

Utilization of Nucleic Acid Amplification Assays for the Detection of Respiratory Viruses

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KEYWORDS

- Nucleic acid amplification assays • Respiratory virus
- Influenza • Respiratory syncytial virus
- Molecular methods • PCR

Viruses are major contributors to morbidity and mortality from acute respiratory infections (ARIs) in all age groups worldwide. In addition to the enormous burden of upper respiratory syndromes caused by them, they are a leading cause of hospitalizations for lower respiratory infections (LRIs) in all age groups. Estimates of the national disease burden of respiratory viruses report that approximately 300,000 children are hospitalized each year in the United States with a specific diagnosis of viral LRI, and an additional 500,000 children are hospitalized with a clinical diagnosis of viral LRI, at a direct cost estimated at nearly \$1 billion per year.¹ Rapid viral diagnosis has been demonstrated to significantly decrease length of hospital stay, additional laboratory testing, and unnecessary antibiotic use; it additionally helps direct specific antiviral therapy.^{2,3}

Accurate identification of the etiologic agent of respiratory tract infections is important for proper patient management. Diagnosis can be problematic, because a range of potential pathogens can cause similar clinical symptoms. Nucleic acid amplification testing is emerging as the preferred method of diagnostic testing. Real-time technology and the ability to perform multiplex testing have facilitated this emergence. Commercial methodologies for nucleic acid amplification testing of respiratory viruses

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include real-time polymerase chain reaction (PCR), nucleic acid sequence-based amplification (NASBA), and loop-mediated isothermal amplification (LAMP). Multiplex PCR with fluidic microarrays or DNA chips is the most recent diagnostic advance. Before the issuance of the guidance document on analyte-specific reagents (ASRs) by the US Food and Drug Administration (FDA),⁴ primers and probes were being developed, packaged, and sold as ASRs. Since then, some of these reagents are no longer commercially available; some have been submitted and received FDA approval as *in vitro* diagnostic devices, and some primer and probe reagents are sold separately without recommendations for use. This article discusses commercially available molecular methods and their performance characteristics for the detection of respiratory viruses.

COMMERCIALY AVAILABLE ASSAYS FOR THE DETECTION OF RESPIRATORY VIRUSES

Influenza Virus

Influenza viruses remain significant causes of ARI every year despite availability of vaccines and increasing efforts to achieve targeted vaccination rates. In 1998, these viruses were estimated to cause 39,000 hospitalizations per year in children nationally in the US.¹ The outpatient visits associated with influenza were reported to be approximately 10, 100, and 250 times as high as hospitalization rates for children 0 to 5 months, 6 to 23 months, and 24 to 59 months of age, respectively.⁵ Substantial burden from influenza-associated hospitalizations and deaths has been ascertained in several studies, most severely affecting those older than 65 years but also being significant in the 50- to 65-year-old adults, in whom hospitalization rates were determined to equal those among children younger than 5 years.⁶ Antigen assays for the detection of influenza are commonly available. The likelihood of a negative result using these assays increases significantly with the increasing age of the patient, whereas virus culture and reverse transcriptase PCR (RT-PCR) do not; for older patients, RT-PCR or culture is necessary to avoid false-negative results.⁷

Molecular methods for detecting influenza viruses were described as early as 1991. Various targets, including the matrix, HA, and N genes have been used. The first commercially available assay was the Hexaplex assay (Prodesse Inc, Waukesha, Wisconsin), an RT-PCR assay that employed enzyme hybridization postamplification detection. The assay was technically demanding, requiring postamplification purification; it was also expensive and took 8 to 9 hours to complete.⁸ This assay had an analytical sensitivity of 10 copies for influenza A and 5 copies for influenza B⁹ and set the standard for the detection of influenza viruses. Since then, it has been redesigned and is now an FDA-approved multiplex real-time RT-PCR assay (Pro-Flu+, Prodesse Inc.) for detecting influenza A (M gene), influenza B (NS1 and NS2 genes), and respiratory syncytial virus (RSV) (polymerase gene). It can be performed using a MagNA Pure LC Instrument (Roche Diagnostics Corporation, Indianapolis, Indiana), or the NucliSens easyMAG System (bioMérieux Inc, Durham, North Carolina) for extraction with amplification and detection on the SmartCycler system (Cepheid, Sunnyvale, California). According to the manufacturer, it has a clinical sensitivity and specificity of 100% and 93% for influenza A and 98% and 99% for influenza B, respectively, and an analytical sensitivity of 10² 50% tissue culture infectious dose (TCID₅₀)/mL for both viruses. The xTAG Respiratory Virus Panel (Luminex Molecular Diagnostics, Toronto, Canada) also is an FDA-approved multiplex panel for detecting respiratory viruses. While capable of detecting 17 respiratory viruses, it is FDA-approved for detecting RSV; parainfluenza 1, 2, and 3; metapneumovirus; adenovirus, and rhinovirus, in addition to influenza. The assay employs multiplex PCR and fluid

microsphere-based array detection on the Luminex x-MAP system (flow cytometer). It has a reported clinical sensitivity of 100% for influenza A¹⁰ and an analytical sensitivity of 10 TCID₅₀.¹¹ In addition to these FDA-approved assays, ASRs for influenza are commercially available from EraGen Biosciences (Madison, Wisconsin) and Cepheid (Sunnyvale, California). These assays have no performance claims made by the manufacturer, and little information is published regarding their performance. The literature is replete with home-brew or in-house developed assays for detecting influenza using various amplification methodologies. In evaluating a home-brew assay or an ASR, an analytical sensitivity of 10 TCID₅₀ is desirable. In comparison with antigen assays and culture, clinical sensitivity of 98% to 99% should be easily attainable, along with the detection of additional viruses missed by culture. An alternate PCR methodology can confirm these as true positive specimens. PCR-based respiratory virus assays have demonstrated significantly better sensitivity than antigen detection assays, both enzyme immunoassays^{7,12} where adults may have a false-negative rate of up to 29%,¹³ and immunofluorescent assays¹⁴ They also have better sensitivity than culture^{15,16} in children¹⁷ and particularly in adults.^{18–20} Several manufacturers have assays available as research use only (RUO) kits. There are several kits, the Multi-Code-PLx (EraGen Biosciences, Inc) and the ResPlex II Panel (Qiagen Inc, Valencia, California), that employ the Luminex system, and these have reported performance claims similar to the xTAG Respiratory Virus Panel.^{21,22,23} Automated solid film microarray assays also are being developed,²⁴ but none are commercially available.

Respiratory Syncytial Virus

Respiratory syncytial virus (RSV) is accepted as the most important respiratory viral pathogen in infants and young children. It long has been recognized to cause the highest number of pediatric hospitalizations from LRI, with estimates of more than 90,000 to 112,000 hospitalizations annually in children younger than 5 years.¹ Subsequent studies have shed light on the enormous outpatient and emergency room burden of illness associated with this virus also.²⁵ Thus, in addition to causing annual winter epidemics of bronchiolitis and pneumonia in young children requiring hospitalization, RSV also leads to significant outpatient disease. The virus further has been established as an important pathogen of respiratory disease in the elderly and high-risk adults.²⁶

Like influenza, the Hexaplex assay was the first commercially available assay for detecting RSV. It had an analytical sensitivity of 42 to 4200 copies⁸ and a clinical sensitivity and specificity of 99% and 97%, respectively, in children¹² and 91% and 99% in a mixed population of adults and children.⁸ It, too, has been redesigned into the FDA-approved multiplex real-time RT-PCR assay, the Pro-Flu+, which, according to the manufacturer, has an analytical sensitivity of 10⁻¹ to 10¹ TCID₅₀ and a clinical sensitivity and specificity of 90% and 95%, respectively. In a study of an earlier version performed on pediatric samples, the assay had a sensitivity and specificity of 99% and 100%.¹⁷ The assay has a sensitivity of 95% as compared with 82% for the enzyme immunoassay and 57% for culture.²⁷ The FDA-approved xTAG Respiratory Virus Panel has an analytical sensitivity of 10² TCID₅₀/reaction¹¹ and a clinical sensitivity of 97%. The ASR commercially available from Cepheid has an analytical sensitivity of 12 copies per reaction and a clinical sensitivity of 100% when compared with enzyme immunoassay. A 22% increase in positive patients, however, was seen with the ASR. This increased positivity rate by RT-PCR is consistent with that reported by others when compared with antigen detection methods.²⁸ In-house assays for detecting RSV have been developed using the F, N, or L polymerase gene targets. These studies report 82% sensitivity of the antigen assay,²⁹ low sensitivity of culture,³⁰

and an increase in detection of positive patients by RT-PCR.^{14,31} This increased sensitivity of RT-PCR has been reported in immunocompromised adults also.³² Nucleic acid amplification assays are the most sensitive method for the detection of RSV³⁰ regardless of the population tested. Specimens with low viral load, as is seen in adults and immunocompromised patients, are more likely to be antigen-negative and RT-PCR positive^{14,27} The MultiCode-PLx RUO assay has reported performance claims similar to the xTAG Respiratory Virus Panel,^{21,34} while the ResPlex II Panel RUO assay has a significantly lower sensitivity of 73%.²² Automated microarrays are being developed for RSV also.²⁴

Parainfluenza

Human parainfluenzaviruses types 1, 2, and 3 (HPIV-1, HPIV-2, and HPIV-3) are important causes of ARI in all age groups. They cause upper respiratory tract infections (croup) in both children and adults, and lower respiratory tract infections in infants, young children, the elderly, the immunocompromised, or those with chronic medical conditions.³⁵ HPIVs are second only to RSV as a cause of hospitalizations for LRI in children^{1,35} The different subtypes are associated with distinct clinical syndromes, age groups, and seasonality.³⁵ The clinical significance and epidemiology of a fourth type (HPIV-4), although discovered more than 40 years ago, are understood less well than the other three types.

The only FDA-approved assay for detecting HPIV-1, 2, and 3 is the xTAG Respiratory Virus Panel. It has an analytical sensitivity 100, 100, and 25 TCID₅₀/reaction¹¹ and a clinical sensitivity of 100%, 92% and 100% for HPIV-1, 2, and 3, respectively¹⁰ The MultiCode-PLx RUO assay has reported sensitivity of 85% to 90%,^{34,23} while the ResPlex II Panel RUO assay has a significantly lower sensitivity of 72% for HPIV-3.²² These evaluations suffer from a low number of positive samples, particularly for HPIV-2. The Pro-Parafly+ assay from Prodesse is also an RUO assay; however, there are no published reports of its performance. The Hexaplex assay had reported sensitivity of 100% for parainfluenzaviruses with an increased detection in the number of positive patients, suggesting that it was more sensitive than culture^{8,12} and that sensitivity of this nature is achievable with the proper primers, probes, and conditions. Other in-house developed assays have reported similar findings.^{14,36} A study comparing an in-house assay with direct fluorescent antigen reported a sensitivity of only 52% for antigen detection and 99% for RT-PCR.¹⁴ Nucleic acid amplification assays are preferable to direct fluorescent antigen assays and culture for detecting parainfluenzaviruses.

Adenovirus

Adenoviruses are ubiquitously distributed viruses that are common causes of self-limiting respiratory, ocular, or gastrointestinal illnesses and outbreaks in immunocompetent children and United States military recruits. They have been reported to cause severe, prolonged, and sometimes fatal illness in immunocompromised hosts. The spectrum of disease in the latter population includes pneumonia, hepatitis, hemorrhagic cystitis, colitis, pancreatitis, myocarditis, meningoencephalitis, and disseminated disease, depending on host and virus characteristics.³⁷ Seven species (A to G) and 52 serotypes have been described, some associated with distinct clinical syndromes involving specific organ systems.

Most nucleic acid amplification assays for detecting adenovirus detect the hexon gene although assays targeting the fiber gene have been reported. The xTAG Respiratory Virus Panel is the only FDA-approved assay for detecting adenovirus from respiratory specimens. It has an analytical sensitivity of 10² TCID₅₀/reaction and a clinical

sensitivity of 78%.³³ Adenovirus also is included in the MultiCode-PLx RUO assay and has reported sensitivity of 100%; however, only a small number of positive samples were tested.²¹ An in-house developed assay targeting the hexon gene has a clinical sensitivity of 98%, with indirect immunofluorescent antigen test having a sensitivity of only 24%. This study also showed a several log difference in the concentration of viruses in specimens positive by PCR alone as compared with specimens positive by both immunofluorescent antigen testing and PCR.¹⁴ Well-designed nucleic acid amplification assays that can detect all of the adenovirus serotypes are the most sensitive method for the detection of adenovirus. Because of the wide spectrum of disease, validation of the assay with a wide range of specimen types is important.

Metapneumovirus

Human metapneumovirus (hMPV), the most well-studied of the new viruses, was discovered in 2001; since then, it has been established as a significant respiratory pathogen in children and adults.^{38,39} It has been reported to be the second most common causative agent in bronchiolitis in infants, and it contributes to a substantial burden of upper respiratory tract infections including acute otitis media in older children. The virus is a common cause of mild upper respiratory infection (URI) or asymptomatic illness in healthy adults. Severe illness resulting in hospitalizations including ICU admissions has been described in the elderly, adults with underlying conditions, and the immunocompromised.³⁹

The xTAG Respiratory Virus Panel is an FD- approved assay for detecting hMPV from respiratory specimens. It has an analytical sensitivity of 0.1 TCID₅₀/mL and a clinical sensitivity of 97%,³³ detecting significantly more positives than the direct immunofluorescent assay. Pro-hMPV+ (Prodesse) is an FDA-approved real-time RT-PCR. According to the manufacturer, it has an analytical sensitivity of 10¹ to 10² TCID₅₀ and a clinical sensitivity of 95%. When compared with the previously available NucliSENS ASR, both assays showed excellent sensitivity.⁴⁰ The MultiCode-PLx RUO assay has reported sensitivity of 100%; however only a small number of positive samples were tested.^{21,23} The ResPlex II Panel RUO assay has a significantly lower sensitivity of 80%.²² There are few reports of in-house developed assays for metapneumovirus, and those suffer from low number of positive samples and lack of a comparator assay.^{14,41}

OTHER RESPIRATORY VIRUSES

Viruses previously considered to be upper respiratory pathogens such as rhinoviruses and coronaviruses (CoVs) more recently have been reported to play a role in respiratory hospitalizations also. Rhinoviruses classically have been associated with URIs, including the common cold, otitis media, and sinusitis, causing illnesses throughout the year but with peaks in early fall and spring.⁴² The clinical significance and precise role of rhinoviruses in LRI in healthy hosts need further investigation. No assay is approved by the FDA for detecting rhinovirus. All three assays employing multiplex PCR and fluid microsphere-based array detection on the Luminex x-MAP system include rhinovirus as a target. The xTAG Respiratory Virus Panel has an analytical sensitivity of 3×10^{-2} TCID₅₀/mL and clinical sensitivity and specificity of 100% and 91%, respectively.³³ The MultiCode-PLx also had a sensitivity of 100%, with some false-positive results.³⁵ In-house developed assays employing NASBA and RT-PCR showed sensitivities of 85% and 83%, respectively. Both assays were significantly more sensitive than culture (45%).⁴³ There appear to be rhinoviruses that are not detected using these molecular methods and positive results that cannot be

confirmed by culture. More sequence data from different rhinovirus serotypes and currently circulating strains are needed to improve primer coverage.

Five CoV species have been described to cause human disease. CoV-229E and CoV-OC43, described in the 1960s are causative agents of the common cold and LRIs in young children and elderly adults.^{44,45} Severe acute respiratory syndrome CoV (SARS-CoV), the causative agent of an outbreak of SARS worldwide from 2002 to 2003 has not been found to circulate in people since 2004. Two new CoVs, however, CoV-NL63 and CoV-HKU1, were described in 2004 and 2005 respectively. These viruses, although newly discovered, subsequently have been shown to have been circulating in people for a long time. These new CoVs have been associated with both URIs and LRIs.⁴⁶ No assay is approved by the FDA for detecting CoV. The xTAG Respiratory Virus Panel is not FDA-approved for CoV but has been used to study the epidemiology of the virus. It has an analytical sensitivity of 50 genome equivalents.³³ The MultiCode-PLx RUO assay includes CoVs except for HKU1. Published reports suffer from inadequate number of positive specimens to assess performance.³⁴

Bocavirus, a new virus discovered in 2005 using nonspecific nucleic acid amplification techniques, has been detected in 2% to 19% of samples from patients with acute respiratory symptoms.⁴⁷ Limited sequence data are available for bocavirus, making selection of conserved regions difficult. Detection using real-time PCR methods, however, has been used to detect bocavirus.⁴⁸ Although an increasing number of studies have reported detection of bocavirus in patients who have respiratory illnesses, determination of a causal relationship in LRI has been difficult given:

The high rates of codetection of other respiratory pathogens

Prolonged detection in some patients raising questions about possible persistence or prolonged infections

Lack of cell culture or serodiagnostic methods that could help differentiate true infection from nucleic acid detection

CONSIDERATIONS FOR IN-HOUSE DEVELOPED ASSAYS

Extraction

An important, yet underemphasized aspect of nucleic acid amplification-based diagnostic methods is the extraction system used. Detection of respiratory viruses cannot be performed adequately without specimen extraction.^{49,50} Respiratory viruses are predominantly RNA genomes; however, adenovirus and bocavirus are DNA viruses that should be included in a diagnostic screening assay. An extract that contains total nucleic acids is most useful for that type of application. Different systems have differing abilities to recover RNA, DNA, or total nucleic acids.^{40,51–54} When determining the performance characteristics of laboratory-developed assays or assays employing ASRs, the performance characteristics of the extraction system also must be documented. FDA-approved kits are approved using a specific method of extraction. Use of a different method by a laboratory requires the user to perform a complete revalidation of the performance characteristics of the kit.

Primer and Probe Design

There are numerous reports in the literature of laboratory-developed assays for detecting respiratory viruses. Many of these reports include the target gene; some include the amino acid position, and few include *in silico* coverage rates for the primers and probes described.⁵⁵ The amplification and detection format employed will determine the number of mismatches permissible while still retaining target detection. It is

important to check the coverage regularly to ensure that newly emerging strains can be detected.

Amplification Method

PCR or RT-PCR are the most commonly used nucleic acid amplification methods for detecting respiratory viruses; many of these employ primers to a single target. Design of multiplex assays requires good primer design and extensive validation to document lack of interference. There are reports of in-house developed multiplex assays and commercially available products. The implementation of touchdown amplification protocols that involve a stepped reduction in the annealing temperature, which introduces an advantage for specific binding, allows for multiplexing and a common amplification protocol.⁵⁶ Isothermal amplification procedures such as NASBA^{16,40} and LAMP⁵⁷ also have been used for detecting respiratory viruses.

Detection

Real-time detection eliminates the manipulation of amplified products, which minimizes problems associated with amplicon, contamination, carryover, and false-positive reactions. It also decreases the turnaround time of the assays. Real-time detection most often employs target specific probes, thus also increasing the specificity of the assay. Various probe chemistries and labels are available. The choice of probe format and label often is determined by the detection platform employed. Spectral overlap of fluorescent labels, however, also limits the number of multiplex target detection options. Thus, real-time detection is extremely useful; however, it is not adaptable to broad range multianalyte screening.

To improve amplification efficiency and expand multiplexing ability, postamplification detection must be employed. Postamplification detection requires handling of amplicon and significantly increasing turn around time. The advantage is the ability to detect multiple pathogens in a single assay. Because of the large number of viruses capable of causing similar symptoms in patients, the diagnosis of respiratory tract infections seems uniquely suited for this application. Solid-phase microarrays allow for a large number of probes to be employed and also may allow for variable hybridization conditions such that detection of a large number of targets or mismatched targets can be accomplished. Suspension microarrays employ a liquid phase bead conjugated array. These allow for rapid hybridization conditions and flexibility in assay design. Bead makeup can be modified easily. Although no automated microarray system is approved by the FDA, these systems have the potential to decrease hands on technologist time; however, it currently is at the cost of turnaround time.²¹

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