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Molecular diagnosis of respiratory viruses

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Summary
The increasing availability of nucleic acid amplification tests since the 1980s has revolutionised our understanding of the pathogenesis, epidemiology, clinical and laboratory aspects of known and novel viral respiratory pathogens. High-throughput, multiplex polymerase chain reaction is the most commonly used qualitative detection method, but utilisation of newer techniques such as next-generation sequencing will become more common following significant cost reductions. Rapid and readily accessible isothermal amplification platforms have also allowed molecular diagnostics to be used in a ‘point-of-care’ format. This review focuses on the current applications and limitations of molecular diagnosis for respiratory viruses.

Key words: Diagnostics, molecular, PCR, respiratory viruses.

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INTRODUCTION
Worldwide, viral respiratory tract infections cause significant morbidity and mortality. In the United States of America (USA), pneumonia and influenza ranks sixth as the cause of hospitalisation among Medicare beneficiaries. In New South Wales, Australia, influenza and pneumonia was responsible for 9.1% of total deaths in 2013. However, these are likely underestimates of the true burden of influenza as unrecognised infection may result in respiratory or cardiovascular hospitalisation and deaths. Similarly, other respiratory viruses may also cause severe respiratory infections, particularly in the young, elderly or immunocompromised.

Laboratory confirmation of the aetiology of viral respiratory tract infection guides treatment, obviates the need for further unnecessary testing and is useful for epidemiological purposes, including planning vaccination strategies. When selecting the most appropriate test, clinicians should consider the availability, performance and turnaround times of the different diagnostic methods. Although viral culture remains the ‘gold standard’ for diagnosis, nucleic acid amplification tests (NAATs) are predominantly used given their increased sensitivity, specificity, breadth, and reduced turnaround time to pathogen detection. NAATs can also be used for typing, subtyping, quantitation of viral loads and detection of antiviral resistance. Nevertheless, NAATs are not perfect, and their role in the laboratory diagnosis of respiratory viruses is constantly evolving.

Herein, we discuss the applications, advantages and limitations of NAATs in the diagnoses and clinical management of respiratory viruses.

THE ROLE OF NUCLEIC ACID AMPLIFICATION TESTING

Qualitative detection and quantitation of respiratory viruses
NAATs are primarily used to determine the respiratory viruses responsible for infection, as the viral aetiology is unlikely to be reliably distinguished on clinical features alone. Table 1 outlines the respiratory viruses that are commonly detected using NAATs.

Although not routinely performed, quantitative detection may provide useful information on the severity and prognosis of viral respiratory infections, efficacy and resistance development during antiviral therapy and the duration of viral shedding to inform infection control measures. In hospitalised adults with influenza infection, viral RNA detection served as a surrogate for persistent isolation of virus, thus enabling the identification of risk factors for severe infection. The pathogenesis of novel or emerging respiratory viruses may be guided by studying viral replication; for example, over time, in different patient groups (adult or paediatric, immunocompetent or immunosuppressed), in relation to symptomatology, in response to treatment, in different tissues, and in different locations of the respiratory tract. In the paediatric population, quantitation of respiratory viruses may be used to differentiate clinically significant infection versus asymptomatic infection or ‘carriage’. Quantitation is also useful to understand the clinical impact of co-infections, including the pathogenicity of frequently detected viruses such as human bocavirus (HBoV) and polyomaviruses. The duration of antiviral therapy for influenza virus infections may also be optimised with viral load measurements. However, accurate quantitation of viral load may be compromised by the non-uniformity in sample volumes, as in samples like nose and throat swabs (NTS).

Detection of antiviral resistance
Although neuraminidase inhibitors (NIs) are widely prescribed for influenza infections, a number of antivirals are currently in phase II or III trials for non-influenza viruses. Detection of antiviral resistance is best described for NIs, with near 100% resistance of seasonal influenza A/H1N1 strains to oseltamivir prior to the influenza pandemic of 2009. This influenza subtype
was then replaced by the A(H1N1)pdm09 virus, an influenza A subtype that has remained mostly NI susceptible. Oseltamivir resistance is more likely to develop in subjects given oseltamivir prophylaxis or in immunocompromised patients with prolonged viral shedding.

Oseltamivir resistance is most often associated with the histidine to tyrosine amino acid substitution at position 275 in the neuraminidase gene (His275Tyr) in influenza A/H1N2 viruses, and the glutamine to valine substitution and arginine to histidine for tyrosine amino acid substitution at position 275 respectively for influenza A/H3N2 viruses. Other substitutions that have been identified include isoleucine to arginine, lysine or valine at position 223 (Ile223Arg/Lys/Val), serine to asparaginase at position 247 (Ser247Asn) and isoleucine to valine at position 117 (Ile117Val). These substitutions, in combination with His275Tyr, confer even higher levels of resistance to NIs.

Antiviral resistance can be determined using various NAAT methods including reverse transcription polymerase chain reaction (RT-PCR), rolling circle amplification and sequencing techniques. High resolution melting (HRM) analysis and pyrosequencing are more widely used in clinical virology laboratories compared to conventional or next-generation sequencing (NGS), which were used to detect the single nucleotide polymorphisms (SNPs) outlined above. Compared to HRM analysis, pyrosequencing has the added advantage of estimating the relative proportions of susceptible wild-type and resistant mutant viruses in mixed population samples.

Epidemiological and phylogenetic typing

NAATs can be used to explore the genomic relationships of existing or novel respiratory viruses. Such analyses allow greater resolution between and within species type to determine the origin and evolution of respiratory viruses, aid outbreak investigations by demonstrating transmission events, advance pathogenic understanding, guide discovery and subsequent detection of antiviral resistance and assess vaccine effectiveness. Molecular typing methods generally involve PCR followed by nucleotide sequencing of partial or whole genomes.

Over a 5 year period, phylogenetic analysis of 156 complete genomes of influenza A/H3N2 viruses demonstrated the presence of multiple clades co-circulating in New York State. Multiple lineages from a common haemagglutinin gene ancestor were circulating following distinct reassortment events. At a 180-bed Japanese hospital, investigators demonstrated nosocomial transmission of two genetically distinct influenza A/H3N2 variants by analysing haemagglutinin sequences over a 5 week period. These data were used to identify lapses in, and reaffirm the importance of stricter infection control measures.

Full length analyses of neuraminidase and haemagglutinin genes of influenza A(H1N1)pdm09 viruses during the 2011 influenza season showed that they were distinct compared to viruses that were circulating during the influenza pandemic of 2009, but associated with viruses collected from Newcastle, Australia, at the time of transmission of oseltamivir resistant A(H1N1)pdm09 viruses in the community.

Similar techniques have also identified the circulation patterns of influenza B viruses, an observation that assists with understanding influenza vaccine composition and effectiveness.

Specimen collection and pre-analytical issues

NAATs can be performed on upper and lower respiratory tract samples including NTS, nasopharyngeal swabs, nasopharyngeal aspirates (NPA), throat gargles, bronchoalveolar lavage fluid and pleural fluid. Sputum is not a preferred specimen due to its viscosity, but a recent study showed higher mean viral loads for influenza A, respiratory syncytial virus (RSV) and human metapneumovirus (hMPV) when sputa were processed using a 'dunk and swirl' method compared to NTS. This method involves dunking a sterile swab into sputum and swirling the swab into sterile water, which is subsequently processed.

Respiratory specimen type and the age of the subject tested can affect the performance of NAATs. The sensitivity of NAATs may be increased when lower respiratory tract or paediatric samples are tested. The detection of viruses from respiratory samples is also affected by the time between the onset of symptoms and specimen collection. Respiratory viruses are more likely to be detected when specimens are collected soon after symptom onset as viral loads are generally higher early in the illness, especially in paediatric samples. The quality of sample collection is especially important in respiratory tract infection, and training in sampling is recommended. However, patient self-collected samples such as throat washings in severe acute respiratory syndrome-coronavirus (SARS-CoV) infection may reduce transmission risks to healthcare workers.

Testing stools may complement testing of respiratory samples for viruses able to replicate outside the respiratory tract. SARS-CoV RNA was detected in stool but not respiratory samples for more than 10 weeks after symptom onset, whilst avian influenza A/H5N1 and A/H7N9 RNA (but not human seasonal influenza viruses) have been detected in 50–78% of stool samples. Quantitation of RSV RNA in blood collected from patients that have undergone haematopoietic stem cell transplantation (HSCT) may also predict poor outcomes and guide antiviral therapy.

The volume and method of nucleic acid extraction from submitted specimens can also affect NAAT performance. Different extraction methods may be more suited to recovering RNA, DNA or total nucleic acids. More recently, commercial extraction-independent assays for the detection of influenza

| Respiratory virus                                      | Virology   | Diagnostic methods |
|--------------------------------------------------------|------------|--------------------|
| Influenza virus                                        | ssRNA (-)  | RT-PCR LAMP        |
| Respiratory syncytial virus (RSV)                      | ssRNA (-)  | RT-PCR LAMP        |
| Human rhinovirus (HRV)                                 | ssRNA (+)  | RT-PCR             |
| Human enterovirus (HEV)                                | ssRNA (+)  | RT-PCR             |
| Parainfluenzavirus (PIV)                               | ssRNA (-)  | RT-PCR             |
| Human metapneumovirus (hMPV)                           | ssRNA (-)  | RT-PCR LAMP        |
| Human adenovirus (HAdV)                                | dDNA       | PCR                |
| Human coronavirus (NL63, HKU1, OC43, 229E)             | ssRNA (+)  | RT-PCR LAMP        |
| SARS-coronavirus (SARS-CoV)                            | ssRNA (+)  | RT-PCR             |
| MERS-coronavirus (MERS-CoV)                            | ssRNA (+)  | RT-PCR LAMP        |
| WU (WUPyV) and Kl (KIPyV)                              | dsDNA      | PCR                |
| Polyomavirus                                           | ssDNA      | PCR                |
| Human bocavirus (HBoV)                                 |            |                    |

(+) positive sense; (-) negative sense; ARI, acute respiratory infection; ds, double-stranded; LAMP, loop-mediated isothermal amplification; RT-PCR, reverse transcription polymerase chain reaction; ss, single-stranded.
and RSV have become available, with comparable performance to conventional extraction-dependent assays. 38,39

**NUCLEIC ACID AMPLIFICATION TESTING METHODS**

Multiple NAAT methods are used in the diagnostic virology laboratory for the diagnosis of viral aetiologies of respiratory infections including PCR, RT-PCR, real-time RT-PCR (rRT-PCR), conventional Sanger sequencing, pyrosequencing, PCR coupled with mass spectrometry and microarrays. Of these techniques, RT-PCR is the most commonly employed qualitative method for detecting respiratory viruses.

**Polymerase chain reaction**

Most commercial rRT-PCR platforms have comparable sensitivity in detecting respiratory viruses, although there are some exceptions. 40,41 Commercial assays that target the 5'UTR of human rhinovirus (HRV) may not reliably differentiate HRV from human enterovirus (HEV) within the Picornaviridae family. Commercial rRT-PCR platforms may also lack sensitivity in detecting certain HRV and human adenovirus (HAdV) subtypes. 40 Nested PCR, where two amplification reactions are performed sequentially with either one (hemi-nested) or two (fully nested) primers located 3' in relation to the first primer set is generally more sensitive, but at the expense of increased risk of contamination. 42,43

In contrast to singleplex or duplex assays, multiplex PCR allows the simultaneous detection of different respiratory viruses. Viruses such as influenza may also be typed or subtyped. 44 Bacterial targets such as Bordetella pertussis, Mycoplasma pneumoniae and Chlamydia pneumoniae have also been included in some commercial multiplex respiratory virus PCRs, 45 and a similar approach can be used to detect bacterial pathogens that complicate respiratory viral infections. 46

Limiting the number of multiplex reactions to three or four targets may improve sensitivity by eliminating primer dimerisation and competition between multiple targets, although newer assays using PCR with melting curve analysis (FilmArray, AusDiagnostics); microcapillary electrophoresis (Seeplex, RespiFinder, ICEPlex); microsphere hybridisation associated with flow cytometer detection, LED camera detection or barcode detection (xTAG RVP, Resplex II, MultiCode-PLx); solid phase hybridisation microarrays (Infiniti, NGEN, Verigene, iCubate, eSensor), and PCR coupled with electrospray ionisation mass spectrometry (PLEX-ID) have allowed multiplexing of over 20 viral targets with good sensitivity. 47

**High resolution melting analysis**

HRM analysis is based on the dissociation behaviour of DNA as it transitions from double- to single-strand status in the presence of a DNA intercalating dye. By detecting differences in PCR amplicons based on their sequence length, base composition (%G + C content) and strand base pairing, HRM analysis can be used for pathogen identification, species identification and genotyping. 48,49 Detection of SNPs can be used for influenza typing and also to determine antiviral resistance by detecting the Hs275Tyr mutation. 50,51 HRM is relatively simple, cheap, and can be performed using standard equipment in most laboratories offering NAATs. However, SNPs in larger amplicons (>200bp) are not readily discerned, compared to smaller fragments where they are consistently detected. A further limitation of HRM is that alleles that differ on the basis of length such as variable number tandem repeats may not be readily resolved if the repeat number variations do not change the %G + C and the amplimer is of appreciable size. 51

**Loop-mediated isothermal amplification**

LAMP relies on auto-cycling strand displacement to generate DNA or RNA (using reverse transcriptase). Isothermal amplification has been used for the detection of several respiratory viruses including human and avian influenza viruses, RSV, hMPV, human coronavirus-NL63 and Middle East respiratory syndrome coronavirus (MERS-CoV). 52-59 Unlike rRT-PCR, LAMP assays cannot optimally detect multiple respiratory viruses simultaneously.

However, influenza virus A and B can be detected using two templates and three enzymes (thermostable DNA polymerase, reverse transcriptase and a thermostable nicking endonuclease). 52 In the absence of thermal cycling using specialised equipment, highly specific LAMP assays can be performed using small footprint, bench-top instruments such as the Alere i Influenza A&B (Alere, USA). Whilst this assay is less sensitive than rRT-PCR, the rapid turnaround time of 15 minutes allows it to be used as a ‘point-of-care’ test. 54,60

A recent evaluation of LAMP using six primers each targeting the matrix gene of RSV A and polymerase gene of RSV B compared to RT-PCR for the detection of RSV showed 100% concordance. 55

**Simple amplification-based, nucleic acid sequence-based and rolling circle amplification assays**

Simple amplification-based assay (SAMBA), nucleic acid sequence-based amplification (NASBA) and rolling circle amplification (RCA) are three other isothermal amplification methods that have been developed, but not widely used for detecting respiratory viruses.

As an alternative to rRT-PCR, influenza A(H1N1)pdm09 amplicons were visually detected using test strips following nucleic acid extraction and simple amplification. 61 The performance of SAMBA was comparable to rRT-PCR, but turnaround times were reduced by 25–50%. 55 A recent study determined that the limit of detecting influenza A and B virus using SAMBA is 95 copies and 85 copies, respectively.

Multiplex NASBA assays have been developed to detect influenza A and B viruses, parainfluenza virus (PIV) 1–4, RSV, rubella virus and Coxsackie viruses using enzyme-linked oligonucleotide capture optical detection. 63 By calculating the time to positivity, quantitation of human rhinovirus was also achieved using NASBA. 64

RCA is another isothermal amplification method that utilises circularisable oligonucleotides to detect target nucleic acid sequences. DNA ligase joins the two ends of juxtaposed probes upon hybridisation of a target DNA or RNA sequence, creating a template for the exponential, rolling circle amplification reaction. This method can be used for the qualitative detection for respiratory viruses and the detection of NI resistance conferred by previously identified SNPs. 55,66

**Polymerase chain reaction coupled with electrospray ionisation-mass spectrometry and matrix-assisted laser desorption ionisation-time of flight sequencing**

Mass spectrometry methods are beyond the scope of this review and not generally used in routine virology diagnostic laboratories. These methods have been detailed previously. 67–69 and
employ PCR prior to mass spectrometry analysis. RT-PCR ESI/MS and MALDI sequencing have been previously used for the identification of viruses, subtyping human and avian influenza viruses, and to elucidate the route of introduction and subsequent transmission of SARS-CoV by studying the evolution of single nucleotide variants in viral isolates. NGS has largely superseded these techniques.

In-house versus commercial assays and quality assurance
NAAT assays for the diagnosis of respiratory viruses are available commercially or may be developed in-house. Qualitative commercial assays are subject to stringent conformity assessments before approval for diagnostic use by the relevant administrative authorities, such as the Food and Drug Administration (FDA) in the USA or the Conformité Européenne (CE) in the European Union. In Australia, in-house developed NAATs are subject to regulatory requirements of the Therapeutic Goods Administration (TGA).

In-house NAAT assays are developed with reference to genomic sequences that have been previously determined. These assays are generally cheaper than commercial NAATs, although the latter are typically easier to operate. Open platform, in-house developed assays have the added advantage of flexibility, adaptability and ease of trouble-shooting. Their flexibility is especially useful when new viruses emerge in human populations. However, they require the use of specialised equipment and technical expertise, making them unsuitable as ‘point-of-care’ tests. Constant review of assay performance is important to ensure optimal sensitivity given the changing epidemiology of circulating viruses; this is particularly important with the annual antigenic drift of influenza viruses. The validation of different targets in multiplex assays during assay development may be challenging.

Commercial ‘black box’ closed platforms are simple to operate and offer convenience for smaller laboratories that lack more sophisticated equipment and expertise, but oligonucleotide primers and probes that are used are typically not published due to commercial confidentiality. Commercial assays are also unlikely to have been developed in the early phases of a newly circulating virus. Some examples of commercial NAATs and the viruses detected are provided in Table 2.

External quality assessment is necessary to ensure satisfactory performance of NAATs. At the time of writing, the Royal College of Pathologists of Australasia (RCPA) does not provide a quality assessment program specifically for the molecular diagnosis of respiratory viruses. However, the Quality Control for Molecular Diagnostics (QCMD) respiratory diseases program includes qualitative assessments for viruses commonly associated with respiratory infections including influenza virus, PIV, RSV, HRV, hMPV, coronavirus and HAdV.

NUCLEIC ACID AMPLIFICATION TESTS FOR THE DIAGNOSIS OF RESPIRATORY VIRUSES: WHY AND WHY NOT?

The argument for
NAATs are more sensitive than the ‘gold standard’ of cell-culture methods for the diagnosis of respiratory viruses and more specific than antigen detection tests including immunochromatographic and immunofluorescence assays. NAATs are crucial where virus culture cannot be performed. High-throughput, automated, easy to use multiplex assays place NAATs at the diagnostic forefront in developed countries where costs may be less of a concern. The use of multiplex PCRs has facilitated studies on the impact of viral and/or bacterial co-infection in severe respiratory infections.66,71

The heterogeneity of assays and populations studied makes it difficult to draw definitive conclusions about the utility of NAATs in the management of viral respiratory infections. The increased sensitivity and significantly reduced turnaround time of NAATs compared to viral culture improves patient care by guiding antiviral therapy.72

Although some studies have demonstrated the benefits of NAATs in reducing the prescription of antibiotics in viral respiratory infections, others have failed to show significant reductions in testing, antibiotic costs and hospital length of stays despite increases in viral diagnostic yields from 21% to 43%.73,74 As yet unexplored is the clinical impact of LAMP assays that have comparable turnaround times to rapid influenza diagnostic tests, which have reduced unnecessary ancillary testing and antibiotic prescriptions but increased antiviral prescription when used in emergency departments.75 Furthermore, the clinical and financial benefits derived from NAATs that benefit clinicians may not be seen by the laboratory.

The argument against
Cost is the main limitation to more widespread use of NAATs for diagnosing viral respiratory infections at present. ‘Black box’ closed platforms may not offer the throughput required, and the range of viruses detected may be inadequate. For example, the single-sample loop-mediated isothermal Alere i Influenza A&B assay is able to detect influenza viruses only.60

The sensitivity of NAATs can be affected by mutations in primer and probe binding regions in viruses subject to genomic ‘drifts’ and potential for reassortments or recombination such as with influenza or enteroviruses. Periodic re-evaluation of assays through quality assurance programs therefore is paramount to ensure optimum performance.

Novel viruses, variants or reassortments of viruses may not always be identified by PCRs that rely on a priori sequence. Viruses generally lack universally conserved genetic regions such as ribosomal RNA that are present in bacteria or fungi, although degenerate primers may occasionally target conserved regions within related virus groups. Although this may be overcome by sequencing methods, isolation of a novel virus is instrumental for developing NAATs or other test methods including serology to facilitate the rapid diagnosis of a pathogen.76,77

NAATs may also lack specificity, particularly in children, where multiple viruses may be detected when multiplex RT-PCR assays are used. In children, NAAT results require cautious interpretation given the high detection rates of respiratory viruses in asymptomatic subjects. By their nature, current NAATs are relatively ‘closed’ in terms of pathogen detection. NGS will assist with this issue, but still remains out of the reach of routine laboratories. Therefore, more ‘open’ tests including virus isolation, electron microscopy and pathogen-specific serology are still needed to detect novel pathogens (e.g., SARS-CoV, MERS-CoV) or subtypes (e.g., influenza A/H5N1). These methods are generally only available in reference laboratories.78 Serology is an option when respiratory sample collection is too late in the clinical course, or for epidemiological studies.78–84
Table 2 Examples of commercial assays used for detecting respiratory viruses

| Technology | Assay type | Pathogens detected | Turnaround times (hours) | Random access | Maximum samples per run | Performance |
|------------|------------|--------------------|--------------------------|---------------|-------------------------|-------------|
| PCR with melting curve analysis | AusDiagnostics | Influenza A/H1 and H3, influenza B, RSV, HRV/HEV, PIV 1–4, HAdV, hMPV, CoV (NL63, HKU1, 229E, OC43) | ~4 h | No | 6 | Good correlation with xTAG RVP and FTD; >92.1% agreement for influenza A, RSV, PIV, Picornaviridae, HAdV, hMPV, CoV, HBV/V | |
| Integrated automated sample preparation and total nucleic acid extraction with nested, multiplex RT-PCR | FilmArray RVP | Influenza A/H1N1, A/H3N2, A(H1N1)pdm09, influenza B, RSV, PIV 1–4, hMPV, HAdV, HRV/HEV, CoV (NL63, HKU1, 229E, OC43), B. pertussis, M. pneumoniae, C. pneumoniae | ~1 h | Yes | 1 | Sensitivity and specificity of 85–100% and 100% respectively for respiratory viruses tested in panel | |
| rRT-PCR | Simplexa Direct Flu A/B and RSV | Influenza A, influenza B, RSV | ~1 h | Yes | 96 | Sensitivity of 91.7% and 97.5% in detecting influenza A and B respectively for non-extracted samples compared to Seeplex RV15 | |
| Microcapillary electrophoresis | Seeplex RV15 | Influenza A/H1N1, A/H3N2, A(H1N1)pdm09, influenza B, RSV A and B, PIV 1–4, hMPV, HAdV, HRV/HEV, CoV (NL63, HKU1, 229E, OC43) | ~7 h | No | 32 | Overall sensitivity of 93.3% (range 82.1–100% depending on the respiratory virus) compared to 87.2% for xTAG RVP | |
| Microsphere hybridisation associated with flow cytometer detection | xTAG RVP | Influenza A/H1, A/H3, influenza B, RSV, hMPV, HRV, HAdV | ~6 h | No | 96 | Less sensitive and longer turn around times compared to FilmArray RVP in paediatric patients | |
| Nanoparticle probes | Verigene RV+ | Influenza A/H1, A/H3, (A(H1N1)pdm09, influenza B, RSV | <2.5 h | Yes | 1 | Sensitivity of 96.6%, 100% and 100% for the detection of influenza A, influenza B and RSV respectively compared to Simplexa Flu A/B & RSV | |

CONCLUSIONS

Although imperfect, high throughput, multiplex NAAT assays targeting different viruses are likely to be the most commonly used method for the diagnosis of viral respiratory infections in the short to medium term. Nucleic acid extraction-independent and LAMP assays are promising, but are limited by the range of viruses that can be detected and the performance of LAMP assays remains suboptimal compared to RT-PCR at present. In the near future, diagnostic virology laboratories will face increasing pressures to provide specific viral aetiologies of respiratory infections within clinically relevant timeframes to facilitate targeted antiviral therapy.

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