Single tube real-time PCR	1 virus	CoV2	20 min
EasyQ SARS-CoV-2 Test**	Real-time RT-PCR	1 virus	CoV2	2
Xpert® Xpress SARS-CoV-2*	RT-PCR	1 virus	CoV2	45 min
Xpert® Xpress CoV-2 plus*	RT-PCR	1 virus	CoV2	20 min
QuantStudio 5 Real-Time PCR System test flu A e B*	Real-time RT-PCR	1 virus	inf A/B	36 min
TaqMan Respiratory Virus Kits**	Real-time RT-PCR	1 virus	RSV	1
TaqPath Menu I GeneProof Adenovirus PCR Kit**	Real-time PCR	1 virus	AdV	1
TaqPath Menu I GeneProof Enterovirus PCR Kit**	Real-time PCR	1	EV	1
TaqPath Menu I GeneProof Bordetella pertussis/ parapertussis PCR Kit**	Real-time PCR	1	*Bp; Bpp*	1
TaqPath Menu I GeneProof Chlamydia pneumoniae PCR Kit**	Real-time PCR	1	*Cp*	1
TaqPath Menu I GeneProof Mycoplasma pneumoniae PCR Kit**	Real-time PCR	1	*Mp*	1
TaqPath Menu I GeneProof Mycobacterium tuberculosis PCR Kit**	Real-time PCR	1	*Mt*	1
Xpert® Xpress Strep A*	Real-time PCR	1	GAS	18 min
iQ-Check Legionella Real-Time PCR Kits**	Real-time PCR	1	*L; Lp*	4
Accula™ CoV2 Rapid PCR Test**	Single-use RT-PCR cassette (CLIA)	2	CoV2; Inf A/B	30 min
Xpert® Xpress Flu/RSV plus*	RT-PCR	2	RSV; Inf A/B	20 min
cobas® CoV2 & Influenza A/B & RSV Test*	Real-time PCR	3	CoV2; Inf A/B; RSV	20 min
Alinity™ m Respiratory Panel*	Real-time RT-mPCR	3	CoV2; Inf A/B; RSV	2
TaqPath™ COVID-19; FluA/B; RSV Combo Kit**	RT-PCR	3	CoV2; Inf A/B; RSV	1.2
Xpert® Xpress CoV-2/Flu/RSV plus*	Real-time RT-mPCR	3	CoV2; Inf A/B; RSV	30 min
TaqPath™ COVID-19; Flu A/B; RSV Combo Kit**	Real-time RT-PCR	3	CoV2; Inf A/B; RSV A/B	45 min
STANDARD™ M10 Flu/RSV/SARS-CoV-2*	Real-time RT-PCR	3	CoV2; Inf A/B; RSV A/B	1

*Integrated system.
**Non-integrated system.
AdV: adenovirus; CoV2: SARS-CoV-2; EV: enterovirus; Inf: influenza virus; RSV: respiratory syncytial virus; Bpp: *Bordetella parapertussis*; Bp: *Bordetella pertussis*; Cp: *Chlamydia pneumoniae*; GAS: group A *Streptococci*; L: *Legionella*; Lp: *Legionella pneumophila*; Mt: *Mycobacterium tuberculosis*; Mp: *Mycoplasma pneumoniae*.

While different targets were evaluated, the nucleocapsid gene exhibited sufficient diversity between the two subgroups (86.0-87.1%) to be differentiated. Despite the widespread use of syndromic panels, ongoing implementation of single PCR for hypervirulent pathogens such as *K. pneumoniae* can enhance the diagnosis and therapeutic options. Commonly, hypervirulence is inferred from a mucoid layer > 5 mm, but not all hypervirulent *K. pneumoniae* strains exhibit hypermucoid colonies. Gene amplification of *iutA, iroN,*

rmpA, and *peg-344* could guide towards a correct diagnosis. In a 1-year study conducted in Italy, 13 hypervirulent *K. pneumoniae* isolates were detected among 354 samples[50]. A second study by Neumann et al.[51] included the *rmpA*, *rmpA2*, *iutA*, and *magA* genes to diagnose hypervirulence among 100 clinical *K. pneumoniae* isolates, and nine were classified as positive. A similar clinical setting is the one of *Pneumocystis jirovecii*. Since the incidence of *P. jirovecii* pneumonia is increasing in critically ill patients admitted to Intensive Care Units, combining a positive result from a singleplex PCR targeting *P. jirovecii* and detection of (1,3)-β-D-glucan in serum helps distinguish colonization from infection; on the contrary, in case of a negative *P. jirovecii* PCR result, *P. jirovecii*-related pneumonia is unlike. The PCR showed 100% sensitivity and negative predictive value for presumptive and proven pneumonia, 65–85% specificity, and 48-65% positive predictive value. Therefore, combining PCR with biochemical parameters could be pivotal, but a diagnostic strategy needs still to be validated[52]. The scientific community agrees that PCR plasticity continuously leads to the development of novel assays and technology, particularly referred to as respiratory infections, and the role of PCR represents the unchangeable diagnostic point of force.

MOLECULAR TESTS BASED ON LAMP AND OTHER ISOTHERMAL NUCLEIC ACID AMPLIFICATION TECHNIQUES (iNAATs)

Molecular techniques based on PCR are widely used to detect bacteria and fungi, and especially viruses. However, these techniques have some limitations. They require complex equipment and facilities and well-qualified personnel to perform them, limiting their accessibility. More accessible nucleic acid amplification techniques, such as the iNAATs, facilitate respiratory pathogen detection (Table 5). Developed in the 1990s, iNAATs amplify nucleic acids at a constant temperature, eliminating the need for PCR's thermal cycles. This simplifies the process, requiring only a thermoblock or a water bath instead of sophisticated equipment[53].

Several iNAAT variants have been developed for pathogen detection, including Nucleic acid sequence-based amplification (NASBA), which amplifies RNA from RNA or DNA using a retrotranscriptase and a pair of primers[54]; rolling circle amplification (RCA) which replicates circular templates with polymerases mimicking bacterial genome or plasmid replication[55]; recombinase polymerase amplification (RPA) which uses recombinase to bind primers to target sites, eliminating the need for thermal cycling[56]; strand displacement amplification (SDA) which enables synthesis despite encountering double DNA strands[57]; helicase-dependent amplification (HDA) where helicases separate DNA strands for primer binding[58]; and transcription-mediated amplification (TMA) which relies on reverse transcriptase and RNA polymerase to generate multiple RNA copies[59].

Some of these techniques have been extensively studied for detecting respiratory pathogens. NASBA has been studied in the detection of different respiratory viruses, such as rhinovirus, influenza A/B, human parainfluenza 1-4, and RSV giving results very similar to those obtained with RT-PCR[60,61]. During the

Table 5. Respiratory pathogens detected by iNAATs

iNAAT	Detected microorganism	References
Nucleic acid sequence-based amplification	Rhinovirus	60,61,63
	Influenza A/B	
	Human parainfluenza virus	
	Respiratory syncytial virus	
	SARS-CoV-2	
	Mycoplasma pneumoniae	
	Chlamydophila pneumoniae	
	Legionella spp.	
Rolling circle amplification	Influenza A/B	64,65
	SARS-CoV-2	
Recombinase polymerase amplification	Respiratory syncytial virus	56, 66
	SARS-CoV-2	
	Influenza A/B	
	Human metapneumovirus	
	Klebsiella pneumoniae	
	Mycoplasma pneumoniae	
	Haemophilus influenzae	
	Pseudomonas aeruginosa	
	Staphylococcus aureus	
	Streptococcus pneumoniae	
Strand displacement amplification	*Mycobacterium tuberculosis*	67-69
	Influenza A/B	
	Mycoplasma pneumoniae	
Helicase-dependent amplification	SARS-CoV-2	71,72
	Staphylococcus aureus	
	Influenza A/B	
	Respiratory syncytial virus	
	Human parainfluenza virus	
Transcription-mediated amplification	SARS-CoV-2	70,73
	Mycobacterium tuberculosis	
Loop-mediated isothermal amplification	*Mycobacterium tuberculosis*	74, 79-82, 84, 87
	Respiratory syncytial virus	
	Parainfluenza virus	
	SARS-CoV-2	
	Influenza A/B	

(Continues)

Table 5. Respiratory pathogens detected by iNAATs (*continued*)

iNAAT	Detected microorganism	References
	Influenza variants H1N1, H3N2, H5N6, H5N1 and H7N9	
	Rhinovirus	
	Human metapneumovirus	
	Parainfluenza virus	
	Acinetobacter baumannii	
	Pseudomonas aeruginosa	
	Stenotrophomonas maltophilia	
	Klebsiella pneumoniae	
	Escherichia coli	
	Haemophilus influenzae	
	Staphylococcus aureus	
	Streptococcus pneumoniae	
	Enterobacter spp.	
	Mycoplasma pneumoniae	
	Legionella pneumophila	
	Pneumocystis jirovecii	

iNAATs: isothermal nucleic acid amplification techniques.

recent pandemic, it was also used for SARS-CoV-2 detection[62]. In addition, it has been applied to detect bacteria such as *M. pneumoniae*, *C. pneumoniae,* and *Legionella* spp[63].

RCA has been used to detect respiratory viruses such as influenza A/B and coronaviruses, including SARS-CoV-2[64,65]. RPA has also been applied to various coronaviruses, including SARS-CoV-2 and VSR, even combined with CRISPR-Cas12 technology[66]. This technique is also capable of detecting influenza A/B, human metapneumovirus, *K. pneumoniae*, *M. pneumoniae*, *H. influenzae*, *P. aeruginosa,* and Gram-positive bacteria such as *S. aureus* and *S. pneumoniae*[56]. SDA has been studied mainly for *Mycobacterium tuberculosis* detection[67] but is also used for influenza A/B, SARS-CoV-2, and *M. pneumoniae* detection[68,69]. The recent pandemic has increased the interest in the detection of SARS-CoV-2, leading to the use of HDA and TMA for this purpose[70]. HDA is also capable of detecting *S. aureus*, Influenza A/B virus, RSV, and human metapneumovirus,[71,72] and TMA has also been studied as a tool for *Mycobacterium tuberculosis* detection[73].

Loop-mediated isothermal amplification (LAMP)

LAMP stands out among iNAAT variants due to its high inhibitor tolerance, use of a single polymerase, and exceptional sensitivity. Its high specificity and easy result interpretation further enhance its relevance. The WHO has even approved it as an alternative molecular method for tuberculosis detection[74]. Developed in 2000 by Notomi[75], LAMP amplifies a specific region of DNA using *Bst* polymerase isolated

from *Geobacillus stearothermophilus*, an enzyme with high strand displacement activity and heat resistance. The reaction needs four primers targeting six regions; in addition, two more primers can be used to maximize the reaction efficiency[76]. A key advantage of LAMP is the ease of result interpretation through various methods, including turbidity observation, electrophoresis, or color changes using pH indicators such as phenol red, neutral red, and cresol red, as well as dyes such as malachite green, and leucocrystal violet, hydroxynaphthol blue, calcein, Eriochrome black T and GeneFinder™. These dyes visually indicate a positive reaction. Fluorescence is also widely used to visualize LAMP results using dsDNA intercalators such as SYBR Green I, Eva Green, Barberine, and SYTO-9, among others. In addition, it can be designed with primers linked to fluorophores[77].

More complex interpretation systems have been developed, including microsensors, microfluidic microchips, and immunological techniques such as lateral flow immunochromatography, LAMP coupled to ELISA, and detection through nanoparticle-based aggregation detection, to name a few[78].

Among iNAATs, LAMP stands out for its advantages, making it widely used in respiratory pathogen detection. For viruses, it can be coupled to reverse transcription (RT-LAMP) to detect RNA sequences. It has been studied for diagnosing RSV subtypes A and B, the leading cause of hospitalizations in children under five[79]. Some LAMP tests detect viruses in under 30 min and with a LoD close to 1×10^2 copies/mL[80]. LAMP can also detect the parainfluenza virus using multiple detection kits[80]. Studies have shown its potential for diagnosing coronaviruses, including SARS-CoV-2, which aims for rapid results and simplicity for PoCT. Tested samples include saliva, serum, nasopharyngeal and oropharyngeal exudates, and even urine[80].

LAMP has also been used as a screening tool mainly for human metapneumovirus and influenza A/B viruses[80], as well as variants such as H1N1, H3N2, H5N6, H5N1, and H7N9[82]. In some cases, the technique was able to detect < 10 copies/µL[83]. For rhinoviruses, LAMP can detect subtypes A, B, and C, and it has also been used to identify parainfluenza virus subtypes 1 and 3[80]. LAMP has been widely used and validated in different studies for detecting pneumonia-causing bacteria, including *A. baumannii*, *P. aeruginosa*, *S. maltophilia*, *K. pneumoniae*, *H. influenzae*, *E. coli*, *S. aureus*, *S. pneumoniae*, *L. pneumophila* and *M. pneumoniae* [74,84-86].

In the case of fungal respiratory infections, especially in immunocompromised patients, multiple studies have also demonstrated that LAMP can be used as a tool to detect *P. jirovecii*[87].

Various multi-detection kits target key bacteria causing respiratory infections using LAMP.[70,83] This is relevant because it simplifies the diagnosis of these pathogens, besides several resistance variants such as *mec* and bla_{KPC}, $bla_{NDM-1.}$ bla_{IMP-1} group and bla_{VIM} genes have been detected[88].

This highlights the potential of LAMP as a PoCT, making it suitable not only for robust healthcare settings but also for resource-limited environments. Early and accurate diagnosis improves patient prognosis, reduces morbidity and mortality, lowers healthcare costs, minimizes environmental impact by using fewer resources, and helps combat antimicrobial resistance by enabling effective treatment at an earlier stage.

NGS: PRESENT AND FUTURE IN DIAGNOSING LRTIs

NGS has revolutionized the field of infectious disease diagnostics. Unlike conventional microbiological techniques (CMT), which rely on culture, serology, or targeted PCR-based techniques, NGS provides an unbiased, hypothesis-free approach to pathogen identification[89]. This is particularly relevant in the diagnosis of LRTIs, where rapid and accurate identification of causative agents is crucial for effective treatment. Despite its potential, several challenges remain in its implementation as a routine clinical diagnostic tool.

NGS includes various methodologies, such as whole-genome sequencing (WGS), targeted NGS (tNGS), and metagenomic NGS (mNGS)[90]. WGS is primarily used for the detailed characterization of cultured pathogens and provides insights into ARGs and virulence factors. While informative, its reliance on cultured isolates limits its direct applicability in acute clinical settings. The tNGS approach amplifies specific genomic regions, such as the 16S rRNA gene for bacteria, directly from clinical specimens. It offers a balance between broad pathogen detection and manageable data analysis, enhancing the identification of bacterial communities present in the samples. Finally, mNGS represents an untargeted approach that sequences all nucleic acids in a sample and can detect a wide array of pathogens – bacteria, viruses, fungi, and parasites – without prior knowledge of the potential causative agents. This comprehensive detection capability is particularly advantageous in identifying co-infections and rare or unexpected pathogens.

State of the art in NGS applications for LRTIs diagnosis

Since respiratory mNGS first emerged in research, efforts have been made to integrate it into clinical practice[91]. The initial challenge was demonstrating its superior diagnostic yield compared to traditional methods[92-94]. The results of a recent meta-analysis focused on severe pneumonia indicated that the mNGS group achieved a significantly higher pathogen detection positive rate (80.48%) compared to the conventional methods group (45.78%)[95]. The next significant challenge was proving its logistical and technical feasibility in real-time clinical settings. Charalampous et al.[96] demonstrated that, despite concerns over lengthy sequencing times, the median diagnostic time for identifying a potential pathogen was 6.7 h (interquartile range: 6.1-7.5 h). The third significant challenge was understanding how real-time results would influence clinical decision-making. Charalampous et al.[96] found that, among patients with positive mNGS results, antibiotics were escalated in 22% of cases and de-escalated in 26%. These findings underscore the specific benefits of non-culture-based pathogen identification methods, particularly when speed and accuracy are critical. The fourth major challenge is assessing whether real-time access to pulmonary metagenomics can lead to improved patient outcomes. In a recent clinical trial, including patients with severe CAP, the authors found that the proportion of patients with clinical improvement within 14 days was significantly higher in the metagenomics group (62.0%) than in the CMT group (46.5%)[97]. Table 6 provides a comprehensive summary of recent studies on the application of mNGS for diagnosing CAP, HAP, and VAP, highlighting the advantages of mNGS in pathogen detection and clinical outcomes.

Workflow of NGS

The mNGS workflow involves both wet lab (laboratory) and dry lab (bioinformatic analysis) procedures (Fig. 2). The wet lab starts with sample collection and nucleic acid extraction. Microbial findings vary depending on the sampling method used to collect respiratory specimens[98]. However, there is a limited number of studies on the optimal respiratory sample selection for mNGS detection of pulmonary infections. The collected sample types primarily included blood, sputum, and BAL fluid[91]. A major challenge of mNGS is the dominance of human host DNA in most patient samples. Various alternative methods are available to deplete human background DNA during the preanalytical phase. Then, library preparation and high-throughput sequencing are carried out. Several sequencing platforms are available[99]. Long-read sequencing platforms, such as Oxford Nanopore Technology (ONT) and Pacific Biosciences single-molecule real-time sequencing, are particularly promising due to their ability to apply mNGS directly to clinical samples, potentially delivering faster results. Short-read sequencing platforms, including Illumina (NovaSeq, HiSeq, NextSeq, MiSeq), BGI (MGISEQ, BGISEQ), and Thermo Fisher (Ion Torrent), are widely recognized for their high accuracy and are supported by extensive bioinformatics tools and analysis pipelines. While long-read sequencing has historically faced concerns regarding accuracy, recent advancements have significantly reduced error rates. Despite these technological improvements, there remains a critical need for practical, rapid, and clinically relevant tools to generate data in real time. After sequencing, dry lab work begins with bioinformatics analysis. This involves quality control and filtering, adapter trimming, removal of human sequences, and microorganism identification by alignment to reference databases (mapping) or *de novo* assembly, as well as analysis of resistance and virulence genes[100].

Advantages and challenges

NGS can simultaneously detect all known and novel pathogens in a single test without requiring prior knowledge of the causative agent. It can identify a broad spectrum of pathogens, including those difficult to culture or unexpected in clinical presentations. NGS can detect multiple pathogens in a single sample, as well as providing a comprehensive overview of the microbial landscape in polymicrobial infections. Moreover, advancements in NGS technologies have reduced sequencing times, enabling faster pathogen identification compared to traditional culture methods. Some platforms, such as ONT, provide results within 6-7 h, enabling same-day clinical decision-making. Such rapid turnaround times are critical for guiding timely antimicrobial therapy and reducing inappropriate antibiotic use. One of the major advancements in mNGS is the ability to detect ARGs, aiding in personalized treatment strategies.

Despite these advantages, several challenges hinder the widespread implementation of NGS[100,101]. The requirement for specialized laboratory infrastructure, complex data interpretation, high costs, and the need for robust clinical validation studies pose significant barriers. NGS's high sensitivity makes it susceptible to detecting contaminants, which can lead to false-positive results if not properly controlled. Most of the reagents used for mNGS

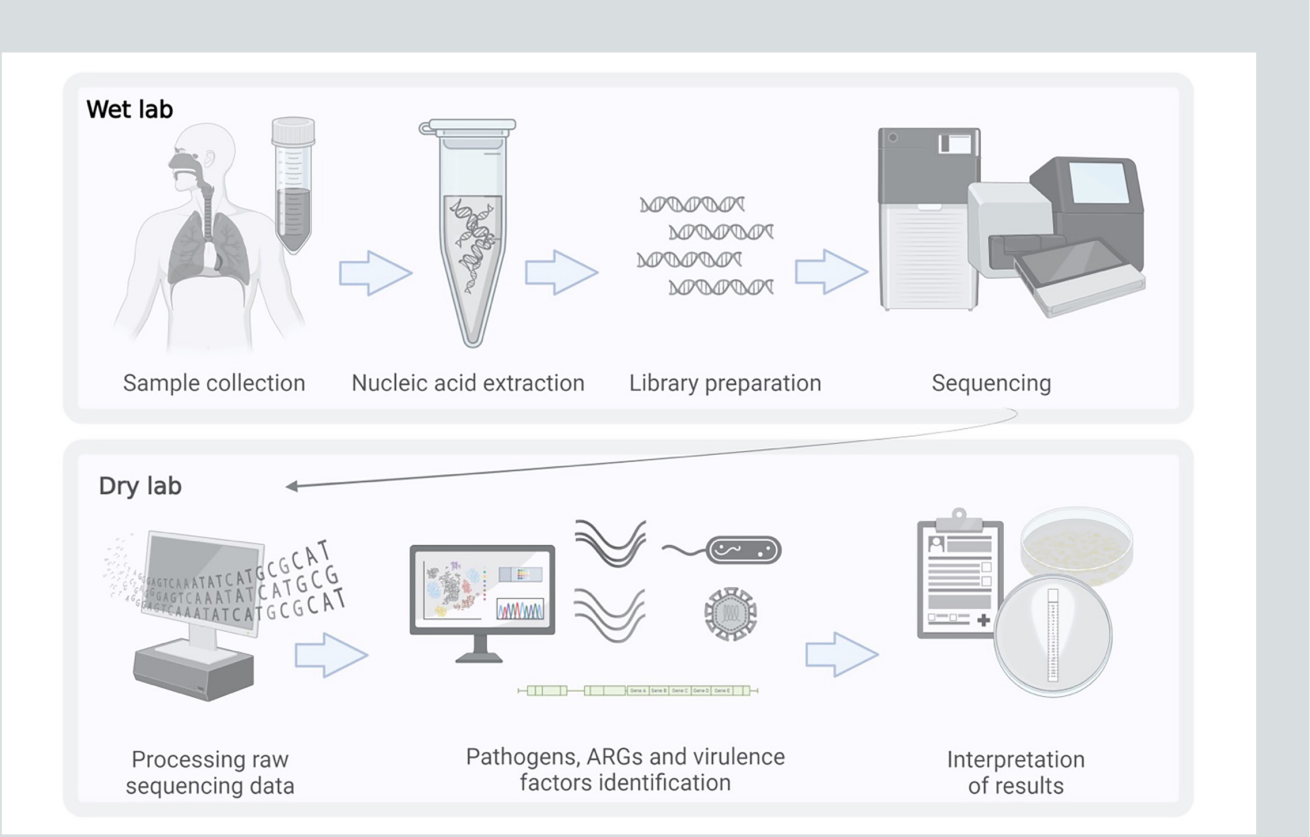

Figure 2. Workflow diagram of a next-generation sequencing experiment encompassing both wet and dry lab processes. The workflow begins with respiratory sample collection, followed by nucleic acid extraction and human DNA depletion. Then, library construction and sequencing are performed. In the dry lab part, raw reads undergo quality filtering before being analyzed for pathogen identification, antimicrobial resistance genes, and virulence factors. The final results are integrated with clinical data and other laboratory findings, including conventional microbiological tests, to ensure accurate interpretation. Created with BioRender.

will also introduce foreign DNA during the sequencing process. This phenomenon is called "kit-ome," which will seriously affect the sample results. Moreover, the variability in sample processing, sequencing protocols, and data analysis pipelines can lead to inconsistent results across different laboratories. Standards and guidelines are essential for ensuring the quality and reliability of mNGS in clinical practice[102]. Regarding ARGs, there is no isolate available to validate genomic resistance predictions through true phenotypic susceptibility testing. As a result, it remains uncertain whether the identification of ARGs correlates with resistance phenotypes. Moreover, despite technological advancements, the time required for sequencing and data analysis may still be longer than desired for critical care scenarios. In addition, the large datasets generated by NGS necessitate robust data storage solutions and raise concerns about patient data privacy and security. Last but not least, distinguishing between pathogenic and colonizing microorganisms remains a significant issue.

NGS represents a significant advancement in the diagnosis of respiratory infections, particularly in complex cases of CAP and

Table 6. Examples of mNGS applications for diagnosing lower respiratory tract infections over the past five years. It includes details on study type, patient cohort size, diagnosis, sample type, sequencing technology, key findings, and turnaround time

Type of study (N)	Diagnosis	Sample	Technology used	Main results	TAT	References
Prospective study (32)	HAP/VAP	BALF	MiSeq (Illumina)	mNGS identified pathogens in culture-negative cases.	45 h	103
Prospective study (72)	VAP	BALF	BGISEQ-500 (BGI)	mNGS had higher sensitivity for bacterial detection (97.1% vs. 42.1%) compared to CT. No significant differences were noted with regard to fungal infections. mNGS exhibited advantages in detecting viruses and identifying mixed infections.	24-48 h	104
Retrospective study (65)	Pulmonary infection	Sputum	MGISEQ-200 (BGI)	mNGS and tNGS were superior to CT for pathogen detection.	mNGS: 38.67 ± 16.53 h and tNGS: 37.68 ± 14.72 h	105
Retrospective study (177)	Pulmonary infection	BALF	HiSeq2500/ NextSeq1000/ NovaSeq/ NextSeq2000	The workflow could serve as adjunctive testing with, but not as a replacement for, standard microbiology techniques.	40 h	106
Retrospective study (113)	HAP/VAP	BALF	MGI-200/2000 (BGI)	The mNGS group had a significantly higher rate of antibiotic adjustments than the CT group, which led to more ventilator-free days within 28 days) and a shorter duration of invasive ventilation.	22 h	107
Retrospective study (207)	CAP	BALF	BGISEQ-50/ MGISEQ-2000 (BGI)	mNGS had higher sensitivity for bacterial (76.74%) and fungal (93.68%) detection compared to CT.	Not specified	108
Retrospective study (151)	CAP	BALF and sputum	NextSeq CN500 (Illumina)	An optimized mNGS workflow was developed, achieving a TAT of just one working day.	24 h	109
Retrospective study (43)	Infants with severe pneumonia	BALF	BGISEQ-50 (BGI)	mNGS significantly improved the sensitivity of pathogen detection in infants with severe pneumonia.	25.6 ± 3.5 h	110
Retrospective study (205)	CAP	BALF, sputum, blood	NextSeq CN500 (Illumina)	mNGS of BALF and sputum samples had a higher sensitivity of pathogen detection than blood	Not specified	111
Prospective study (66)	CAP	BALF	Nextseq 550 DX (Illumina)/ GridION X5 (ONT)	Species detected at an hour and 4 h through Nanopore were consistent to some extent, and TAT was significantly shorter than that by Illumina.	20/14 h	112
Multicentre prospective study (171)	Pulmonary infection	BALF, sputum	GridION X5 (ONT)	mNGS achieved 96.6% sensitivity and 88.0% specificity and identified pathogens in 39.1% culture-negative samples.	6.4 ± 1.4 h	113

(Continues)

Table 6. Examples of mNGS applications for diagnosing lower respiratory tract infections over the past five years. It includes details on study type, patient cohort size, diagnosis, sample type, sequencing technology, key findings, and turnaround time (*continued*)

Type of study (N)	Diagnosis	Sample	Technology used	Main results	TAT	References
Prospective study (10)	HAP	BALF	MGISEQ-2000 (BGI)/MinION (ONT)	The agreement rate between ONT sequencing and the clinical composite diagnosis was 73.3% (kappa value = 0.737).	24.7 ± 2.7 h / 9.6 ± 0.7 h	114
Prospective study (83)	VAP	ETA	MinION (ONT)	Sensitivity of mNGS was higher than that of CT (89.15% vs. 37.77%), and the average specificity was 98.8%.	5-6 h	115

N: number of included patients; TAT: turnaround time; mNGS: metagenomic next-generation sequencing; CT: conventional testing; ONT: Oxford Nanopore Technologies; ETA: endotracheal aspirate; HAP: hospital-acquired pneumonia; CAP: community-acquired pneumonia.

HAP. Its ability to provide comprehensive and rapid pathogen identification, including antimicrobial resistance profiling, holds the potential to enhance patient management and outcomes. However, its routine clinical application requires overcoming challenges related to cost, standardization, and clinical validation. Numerous studies have reported improved diagnostic yields with mNGS compared to conventional methods. However, evidence demonstrating its direct clinical benefit remains limited, as few studies have systematically assessed its impact on patient outcomes relative to standard diagnostic practices. mNGS could serve as a valuable complement to conventional methods for LRIs diagnosis, particularly in cases involving rare pathogens and complex infections.

LIMITATIONS OF THE MOLECULAR BIOLOGY TOOLS IN THE DIAGNOSIS OF RTIS

Despite the key advantages of molecular biology tools in diagnosing RTIs – such as their rapidity, high sensitivity and specificity, and independence from prior antibiotic treatment – several limitations must be considered. These include the fact that not all pathogens responsible for RTIs are included in syndromic panels, the inability to detect certain antimicrobial resistance determinants in some pathogens, and the potential for false-positive results.

Most studies conducted to date to evaluate the implementation of these rapid molecular diagnostic tools have certain limitations, including: 1) The most appropriate gold standard for comparison is the quantitative culture of BAL, which is not the most commonly used respiratory sample; 2) Sample quality is a critical issue; therefore, both Gram staining and assessment based on the Murray–Washington criteria should be included; 3) Prior antibiotic treatment of patients should be evaluated to compare molecular biology tools, which detect nucleic acids, with conventional cultures, which detect viable bacteria; 4) The analysis of the clinical impact on antibiotic stewardship; 5) The lack of cost-effectiveness analyses[116].

Although well-designed, in-depth studies addressing these limitations are needed, it can be stated that molecular biology tools are beneficial for managing patients in whom HAP or CAP caused by MDR bacteria is suspected[117]. Similarly, while NGS has shown improvements, further studies are required to assess its real clinical impact, taking into account the same issues mentioned above.

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