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Emerging Advances in Rapid Diagnostics of Respiratory Infections

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Diagnostic laboratories play a central role in the recognition of new and emerging infections. The identification of the severe acute respiratory syndrome (SARS) coronavirus in 2003 highlighted how modern diagnostic tools and collaboration between clinicians, public health professionals, and laboratorians can lead to the rapid characterization of a new respiratory pathogen.[1] Similarly, the development and rapid dissemination of a polymerase chain reaction (PCR) method to detect the novel H1N1 influenza A strain by the US Centers for Disease Control and Prevention in 2009, relied on the most recent diagnostic technology and played an important role in the response to the latest influenza pandemic.[2] These events remind us of how much recent developments in diagnostics have improved our ability to identify respiratory

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viruses. For nonviral respiratory pathogens, developments in laboratory technology have been less profound in general, but have still led to modest improvements in the diagnostic capability.

For the diagnostic microbiology laboratory, the routine evaluation of patients with suspected respiratory infections continues to rely on methods that have been used for a long time: microscopy and culture of respiratory tract specimens, blood cultures, detection of antigens in urine and upper respiratory specimens, and serology. Recent advances in pneumonia diagnostics have mostly occurred in the areas of antigen and nucleic acid detection. Despite these technological advances, there remain several major challenges that hinder the search for the causes of respiratory infections, particularly for pneumonia.[3] These challenges include difficulty collecting lower respiratory tract specimens, problems distinguishing colonization from infection, poor clinical (diagnostic) sensitivity of assays, and often inadequate evaluation of new diagnostics.

This review focuses on recent advances in laboratory diagnostics that enable rapid identification of respiratory pathogens.

ANTIGEN DETECTION

Assays to detect microbial antigens in body fluids have been used for the diagnosis of respiratory infections for many years, using various formats such as immunofluorescence, enzyme-linked immunosorbent assay (ELISA), latex agglutination, coagulation, and chromatographic immunoassay. These methods are the diagnostic tools most easily applied as near-patient tests, but development is reliant on the identification of suitable antigens that are present in detectable quantities in clinical specimens. To date, commercial assays have been developed only for a limited range of pathogens. The most widely available assays have focused on the detection of selected bacterial pathogens in urine and the detection of viruses in respiratory specimens.

Among bacterial respiratory pathogens, assays for *Streptococcus pneumoniae* and *Legionella pneumophila* are the most developed. A newer generation immunochromatographic test that detects the C-polysaccharide cell wall antigen in urine (NOW) has been an important advance in the diagnosis of pneumococcal disease.[4] This test has a sensitivity of 70% to 80% and a specificity of greater than 90% compared with conventional diagnostic methods for detection of pneumococcal pneumonia in adults. Unfortunately, the NOW test cannot be used reliably in children as it also detects pneumococcal carriage.[5] Alternative pneumococcal antigens for diagnostic purposes, such as pneumolysin, have shown promising results, although none has been demonstrated to perform better than existing commercial C-polysaccharide antigen assays.[6–8] The combination of a pneumolysin-specific antigen detection ELISA together with the NOW test may result in a better diagnostic yield because of the higher specificity of the pneumolysin detection ELISA.[7]

Detection of soluble *Legionella* antigen in urine is an established and valuable tool for the diagnosis of Legionnaires' disease, although current commercial assays can only reliably detect infection caused by *Legionella pneumophila* serogroup 1.[9] Some assays have been intended to detect other legionellae,[10] although the performance is not as good as for *L pneumophila* serogroup 1.

Detection of respiratory viral antigens in respiratory secretions has become an important diagnostic tool.[11–13] Antigen detection using immunofluorescent techniques were pioneered in the 1970s, and commercial reagents are now widely used for the detection of influenza viruses, respiratory syncytial virus (RSV), parainfluenza viruses, adenoviruses, and human metapneumovirus. These assays require technical expertise and have the advantage of allowing direct evaluation of specimen quality. More

recently, commercial rapid diagnostic tests (RDTs) have become widely used for the detection of influenza or RSV directly in respiratory specimens. These diagnostic test kits, produced as dipsticks, cassettes, or cards, contain internal controls, and a positive result is signaled by a color change. Results are produced by these tests within 5 to 40 minutes.

The sensitivity of rapid tests for the detection of seasonal influenza in clinical specimens ranges from 10% to 96%,[14,15] and varies with virus type or subtype, timing of specimen collection, specimen type, patient age, and the test comparator.[16,17] With the emergence of the pandemic influenza A (H1N1) 2009 virus, RDTs have been widely used for patient triaging, although there are limited data available on their clinical accuracy.[18,19] The sensitivity of these assays for detecting this new strain is 10% to 69% compared with real-time PCR.[20–22] Specificity of RDTs for seasonal influenza is 90% to 100% according to the available data for H1N1 2009.[22] Commercial RSV RDTs have sensitivities of 71% to 95% and specificities of 80% to 100% compared with culture.[23–25] Poorer performance has been observed in adults,[26] which may be related to the decreased viral titer in adults compared with children.

To correctly interpret results of RDTs, the prevalence of influenza or RSV disease in a community must be considered.[15] During peak disease activity, positive predictive values are highest, but false-negative results more likely. The opposite is true during times of low disease activity.[17] When the disease prevalence is low or unknown, RDT results become difficult to interpret and of limited use.[17]

NUCLEIC ACID AMPLIFICATION TESTS

The use of nucleic acid amplification tests (NAATs) has transformed our understanding of respiratory infections, demonstrating the relevance of new agents such as human metapneumovirus, and providing new insights into previously recognized ones such as rhinoviruses. The progressive commercialization and clinical application of these methods is placing them at the forefront of respiratory diagnostics.

NAATs possess several advantages over more traditional techniques for the detection of respiratory pathogens.[27] These tests have improved sensitivity for detecting organisms that are fastidious, no longer viable, or present in small amounts. NAATs can provide rapid genetic information regarding sequence evolution, geographic variation, or the presence of virulence factors or antibiotic resistance. Their rapid turnaround times allow them a more prominent role in patient management, and the ability of NAATs to test for multiple pathogens simultaneously has aided in the diagnosis of nonspecific respiratory syndromes, such as in outbreak settings. Within the laboratory, NAATs offer enhanced opportunities for automation, and have a lower safety risk than culture for the detection of highly virulent pathogens.

Among the NAATs, the PCR is the most common and thoroughly evaluated method.[28,29] The PCR formats most relevant for respiratory diagnostics can be classified into conventional, real-time, and multiplex platforms, with various amplicon detection methods such as gel analysis, ELISA, DNA hybridization, or the use of fluorescent dyes or chemical tags. Real-time PCR has several features that place it at an advantage. First, the two steps of amplification and detection are combined in one reaction, increasing the speed and efficiency of testing and reducing the risks of operator error and cross-contamination. Real-time PCR also allows for the possibility of quantifying the amount of starting nucleic acid material.

Multiplex PCR systems, in which multiple PCR targets are sought after simultaneously in one reaction, have gained wider acceptance, particularly among commercial assays. These systems have the advantage of increasing the number of

pathogens tested for, without increasing the required amount of operator time or specimen material. Multiplex assays have broadened the scope of respiratory surveillance studies, and have also led to the increasing recognition of dual or triple infections in the same individual.[30] As noted in **Table 1**, these assays can be differentiated by either their amplification or detection steps. In the amplification step, all multiplex platforms must balance the competing optimal PCR conditions for each individual target, and must overcome problems of competition and inhibition among the various primers and probes. Each platform uses a unique method to address these issues, such as nested primer combinations,[30,31] complex primer structures,[32,33] and nontraditional nucleotides.[34–36]

These assays are even more varied in their detection stages, where the common task is to differentially detect and report distinct populations of amplified targets. Several platforms involve solid-phase arrays, such as polystyrene microbead suspensions that use fluorescent dyes to differentiate targets,[34,37,38] or the microchip formats that identify targets by binding to a specific physical location.[39–41] The former has been developed into platforms detecting 17 to 20 targets, whereas the latter can identify between a few dozen to thousands of targets. The increased breadth of targets afforded by microarrays, however, comes at the expense of decreased sensitivity.[42] Multiplex PCR products can also be distinguished by their size, using resolution techniques such as agarose gel electrophoresis to differentiate by weight, and capillary-based auto-sequencers that identify targets by length and sequence.[32,33] Mass spectrometry can also be used for identification, either by the attachment of high molecular weight tags to primers[43–46] or by the analysis of specific nucleotide base ratios that can be resolved by molecular weight.[47]

Regardless of the platform, all PCR assays require good primer design taking into consideration gene target, gene number, mobility of genes between species, stability of gene, and the presence of mutations. Bacteria have large genomes with many genes for a fully functional organism, including their own genes for replication and enzyme product. Owing to the large genome size there are many targets available for specific detection of a bacterial species. Housekeeping genes, those genes that are essential for the survival of the organism, are desirable gene targets because they have conserved regions and hypervariable regions (eg, the 16S rRNA gene). Genes found in multicopies will also increase the sensitivity of PCR assays. The choices of viral pathogen gene targets are limited because of the limited size of the viral genome. Genes that are highly conserved are desirable targets for PCR because they allow the detection of many strains. Other genes that process areas of nucleotide hypervariability caused by genetic mutation should be avoided because changes over primer and probe sites can cause poor PCR efficiency and the potential for false-negative results. **Table 2** lists some of the more common respiratory pathogen target genes.

The published literature on NAATs can be difficult to interpret, because study designs vary and rarely involve head-to-head comparisons among the different assays. Calculation of clinical sensitivity and specificity is complicated because NAATs often are more sensitive than the reference culture-based standards. Comparisons of study results are also problematic because of the use of different specimen types that may have differential yields for pathogens.[48] Finally, several NAAT platforms require investment in specialized equipment, the cost of which can only be recovered through high-volume testing. Therefore, few NAAT assays for respiratory diagnosis are licensed for clinical use, and their daily use in clinical practice remains uncommon. To promote widespread adoption in the future, developers of NAAT diagnostics will need to standardize evaluation methods, particularly in comparison with reference

techniques, reduce complexity and cost, and better demonstrate their utility in the clinical environment.

NAATs for Specific Respiratory Pathogens

Although NAATs have been developed for all important respiratory pathogens, the clinical application of these tests varies. Perhaps the area that NAATs have had the greatest impact is for the diagnosis of infections caused by respiratory viruses.[29,49,50] For most, if not all, respiratory viruses, detection of viral nucleic acid is the most sensitive diagnostic approach, and current "gold standards" (namely, culture and direct immunofluorescence) will be eventually replaced by NAATs.[50] PCR has become the diagnostic test of choice for some respiratory viral infections (eg, for influenza during the current influenza H1N1 pandemic), and is a useful epidemiologic tool for characterizing the role of viruses in various disease states.[51] NAATs can provide results rapidly and are able to detect many viral pathogens that are unable to be readily detected by culture. Perhaps more so than for other respiratory pathogens, considerable effort has been directed toward the development of multiplex assays to enable the simultaneous detection of multiple viral pathogens. Given the increasingly large number of respiratory viruses, this can be a challenging task.

The need for improved diagnostic tools for pneumococcal disease has lead to the evaluation of several NAATs. For pneumonia, PCR has a sensitivity for detecting *S pneumoniae* in blood samples ranging from 29% to 100%,[27] with a tendency for higher sensitivity in children than adults. The finding of positive pneumococcal PCR results from asymptomatic control subjects complicates interpretation.[52–54] When testing sputum samples from adults with pneumonia, PCR positivity has ranged from 68% to 100%,[27] although it is unclear how often this reflects upper respiratory tract colonization rather than infection.[55] Further refinement of PCR assays, including the use of multiple targets, has increased the specificity,[56] with *lytA* assays potentially offering advantages over other assays.[57,58] Quantification of *S pneumoniae* DNA load may provide additional diagnostic and prognostic information. Quantitative PCR may help distinguish colonization from infection, with a higher bacterial burden in pneumococcal disease than in a carrier state.[59] High pneumococcal DNA loads in blood have been recently shown to be associated with severe disease in various settings.[60–62]

NAATs have improved the ability of diagnostic laboratories to detect respiratory pathogens that are difficult to culture, such as *Mycoplasma pneumoniae*, *Legionella* species, and *Chlamydophila pneumoniae*. An extensive evaluation of 13 antibody detection assays using PCR as the comparator standard concluded that few commercial serologic assays for detection of *M pneumoniae* performed with sufficient sensitivity and specificity, and highlighted the increasing importance of NAATs.[63] Indeed PCR is considered by many to be the method of choice for detection of *M pneumoniae* infection.[64] Both upper and lower respiratory tract samples are suitable for testing for *M pneumoniae* by PCR, although throat swabs and nasopharyngeal samples may be preferred because of high sensitivity, high specificity, and convenience. In practice, PCR has been successfully used to rapidly diagnose mycoplasma pneumonia during outbreaks, and was particularly useful in children, immunocompromised patients, and in early-stage disease.[65,66]

Legionnaires' disease can be difficult to diagnose, and NAATs have proven a useful adjunct to culture and antigen detection.[9] PCR has repeatedly been shown to have sensitivity equal to or greater than culture when testing lower respiratory specimens.[67–73] *Legionella* DNA can also be detected in nonrespiratory specimens, such as urine, serum, and peripheral leukocytes,[9] although testing these specimen types is not well established.

Table 1
Comparison of nucleic acid amplification platforms

Platform	Targets Included	Assays Available	Analytical Performance	Clinical Performance	Development Timeframe	Flexibility	Turnaround Time	Specimen Requirements	Quantitation	Licensing Status
Real-time PCR (rtPCR)	Variable; maximum of 4–5 targets per assay, can run parallel reactions, limited by sample volume	Various in-house protocols	Likely highest sensitivity, eg, 1 pfu/mL, 10 copies/reaction	Good; singleplex rtPCR is often the gold standard molecular diagnostic	Depends on the originating laboratory	Multiplex must be optimized for each additional target, limited to 5 total targets	Half-day	None	Yes	Some approved for in vitro diagnosis (IVD), others research use only (RUO)
Microbead array	17–20 viral or bacterial targets in each assay	Qiagen (Resplex I [bacterial] and II [viral]), Luminex, and Eragen Biosciences	Varies by kit and pathogen; limits of detection reported at 60 copies/reaction, or 0.1–100 $TCID_{50}$/mL	Sensitivity 72%–100% compared with culture plus rtPCR; varies by target, reduced by dual infections	All are commercially available	Multiplex must be optimized for each additional target; up to 30 can be detected at once; commercial kits may be slow to modify	6–8 h		No	IVD (Luminex); RUO (Qiagen, Eragen)
Mass spectrometry	All main respiratory viruses and bacteria	MassTag, IBIS	MassTag: 500–1000 copies/reaction, 1 $TCID_{50}$/mL; IBIS: 50 copies/well (basically singleplex)	Not rigorously evaluated	Unknown	Requires optimization of multiplex; detection methods unrestricted	Half to 1 day		No	RUO

Commercial multiplex	All main respiratory viruses and bacteria	Seegene (Seeplex)	10–100 copies/reaction	96%–100% concordance with DFA and sequencing	Commercially available	Multiplex apparently tolerant of additional targets; detection method determined separately	6–8 h	No sputum	No	IVD in Europe and Canada, RUO in USA
Microarray	Several thousand viruses, bacteria, fungi, and parasites	Greenechip, VIrochip, Autogenomics	10–10,000 copies/reaction	No data	Unknown	Not generally customizable	No data	No data	No	RUO
16S rRNA	All bacteria and mycobacteria	Viruses	Can detect low abundance organisms	For bacteremia, 87% sensitivity, 86% specificity vs blood culture	Assay for respective pathogens is in development	Not applicable	PCR 3–4 h, analysis time 3–4 h	ND	No	RUO
Ultrahigh throughput screening	All microbes	None	Limit of detection 5500 copies/mL	No data	No data	Not applicable	ND	ND; likely none	No	RUO

Abbreviations: DFA, direct fluorescence assay; ND, not determined; $TCID_{50}$, tissue culture infective dose needed to produce 50% change.

Table 2
Common gene targets for nucleic acid amplification tests

Organism	Genome	Size (nt)	Target	Function
Streptococcus pneumoniae	dsDNA	~2,040,000	*ply* *lytA* *psaA* *16S rRNA*	Detection
Haemophilus influenzae	dsDNA	~1,830,138	*16S rRNA* *BexA*	Detection
Moraxella catarrhalis	dsDNA	~1,940,000	*16S rRNA*	Detection
Legionella	dsDNA	~3,576,470	*16S rRNA* *mip*	Detection
Mycoplasma pneumoniae	dsDNA	~816,394	*16S rRNA* *P1* adhesion gene CARDS toxin	Detection
Chlamydophila pneumoniae	dsDNA	~1,225,935	*omp-2* gene	Detection
Bordetella pertussis	dsDNA	~4,086,189	*IS481* *adenylate cyclase toxin (ACT)* gene	Detection
Mycobacterium tuberculosis	dsDNA	~4,411,532	*IS6110* *16S rRNA*	Detection
Pneumocystis jiroveci	dsDNA	~8,400,000	*18S rRNA,* *mitochondrial (mt) rRNA* *5S rRNA*	Detection
Adenovirus	dsDNA	~36,000	Hexon gene Fiber gene	Detection and genotyping
Enterovirus	ssRNA (+)	~7500	5′UTR VP1,2	Detection Genotyping

Rhinovirus	ssRNA (+)	~7500	5′UTR VP1,2	Detection Genotyping
Coronavirus 229E, OC43, SARS, NL63, HKU-1	ssRNA (+)	~30,000	Polymerase gene Nucleocapsid gene ORF1	Detection
Influenza A	ssRNA (−)	~12,000	Matrix protein gene	Detection
Influenza B	ssRNA (−)	~12,000	Hemagglutinin gene	Detection
Parainfluenza virus 1, 2, 3, and 4	ssRNA (−)	~15,600	Hemagglutinin gene	Detection
RSV	ssRNA (−)	~10,000	Fusion protein (F) Nucleoprotein (N)	Detection
hMPV	ssRNA (−)	~14,000	Fusion protein Nucleoprotein Large polymerase (L) protein gene	Detection
Bocavirus	ssDNA	~5,500	Viral protein (VP1) Nonstructural protein (NP1)	Detection

Abbreviations: ORF, open reading frame; UTR, untranslated region.

PCR has been extensively evaluated for the rapid diagnosis of *C pneumoniae* infection using various assays.[74] A standardized approach to *C pneumoniae* diagnostic testing was published in 2001 by the US Centers for Disease Control and Prevention and the Canadian Laboratory Center for Disease Control.[75] However, there are still few evaluations that have extensively used clinical samples, and the great variety in the methods used makes it difficult to make firm conclusions about performance. To further complicate matters, significant interlaboratory discordance of detection rates have been recorded for some assays.[76,77]

The diagnostic yield from PCR is consistently greater than for culture when testing nasopharyngeal samples for *Bordetella pertussis*.[27] PCR remains positive for a longer period after the onset of symptoms and thus is useful for individuals who present late in their illness.[78] In the investigation of a pertussis outbreak, the combination of PCR and culture for samples obtained 2 weeks or less after illness onset and PCR alone for samples obtained more than 2 weeks after illness onset proved to be the most diagnostically useful.[79]

PCR has greater sensitivity than cytologic methods for the detection of *Pneumocystis jiroveci*, although it has been difficult to interpret the common finding of PCR-positive samples that are negative by standard methods.[27] The latter may reflect *P jiroveci* colonization of uncertain clinical significance. The performance of PCR has been shown to vary with different assays,[80] although the results correlate well with clinical evidence of pneumocystis pneumonia.[81]

The need for improved diagnostic methods for tuberculosis has focused attention on the potential role of NAATs. Advances in this area have been relatively slow, with NAATs for mycobacteria failing to provide greater sensitivity than culture-based methods. The relatively high false-negative rate with NAATs for *Mycobacterium tuberculosis* probably reflects a combination of the paucibacillary nature of samples, presence of inhibitors in samples, and suboptimal DNA extraction methods. The situation is changing, with new developments in rapid diagnosis and antibiotic susceptibility testing.[82] For direct detection of *M tuberculosis* in respiratory samples, all commercial assays have high specificity (>98%), but variable sensitivities: 90% to 100% for smear-positive samples and 33% to 100% for smear-negative samples.[27] Consequently, it is recommended that use of these tests is restricted to only smear-positive samples. Evaluations of PCR for the diagnosis of tuberculosis in high-prevalence populations have been promising.[83–86] Alternative strategies under development to diagnose tuberculosis by molecular tools include detection of mycobacterial DNA in urine[87,88] and direct detection in respiratory specimens by microarray.[89]

New Pathogen Discovery

The NAATs discussed herein target known pathogens. When NAATs fail to identify an agent, additional tools are needed to pursue an etiologic diagnosis as might be indicated in an outbreak setting. These additional methods include microarrays and high-throughput sequencing.[42,90,91] Proteomics also has a potential for being developed as a tool for pathogen discovery.[92]

BREATH ANALYSIS

Breath analysis is an exciting new area with enormous diagnostic potential.[93–95] Alveolar breath contains many biomarkers derived from the blood by passive diffusion across the alveolar membrane,[93] and also contains direct markers of lung injury.[96–98] Breath testing is noninvasive, easily repeatable, and requires minimal specimen

workup. Various testing methodologies and sample types have been used in breath research, usually involving the measurement of exhaled permanent gases, detection of volatile organic compounds, or analysis of exhaled breath condensate.

The use of breath analysis for the investigation of respiratory infections has not yet been extensively evaluated. Electronic nose devices detect volatile molecules as they interact with chemical sensor assays.[99–101] Based on the reactivity of multiple sensors to the volatile molecules, an electronic signature is generated. Testing of exhaled breath by a portable electronic nose has been used to diagnose pneumonia in mechanically ventilated patients.[102–104] The clinical impact of this device needs further evaluation, but it could be used as a trigger for further diagnostic studies in pneumonia such as bronchoscopy.

Microorganisms produce volatile metabolites that may be used as biomarkers.[105] Detection of these biomarkers in breath samples by gas chromatography/mass spectroscopy or similar methods may provide an etiologic diagnosis of respiratory tract infection. Ideally, specific biomarkers need to be identified, and it may be difficult to discover unique markers for each pathogen produced in sufficient quantities to enable detection. Potential biomarkers have been reported for some respiratory pathogens, such as *Aspergillus fumigatus*[106,107] and *M tuberculosis*,[108,109] but it is still uncertain whether they will prove to be useful as clinical diagnostic tools.

FUTURE PROSPECTS

Diagnostic tests for respiratory infections will continue to evolve and become more user-friendly. Antigen-detection assays in immunochromatographic or similar formats are rapid, simple to perform, and are most easily developed as near-patient tests. These methods are among the most attractive diagnostic tools, but further development is reliant on the discovery of suitable antigens that can be reliably detected in readily obtained specimens. NAATs have now been developed to a stage where multiplex assays that detect the common respiratory pathogens are commercially available, although not all have been rigorously evaluated in clinical settings. Further improvements in design and performance are expected, and an emphasis should be placed on clarifying the clinical usefulness of NAATs, developing standardized methods, producing even more user-friendly platforms, and exploring the role of quantitative assays. New approaches for respiratory pathogen detection are desperately needed. Breath analysis is an exciting new area with enormous potential, and it will be interesting to follow progress in this area over the next few years.

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