Mol Diagn Ther 2010; 14 (5): 283-293
1177-1062/10/0005-0283/$49.95/0

© 2010 Adis Data Information BV. All rights reserved.

Molecular Diagnostic Assays for Detection of Viral Respiratory Pathogens in Institutional Outbreaks

Nick A. Antonishyn and *Paul N. Levett*

Saskatchewan Disease Control Laboratory, Regina, Saskatchewan, Canada

Contents

Abstract . . . 283
1. Conventional Detection Methods . . . 284
  1.1 Rapid Detection of Respiratory Viruses . . . 284
  1.2 Virus Culture . . . 284
2. Molecular Diagnostic Assays . . . 285
  2.1 Recognition of Outbreaks . . . 285
  2.2 Conventional versus Molecular Methods . . . 285
  2.3 Advantages of Molecular Assays . . . 286
3. Probe-Based PCR Assays . . . 286
4. Commercial PCR-Based Applications . . . 289
  4.1 Seeplex® . . . 289
  4.2 Infiniti® Microarray . . . 289
  4.3 xMAP Technology . . . 289
  4.4 Plex-ID . . . 290
  4.5 MassTag PCR . . . 291
  4.6 FilmArray™ . . . 291
5. Conclusion . . . 291

Abstract

Outbreaks of viral respiratory disease in institutions may be associated with high morbidity and mortality, depending upon the viral etiology and the age and immune status of the affected patients. Control of outbreaks may include isolation and/or cohorting, and prohylaxis or treatment with specific antiviral agents may be indicated, all dependent upon the specific cause of the outbreak. Conventional methods of viral diagnosis detect only a limited number of the viruses that are known to cause outbreaks. The availability of sensitive and specific molecular assays has facilitated rapid diagnosis of a wider range of viruses from respiratory outbreaks. Molecular methods have distinct advantages over conventional methods, including the ability to rapidly develop assays for emerging viruses and new variants of existing viruses. In addition, molecular testing allows rapid detection of resistance to antiviral agents or mutations leading to increased virulence. However, high-throughput molecular testing requires batch processes that may compromise the ability to respond quickly to urgent testing demands.

Outbreaks of viral respiratory disease in institutions present significant challenges for infection control. Morbidity and mortality may be high, depending upon the viral etiology and the patient population that is affected.[1-4] Control of outbreaks may include isolation and/or cohorting, and prophylaxis or treatment with specific antiviral agents may be indicated, all dependent upon the specific cause of the outbreak.[5] Traditionally, influenza viruses, parainfluenza viruses, respiratory

syncytial virus (RSVs), enteroviruses, and adenoviruses have been regarded as important pathogens causing nosocomial outbreaks. In recent years, the role of rhinoviruses, coronaviruses, and human metapneumovirus (hMPV) in outbreaks has also been increasingly recognized.[6-9] The importance of the latter group of viruses has become clear only with widespread availability of molecular diagnostic tests. More recently, human bocavirus has been added to the list of potential etiologies.[10,11]

Surveillance of respiratory disease outbreaks in long-term care facilities has shown that outbreaks of influenza are clinically indistinguishable from other viral outbreaks.[12] The management of outbreaks is beyond the scope of this review but may include isolation of affected cases and cohorting of contacts, in addition to prophylaxis and/or treatment with antiviral agents. The duration of isolation or cohorting and the initiation and cessation of anti-viral administration, as well as the selection of the specific agent, will be mandated by the identity of a specific viral etiology, particularly if influenza is detected.[13]

Conventional methods of viral diagnosis are limited both by the ability to isolate only a limited range of the viruses causing respiratory diseases and by the restricted range of viruses that can be diagnosed rapidly by antigen detection. The availability of sensitive and specific molecular assays has facilitated more rapid diagnosis of a much wider range of viruses from respiratory outbreaks and has enhanced our understanding of the viral etiology of such outbreaks. Both multiplex and single-target molecular assays enhance sensitivity, compared with traditional methods.[14,15] Furthermore, it has been shown that the use of sensitive and broad-spectrum molecular assays can enhance diagnosis through detection of a respiratory virus in cases where the presence of the agent was not suspected or a test for the etiologic agent was not specifically ordered by the clinician.[16,17] Nevertheless, further validation of emerging technologies for multi-targeted molecular diagnosis of viral infections will be needed to ensure broad virus amplification and detection.[18,19]

This review presents a critical analysis of molecular methods available for use in the investigation of viral outbreaks. For the purposes of this review, diagnostic assays for detection of viral pathogens are divided into three broad categories: traditional methods; commercial molecular assays; and in-house-developed or home-brew molecular tests. This review is not intended to be a meta-analysis of available in-house and commercial assays for molecular diagnostics of respiratory viruses; rather, it is intended to present a succinct, high-level categorization of the different molecular approaches, based on the multiplex/demultiplex approach that is used, with examples that will serve to illustrate the advantages and disadvantages of each category for outbreak investigation in the context of common tests in use today, from the point of view of a reference or public health laboratory.

1. Conventional Detection Methods

1.1 Rapid Detection of Respiratory Viruses

Rapid detection of influenza and parainfluenza viruses and RSV (and, more recently, hMPV) is conventionally performed using direct fluorescent antigen staining and enzyme immunoassays. Immunofluorescence assays that are directed against enteroviruses have also been applied to picornavirus detection in respiratory specimens.[20] In many cases, this approach remains the most rapid means of detecting an etiologic agent in an outbreak, despite having lower sensitivity than molecular methods.

In addition, rapid antigen tests are available for influenza and RSV, but these assays have low sensitivity compared with culture and molecular methods.[21,22] The sensitivity of antigen tests for influenza viruses may be as low as 25%, compared with culture,[23] and it is significantly lower than the sensitivity of direct fluorescent-antibody assays. The analytical sensitivity of rapid antigen methods for detection of influenza A is approximately four 50% tissue culture infective doses ($TCID_{50}$).[24]

Electron microscopic detection of viruses has been replaced in many laboratories by other methods. This approach has disadvantages in terms of the high cost of the equipment, the significant expertise required to prepare specimens and interpret results, and lower sensitivity than molecular methods.[25] However, examination of negative-stained specimen preparations offers the most rapid means of scanning for a wide range of viruses,[26] which may not be detectable by specific molecular assays or staining procedures. In addition, electron microscopy may be useful for identification of viruses grown in cell cultures,[27] particularly if these are previously unrecognized viruses.[28]

1.2 Virus Culture

Conventional cultures for isolation of respiratory viruses usually involve inoculation of specimens onto at least three cell lines, including primary monkey kidney cells, human diploid fibroblast cells, and a human heteroploid cell line. Inoculated cultures are usually incubated for at least 10–14 days. Cultures of respiratory specimens are usually incubated in a rotating drum, which encourages faster growth of respiratory viruses and earlier detection of cytopathic effects.[29]

© 2010 Adis Data Information BV. All rights reserved.

Cultures performed in shell vials after centrifugation are much more rapid than conventional tube monolayers,[30] usually giving results within 24–48 hours. Similarly, co-cultured mixtures of cell types have been used for more rapid detection of respiratory viruses.[31] These approaches have the disadvantage that fixation and staining limit the range of viruses that can be detected in a single culture. As reagents become available for newly recognized viral pathogens,[32,33] the number of shell vials that are inoculated can be increased, with additional costs.

2. Molecular Diagnostic Assays

2.1 Recognition of Outbreaks

The decision to apply a multiplex panel of tests to specimens must be triggered by the recognition of an outbreak.[34] Given the relatively high per-specimen costs of multiplex molecular assays, it is often impractical to apply these assays to all specimens from potential outbreaks. Therefore, it is becoming standard practice to test only a sample of specimens from large outbreaks. The basis for limiting testing to a small number of samples from an outbreak is derived from studies of norovirus outbreak detection.[35,36] However, early specimen collection is important if the sensitivity of this approach is to be exploited. Excretion of virus, and detection rates, decline after the first 5 days of illness.[37,38]

2.2 Conventional versus Molecular Methods

Conventional virologic methods that have been used for detection and identification of viral respiratory pathogens remain useful. In some circumstances, conventional methods can be preferable to molecular methods, for a range of biologic and technical reasons.[19] For example, the culture of viruses permits full identification and characterization of any virus that can be isolated, including phenotypic confirmation of antiviral susceptibilities. Moreover, culture can often detect novel strains or emerging viruses. Conversely, molecular methods are often more sensitive but are limited to the intended target virus, requiring knowledge of the genome sequence to provide a complete template from which molecular assays can be developed rapidly. Thus, failure to detect any of the targets in a molecular assay does not rule out the absence of a virus that is not included in the assay. This is countered in practice by the ability to rapidly design and detect new viruses, using degenerate primers.[28]

The greater sensitivity offered by molecular methods also comes with a cost of higher quality control standards and assurance programs to prevent and detect false positives due to contamination. Laboratories that use nucleic acid amplification technologies must take precautions to prevent false positive results that might arise from contamination of specimens by amplification products of previously analyzed specimens. To avoid contamination, the workflow must be strictly unidirectional with no movement of specimens or materials in the reverse direction. Ideally, procedures should be carried out in four physically separated rooms for dedicated tasks of reagent preparation, specimen preparation, reaction set-up, and post-amplification analysis.[39] Most importantly, the post-amplification room should have 100% exhaust air with negative pressure relative to that in all other laboratories. The reagent preparation room should have 100% supply air with positive pressure relative to that in all other laboratories. Each laboratory should have dedicated equipment, supplies, and lab coats. Use of sticky mats, dedicated personnel, and strict hand-washing policies are helpful. Unwanted spread of amplicons outside the post-amplification room can be monitored with environmental swabs. In comparison, conventional virology is less prone to contamination issues than molecular methods.

In-house or home-brew assays also require user validation for both analytical limits of detection and, more importantly, clinical sensitivity and specificity.[40,41] In general, for manufactured assays, the performance specifications that are stated by the manufacturer must be verified. Modification of the original assay must always be followed by re-establishment of the performance specifications. For in-house assays, establishment of the performance specifications of the assay must be done with the appropriate sample type. Supplier and lot differences for any analyte-specific reagents typically lack performance specifications, and therefore their effect on assay performance must be re-established by the user after any change in the reagent supplier or even in the reagent lot from the same supplier. Typically, the supplier of manufactured assays provides these quality control assurances.

Another disadvantage that most molecular methods have over conventional virologic techniques is that the molecular approach often requires batch processing of specimens. Once batched, the specimens are often queued and processed in a 96-well format for the necessary extraction process and subsequent amplification and detection steps. Conversely, rapid antigen detection assays and direct fluorescent antibody methods can be completed in less than 1 hour, and testing can be started as specimens arrive at the laboratory. The processing steps can be done individually and therefore are not driven by the same requirement for batch processing that are common to high-throughput molecular methods.

© 2010 Adis Data Information BV. All rights reserved.

2.3 Advantages of Molecular Assays

Despite the limitations of molecular assays discussed above, classical methods such as culture are now becoming secondary tiers in the triage of diagnostic options for investigation of institutional outbreaks. This is largely driven by the increased sensitivity and specificity that molecular diagnostic assays afford. For example, an influenza A molecular assay has been shown to be sensitive to as little as two virus particles per microliter.[42] However, molecular assays will be found to be even more sensitive in practice, since amplification technologies can detect nonviable virus. This makes molecular diagnostic assays several-fold more sensitive than culture in outbreak investigations, where specimen collection, handling, and transport conditions can be less than ideal. In comparison with culture, molecular assays can also provide a much faster time to result, which is vital for effective intervention during an outbreak investigation.

Consequently, multi-targeted molecular assays are now becoming more cost effective, with the increased cost of testing being offset by the reduction in patient care costs that is possible with an earlier diagnostic result.[43] This economic advantage can be even more pronounced with outbreak investigations in which the time to result is of the utmost importance to prevent the spread of disease within institutions. However, the specificity of molecular assays means that laboratories must circumvent this potential limitation, either with a multi-targeted approach or by setting up several individual molecular assays, each targeting a different virus or class of viruses.

Multiplexed PCR assays for respiratory viruses can retain good sensitivity with proper validation.[44-46] However, single-target PCRs are the easiest assays to validate and have the best sensitivity and specificity. This is because of the lack of competitive inhibition effects and compromised buffer and thermal cycling conditions that are often necessary to optimize multiplex PCR. Moreover, compared with single-target assays (which may be referred to as uniplex, monoplex, or single-plex assays), multiplex assays require more quality controls, complex performance evaluations and data analysis algorithms, and more complex reporting of results.[47] Essentially, the increased number of reactions in multiplex assays can entail more stringent requirements for sample purity, sample input, reagents, and instruments to avoid non-specific amplification and obscuring background signals.

The multi-targeted approach is often accomplished with multiplexed amplification of the targeted nucleic acid. Several nucleic acid amplification technologies have been established and have been reviewed elsewhere.[18,48,49] For example, amplification can be performed using a multiplexed version of PCR, and then products or amplicons of this reaction need to be demultiplexed for proper detection and identification of the virus.[50]

This demultiplexing can be done by a variety of technologies including, but not limited to, probe-based fluorescent differentiation,[51] capillary and gel electrophoresis,[52] microarray technology,[53] Luminex beads with flow cytometric analysis,[54] or mass spectrophotometry.[55,56] The following sections highlight each of these approaches, with specific examples that are currently available and useful for investigation of respiratory viral outbreaks. Extensive reviews covering a broader scope of molecular assays that are useful for outbreak-related viral detection and characterization have been published.[57,58]

Use of molecular assays has generated significant information about the epidemiology of respiratory viral outbreaks that was not readily obtained using conventional methods. Co-infections involving two or more viruses were previously not detected often,[59] but are now detected frequently through the use of molecular methods (table I).[60-62] Many of these involve viruses that were formerly undetectable or were simply not sought using conventional methods.[63] In addition, many outbreaks can be shown to involve multiple viruses circulating within an institution.[64] In our initial study of 22 institutional outbreaks using a multiplex approach, multiple viruses were detected in eight outbreaks (36%).[65]

3. Probe-Based PCR Assays

PCR assays developed for outbreak investigations typically fall into two categories: multi-targeted PCR using consensus or universal primers; and several single-plex PCRs, which are then used in a triaged manner. For example, detection of novel strains of influenza A has been done with universal primers[66] and with more type-specific single-plex PCRs in a reflexed manner.[67] Typically, a combination of universal primers and specific primers is most useful for outbreak investigation. A good example is an influenza A screening approach whereby laboratories start with a broad-spectrum single-plex PCR, based on the highly conserved matrix gene, and follow-up the typing of positively identified influenza A specimens using type-specific PCRs against the hemagglutinin gene.[68,69] In either case, the combination of primers or the array of single-plex PCRs are designed to provide a process of elimination to hone in on the viral pathogen of interest, thereby identifying the causative outbreak agent.

The combination of PCR chemistry with fluorescent probe detection of the amplified product in the same reaction tube is

© 2010 Adis Data Information BV. All rights reserved.

© 2010 Adis Data Information BV. All rights reserved.

Table I. Examples of assays with potential utility for viral outbreak investigation of institutions

Assay	Supplier[a]	Amplification step	Demultiplex step	Advantages	Limitations	Available targets for outbreaks	Batch size	Time per batch[b]	Licensing status
Real-time PCR	In-house and several commercial suppliers, including Prodesse	PCR with target-specific primers	Probes and up to 5 unique fluorescent channels or wavelengths	In-house methods are easily adaptable to new strains; very sensitive; same-day results	Limited multiplexing capacity; requires heavy capital investment; requires in-house validation and quality control	Full viral respiratory and gastroenteritis primer/probe sets available in the literature	Up to 348, typically 96	<2 h	ProFlu+ has FDA, CE and HC approval
Seeplex® RV-15; Seeplex® Diarrhea-V ACE Detection	SeeGene Inc. (Seoul, Korea)	Multiplex PCR with DPO primers	Size differentiation by electrophoresis	Highly specific multiplexing	Preset mix of targets	Adenovirus; influenza A and B; RSV A and B; parainfluenza 1–4; coronaviruses OC43, NL63 and 229E; hMPV; rhinovirus; bocavirus; enterovirus; rotavirus; norovirus (genogroups I and II); adenovirus; astrovirus	Up to 96	2 h	RV-15 has CE approval; Diarrhea-V has CE and HC approval
Infiniti® Analyzer	AutoGenomics	Multiplex PCR	Random access microarray	Integrates and automates all discrete test processes; limited hands-on time	Preset mix of targets	Influenza A swine H1N1; influenza B; parainfluenza 1–4; rhinovirus A and B; enterovirus A–D; coronaviruses HKU1, OC43, NL63, and 229E; hMPV A and B; RSV A and B; adenovirus A, B, C, and E	Non-scalable 24 samples per batch	4 h for first result, 6 h for all 24	RVP Plus is for RUO
Plex-ID	Abbott	Universal PCR	ESI-TOF MS	Almost unlimited target/virus detection	High capital costs; limited databases	All human adenovirus types A–F (including types 3, 4, 7, 21, and newly emerging types such as 14); hMPV; all influenza viruses (including common human strains and avian influenza H5N1); RSV	60 samples	6 h for first result, 12 h for all 60	RUO
xTAG® RVP	Abbott	Multiplex PCR	Luminex beads with flow cytometry	Potential for up to 100 unique assays within a single sample	Preset mix of targets; however, open-platform user-developed assays possible	Influenza A, A H1 (seasonal), A H3 (seasonal), and A H5; influenza B; RSV A and B;	96 samples	5 h for all 96	FDA- and CE-approved versions available

Continued next page

Table I. Contd

Assay	Supplier[a]	Amplification step	Demultiplex step	Advantages	Limitations	Available targets for outbreaks	Batch size	Time per batch[b]	Licensing status
						parainfluenza 1–4; coronaviruses NL63, OC43, HKU1, 229E, and SARS-CoV; hMPV; enterovirus/rhinovirus; adenovirus			
ResPlex	Qiagen	Multiplex PCR	Luminex beads with flow cytometry	Potential for up to 100 unique assays within a single sample	Preset mix of targets; however, open-platform user-developed assays possible	Influenza A and B; RSV A and B; parainfluenza 1–4; enteroviruses; SARS-CoV; hMPV; rhinovirus in ResPlex II panel; adenovirus in ResPlex I panel	96 samples	5 h for all 96	RUO
MultiCode-PLx RVP	EraGene	Multiplex PCR	Luminex beads with flow cytometry	Potential for up to 100 unique assays within a single sample	Preset mix of targets; however, open-platform user-developed assays possible	Influenza A and B; RSV A and B; parainfluenza 1–4; coronaviruses NL63, OC43, and 229E; hMPV; rhinovirus; adenovirus B, C, and E	96 samples	5 h for all 96	RUO
MassTag	Qiagen Genomics	Multiplex PCR	APCI MS	Potential for up to 64 unique assays within a single sample	High capital costs, preset mix of targets; however, open-platform user-developed assays possible	Influenza A and B; human RSV A and B; human coronaviruses OC43, 229E, and SARS-CoV; human parainfluenza 1–3; hMPV; enterovirus; human adenovirus; *M. pneumoniae; L. pneumophila; C. pneumoniae; S. pneumoniae; H. influenzae; N. meningitidis*	96 samples	8 h for all 96	RUO
FilmArray™	Idaho Technologies	Nested multiplex PCR	Pre-spotted array with a single set of primers	Completely hands-free and fast	Limited to 1 sample at a time	Adenovirus; bocavirus; coronaviruses 229E, HKU1, OC43, and NL63; influenza A, A H1 (seasonal) and A H3 (seasonal); influenza B; hMPV; parainfluenza 1–4; RSV; rhinovirus	1 sample	<1 h	RUO

a Examples given only, and not necessarily an exclusive manufacturer listing.

b Excludes extraction time, which is common to all processes.

APCI = atmospheric pressure chemical ionization; **CE** = CE-marking for the EU; **DPO** = dual priming oligonucleotide; **ESI** = electrospray ionization; **HC** = Health Canada; **hMPV** = human metapneumovirus; **MS** = mass spectrometry; **RSV** = respiratory syncytial virus; **RUO** = research use only; **RV** = respiratory virus; **RVP** = respiratory viral panel; **SARS-CoV** = acute respiratory syndrome coronavirus; **TOF** = time-of-flight.

© 2010 Adis Data Information BV. All rights reserved.

arguably the fastest and most robust in-house PCR technique. It provides real-time results with the appropriate instrumentation, contamination containment with closed-tube amplification that requires no post-PCR manipulation, and reduced implementation costs due to its robust nature. Several real-time PCR probe technologies exist, such as 5′ nuclease (TaqMan®) probes (Life Technologies Corporation, Carlsbad, CA, USA), molecular beacons, and fluorescence resonance energy transfer (FRET) hybridization probes.[70] However, multiplexing is further challenged with the need to avoid fluorescent cross-talk. Analysis of the fluorescent signal is aided by spectral calibration, which associates the target probe with its proper wavelength, but this is theoretically limited to five targets. In diagnostic practice, however, real-time multiplexing is difficult to optimize and is often seen with fewer than five targets.[71] More commonly, real-time PCRs are used as single-target assays to avoid competitive inhibition, whereby one or more of the targets are preferentially amplified over one of the other multiplexed targets. There are several examples of commercially available assays in this category, but perhaps the best studied and validated example is the ProFlu+ Assay (Gen-Probe Inc., San Diego, CA, USA). This assay is a multiplexed real-time reverse transcription (RT)-PCR that detects influenza A virus, influenza B virus, and RSV.

Although assays that are developed in-house can offer the best combination in terms of per-specimen cost and flexibility, it is important to note that these assays must also pass rigorous validation studies before implementation. False negatives due to low sensitivity and false positives due to low specificity must be eliminated before implementation in any outbreak investigation. Consequently, the start-up cost for in-house methods is greater than that for commercially available molecular methods and may mean higher initial capital costs for the necessary equipment, whereas commercially available assays can often be established without any equipment purchase.

4. Commercial PCR-Based Applications

4.1 Seeplex®

Seeplex® technology (Seegene Inc., Seoul, Korea) is a good example of a multiplex-targeted molecular assay that uses electrophoresis as its means for demultiplexing its multiple targets.[52] The Seeplex® Respiratory Virus (RV) assay uses a multiplexed PCR with a novel primer design, called the dual priming oligonucleotide (DPO) system. Compared with conventional primers, the DPO system has the advantage of a wider range of suitable annealing temperatures, while at the same time maintaining high specificity.[52] This is accomplished by using two priming regions, which are joined by a polydeoxyinosine linker, instead of the usual single priming region.

The RV-12 assay is the first to use the DPO system and was designed to amplify and detect adenovirus, influenza virus (types A and B), RSV (subgroups A and B), parainfluenza virus (types 1, 2, and 3), coronavirus (types OC43, NL63, and 229E), hMPV, and human rhinovirus. The usefulness of the RV-12 assay has already been demonstrated in outbreak investigations.[72] An RV-15 assay is now available with the addition of rhinovirus C, bocavirus (types 1, 2, 3, and 4), parainfluenza virus 4, and enterovirus targets.

4.2 Infiniti® Microarray

An example of microarray technology is the Infiniti® Respiratory Viral Panel (RVP) Plus (AutoGenomics Inc., Carlsbad, CA, USA), which is designed to detect 25 common respiratory viruses; influenza A (swine H1N1), influenza B, parainfluenza virus (types 1, 2, 3, and 4), rhinovirus (types A and B), enterovirus (types A, B, C, and D), coronavirus (strains HKU1, OC43, NL63, and 229E), hMPV (types A and B), RSV (subgroups A and B), and adenovirus (types A, B, C, and E). The assay works on the foundation of multiplex PCR but is coupled to a target-specific primer extension (TSPE) process using ‘tagged’ primers and fluorescent nucleotides. The tagged extension products are then hybridized to anti-tags that are immobilized on a BioFilmChip™ microarray (AutoGenomics Inc.). The BioFilmChip™ is a novel, film-based microarray, which consists of multiple 8–10 μm layers of hydrogel matrices on a solid support.

A comparison between the Infiniti® RVP assay and a set of 25 single-plex real-time PCRs (TaqMan®) for the same viruses showed that although the TaqMan® assays were more sensitive, there was 94% concordance of the results.[53] In outbreak investigations, with low numbers of samples and the need for same-day results, the microarray approach may not be the best choice. However, considering the substantial reduction in hands-on time, microarray-based assays are a very viable option for higher volumes of samples.

4.3 xMAP Technology

The xTAG® RVP from Luminex® (Abbott Molecular Diagnostics, Abbott Park, IL, USA), ResPlex panels (Qiagen Inc., Mississauga, ON, Canada), and MultiCode-PLx RVP (EraGen Biosciences, Madison, WI, USA) are examples of post-PCR demutiplexing systems using Luminex® beads with flow

© 2010 Adis Data Information BV. All rights reserved.

cytometric analysis. These systems operate on the Luminex xMAP® instrument, which is a detection platform that uses lasers to read color-coded microspheres that attach to specific nucleic acid sequences. Viral nucleic acids are extracted from the sample, and a multiplex RT-PCR reaction is carried out under optimized conditions, resulting in amplicons for each of the viruses/subtypes present in the sample. Each RT-PCR product is treated with shrimp alkaline phosphatase and with exonuclease I before incorporation of biotin-deoxycytidine triphosphate (dCTP) during a TSPE reaction. TSPE effectively helps to detect viral DNA that is present in the sample, using a unique sequence tag. After TSPE, the reaction is added directly to microwells containing bead-immobilized anti-tags, which are the complements of the sequence tags on the primers. The beads are spectrally distinguishable from each other using flow cytometry, so that each uniquely colored bead represents a specific virus through the bead/anti-tag/tagged primer association.

The xTAG® RVP is perhaps the best-validated commercially available assay using Luminex® beads with flow cytometric analysis. The original CE-marked version of the xTAG® RVP is a qualitative nucleic acid multiplex test with 14 primer pairs that permits simultaneous detection and identification of multiple respiratory virus nucleic acids in nasopharyngeal swabs, nasal aspirates, and bronchoaveolar lavages from suspected respiratory tract infections. The following virus types and subtypes are identified using the classic version of the CE-marked xTAG® RVP assay: influenza A (subtypes H1 [seasonal], H3 [seasonal], and H5), influenza B, RSV (subgroups A and B), parainfluenza virus (types 1, 2, 3, and 4), coronavirus (strains NL63, OC43, HKU1, and 229E), acute respiratory syndrome coronavirus (SARS-CoV), hMPV, entero-rhinovirus, and adenovirus.

A faster, 1-day version of the RVP assay has recently been released. It retains the ability to detect most of the original panel, including RSV, but it no longer distinguishes RSV A from RSV B. The newer version of the assay can still detect all influenza A strains but no longer includes influenza A H5 as a specific target. The Fast version also drops the target for SARS-CoV and instead detects human bocavirus. Either the classic or the Fast version of the RVP assay would be useful for viral investigations of institutional outbreaks of a respiratory nature, and are currently the method of choice for our laboratory.

Both versions of the RVP assay will detect influenza A pandemic H1 (pH1) but can only identify it as influenza A, with no indication of its subtype.[73] This effectively makes the RVP assay a sentinel test for the novel non-seasonal influenza A virus of recent importance and not a full diagnostic test for pH1 outbreaks. Furthermore, the RVP assay has limited ability to detect adenovirus species and cannot distinguish between rhinoviruses and human enteroviruses.

Other commercially available assays using the same demultiplexing technology as the xTAG® RVP are the ResPlex assay (Qiagen) and MultiCode-PLx RVP (EraGen). The ResPlex II assay simultaneously targets 12 viruses: influenza A, influenza A pH1, influenza B; RSV (subgroups A and B), parainfluenza virus (types 1–4), enteroviruses; SARS-CoV; hMPV; and rhinovirus. Adenovirus has been multiplexed with respiratory bacterial pathogens in the ResPlex I panel. The ResPlex panels were used to demonstrate that co-infections with more than one respiratory pathogen are medically relevant.[60] The MultiCode-PLx RVP assay simultaneously targets 17 respiratory viruses and subtypes.[74] It detects influenza A (no subtyping), influenza B, RSV (subgroups A and B), parainfluenza virus (types 1–3; and subtypes 4a and 4b), coronaviruses (NL63, OC43, 229E), hMPV, rhinovirus, and adenovirus (types B, C, and E).

4.4 Plex-ID

Plex-ID (Abbott Laboratories) is an example of a molecular technology that uses electrospray ionization-mass spectrometry (ESI-MS) for the demultiplexing of PCR.[75] In essence, broadly conserved primer sites are chosen to permit identification of target microbe(s), based on the difference between the nucleotide compositions within the amplified region. The ESI-MS component of the process helps to differentiate a mixture of amplicons after PCR with sufficient accuracy to permit the determination of the base composition (i.e. A, G, C, and T) of individual PCR products. Consequently, this technology appears to have good potential for outbreak investigations with the right mixture of broad-spectrum PCRs and a good database to permit identification.

This approach is theoretically more specific than post-PCR analysis of amplicons, using either size differentiation by electrophoresis or probe-based analysis such as real-time PCR, but is less specific than true DNA sequencing, which determines not just the base composition but also the actual ‘sequence’ of the amplicon. However, the most promising advantage of using mass spectrophotometry for the demultiplexing stage of PCR is the speed and cost effectiveness of this method and its ability to deal with a mixture of amplicons, which challenges traditional DNA sequencing. The weakness to date has been the lack of a public-domain database of base compositions, with attention given to potential mutation-associated differences that will dramatically affect the accuracy of identification. Allelic difference and mutations are less devastating to identification by

© 2010 Adis Data Information BV. All rights reserved.

DNA sequencing. Base composition databases, which are required for analysis, have not yet been developed fully, unlike DNA sequencing data, which are currently available in several well established databases such as GenBank, with over 300 000 organisms at the genus level or lower.[76]

It may be possible to use RNA-dependent RNA polymerase as a target for viral identification, using mass spectrophotometry which, in theory, could provide sufficient coverage for outbreak investigations.[55] The Ibis T5000 (Abbott Laboratories) has been used to effectively detect and type adenoviruses, influenza viruses, alphaviruses, coronaviruses, and orthopoxviruses.[75] The potential power of this approach was shown by a recent study of influenza strain emergence.[77]

4.5 MassTag PCR

Another example of the use of mass spectrophotometry as a means of demultiplexing a highly multiplexed PCR is called MassTag PCR.[56] In this type of assay, a set of primers is used to detect a broad range of pathogens in a multiplex PCR, but in this instance the primers are made with Masscode Tags (Qiagen Genomics). Consequently, the primer sets are labeled with distinct molecular weight tags, which are cleaved after PCR and purification of unused primers. These tags are analyzed in a single quadrapole mass spectrometer using positive-mode atmospheric pressure chemical ionization.[56]

MassTag PCR technology has already proven useful in a retrospective examination of respiratory specimens from influenza-like illnesses that were negative by culture and PCR for influenza A and influenza B.[78,79] The MassTag PCR assay can detect the following respiratory pathogens in one specimen: influenza A and B, human RSV (subgroups A and B), human coronaviruses (OC43 and 229E, and SARS-CoV), human parainfluenza viruses 1–3, hMPV, enterovirus, human adenovirus, *Mycoplasma pneumoniae, Legionella pneumophila, Chlamydophila pneumoniae, Streptococcus pneumoniae, Haemophilus influenzae,* and *Neisseria meningitidis*.

4.6 FilmArray™

The FilmArray™ instrument (Idaho Technology Inc., Salt Lake City, UT, USA) is an example of a molecular method that does not require batching but instead is designed to take one sample to a molecular result in less than 1 hour, using a completely automated hands-free method.[79] Using the FilmArray™ system, all necessary reagents for sample preparation, PCR, and detection are stored freeze-dried in a pouch containing several pockets for the various steps. Once the reagents are hydrated and the sample is added, the instrument moves the sample and then the products of the steps through an array of wells that are designed for each process and are specific for the target panel of viruses or bacteria. Currently, two panels are in the evaluation stages of product development, including a viral panel that detects adenovirus, bocavirus, coronavirus (229E, HKU1 OC43, and NL63), influenza A (including seasonal subtypes H1 and H3), influenza B, hMPV, parainfluenza virus types 1–4, RSV, and rhinovirus.

5. Conclusion

Molecular methods have distinct advantages over conventional methods, including the ability to rapidly develop and implement specific assays for newly recognized viruses or newly emerging variants, coupled with the capacity to process larger numbers of samples and obtain results in less time than conventional diagnostic approaches. However, the ability to process large numbers of samples imposes the need to handle these samples in large batches to accommodate microplate technology. While this approach allows the use of automated processes and reduces errors, the flexibility to handle individual samples that require urgent testing may be lost, unless some conventional testing capacity is retained or either single-specimen molecular assays or random-access molecular analyzers are developed.

Acknowledgments

No sources of funding were used to prepare this manuscript. The authors have no conflicts of interest that are directly relevant to the content of this review.

References

1. Loeb M, McGeer A, McArthur M, et al. Surveillance for outbreaks of respiratory tract infections in nursing homes. CMAJ 2000; 162 (8): 1133-7
2. Tregoning JS, Schwarze J. Respiratory viral infections in infants: causes, clinical symptoms, virology, and immunology. Clin Microbiol Rev 2010; 23 (1): 74-98
3. Vayalumkal JV, Gravel D, Moore D, et al. Surveillance for healthcare-acquired febrile respiratory infection in pediatric hospitals participating in the Canadian Nosocomial Infection Surveillance Program. Infect Control Hosp Epidemiol 2009; 30 (7): 652-8
4. Raboni SM, Nogueira MB, Tsuchiya LR, et al. Respiratory tract viral infections in bone marrow transplant patients. Transplantation 2003; 76 (1): 142-6
5. Monto AS, Rotthoff J, Teich E, et al. Detection and control of influenza outbreaks in well-vaccinated nursing home populations. Clin Infect Dis 2004; 39 (4): 459-64
6. Hicks LA, Shepard CW, Britz PH, et al. Two outbreaks of severe respiratory disease in nursing homes associated with rhinovirus. J Am Geriatr Soc 2006; 54 (2): 284-9

© 2010 Adis Data Information BV. All rights reserved.

7. Kim S, Sung H, Im HJ, et al. Molecular epidemiological investigation of a nosocomial outbreak of human metapneumovirus infection in a pediatric hemato-oncology patient population. J Clin Microbiol 2009; 47 (4): 1221-4
8. Cheng VC, Wu AK, Cheung CH, et al. Outbreak of human metapneumovirus infection in psychiatric inpatients: implications for directly observed use of alcohol hand rub in prevention of nosocomial outbreaks. J Hosp Infect 2007; 67 (4): 336-43
9. Gagneur A, Sizun J, Vallet S, et al. Coronavirus-related nosocomial viral respiratory infections in a neonatal and paediatric intensive care unit: a prospective study. J Hosp Infect 2002; 51 (1): 59-64
10. Allander T. Human bocavirus. J Clin Virol 2008; 41 (1): 29-33
11. Bastien N, Brandt K, Dust K, et al. Human bocavirus infection, Canada. Emerg Infect Dis 2006; 12 (5): 848-50
12. Gravenstein S, Ambrozaitis A, Schilling M, et al. Surveillance for respiratory illness in long-term care settings: detection of illness using a prospective research technique. J Am Med Dir Assoc 2000; 1 (3): 122-8
13. Monto AS. Occurrence of respiratory virus: time, place and person. Pediatr Infect Dis J 2004; 23 (1 Suppl.): S58-64
14. Gooskens J, Swaan CM, Claas EC, et al. Rapid molecular detection of influenza outbreaks in nursing homes. J Clin Virol 2008; 41 (1): 7-12
15. Weigl JA, Puppe W, Grondahl B, et al. Epidemiological investigation of nine respiratory pathogens in hospitalized children in Germany using multiplex reverse-transcriptase polymerase chain reaction. Eur J Clin Microbiol Infect Dis 2000; 19 (5): 336-43
16. Li H, McCormac MA, Estes RW, et al. Simultaneous detection and high-throughput identification of a panel of RNA viruses causing respiratory tract infections. J Clin Microbiol 2007; 45 (7): 2105-9
17. Mahony JB. The clinical need for the RVP test. J Clin Virol 2007; 40 Suppl. 1: S36-8
18. Fox JD. Nucleic acid amplification tests for the detection and analysis of respiratory viruses: the future for diagnostics? Future Microbiol 2007; 2: 199-211
19. Fox JD. Respiratory virus surveillance and outbreak investigation. J Clin Virol 2007; 40 Suppl. 1: S24-30
20. Barbani MT, Gorgievski-Hrisoho M. Rapid detection of respiratory picornaviruses in nasopharyngeal aspirates by immunofluorescence assay. J Clin Virol 2009; 45 (3): 245-8
21. Ginocchio CC. Detection of respiratory viruses using non-molecular based methods. J Clin Virol 2007; 40 Suppl. 1: S11-4
22. Sandora TJ, Smole SC, Lee GM, et al. Test characteristics of commercial influenza assays for detecting pandemic influenza A (H1N1) in children. Pediatr Infect Dis J 2010; 29 (3): 261-2
23. Ginocchio CC, Zhang F, Manji R, et al. Evaluation of multiple test methods for the detection of the novel 2009 influenza A (H1N1) during the New York City outbreak. J Clin Virol 2009; 45 (3): 191-5
24. Chan KH, Lai ST, Poon LL, et al. Analytical sensitivity of rapid influenza antigen detection tests for swine-origin influenza virus (H1N1). J Clin Virol 2009; 45 (3): 205-7
25. Hazelton PR, Gelderblom HR. Electron microscopy for rapid diagnosis of infectious agents in emergent situations. Emerg Infect Dis 2003; 9 (3): 294-303
26. Boivin G, Mazzulli T, Petric M, et al. Diagnosis of viral infections. In: Richman DD, Whitley RJ, Hayden FG, editors. Clinical virology. 3rd ed. Washington, DC: ASM Press, 2009: 265-94
27. Petric M, Szymanski M. Electron microscopy and immunoelectron microscopy. In: Specter S, Hodinka L, Young SA, editors. Clinical virology manual. 3rd ed. Washington, DC: ASM Press, 2000: 54-65
28. van den Hoogen BG, de Jong JC, Groen J, et al. A newly discovered human pneumovirus isolated from young children with respiratory tract disease. Nat Med 2001; 7 (6): 719-24
29. Landry ML, Hsiung GD. Primary isolation of viruses. In: Specter S, Hodinka RL, Young SA, editors. Clinical virology manual. 3rd ed. Washington, DC: ASM Press, 2000: 27-42
30. Leland DS, Ginocchio CC. Role of cell culture for virus detection in the age of technology. Clin Microbiol Rev 2007; 20 (1): 49-78
31. Fong CK, Lee MK, Griffith BP. Evaluation of R-Mix FreshCells in shell vials for detection of respiratory viruses. J Clin Microbiol 2000; 38 (12): 4660-2
32. Landry ML, Ferguson D, Cohen S, et al. Detection of human metapneumovirus in clinical samples by immunofluorescence staining of shell vial centrifugation cultures prepared from three different cell lines. J Clin Microbiol 2005; 43 (4): 1950-2
33. Percivalle E, Sarasini A, Visai L, et al. Rapid detection of human metapneumovirus strains in nasopharyngeal aspirates and shell vial cultures by monoclonal antibodies. J Clin Microbiol 2005; 43 (7): 3443-6
34. Zaza S, Jarvis WR. Investigation of outbreaks. In: Mayhall CG, editor. Hospital epidemiology and infection control. Baltimore (MD): Williams & Wilkins, 1996: 105-13
35. Fisman DN, Greer AL, Brouhanski G, et al. Of gastro and the gold standard: evaluation and policy implications of norovirus test performance for outbreak detection. J Transl Med 2009; 7: 23
36. Duizer E, Pielaat A, Vennema H, et al. Probabilities in norovirus outbreak diagnosis. J Clin Virol 2007; 40 (1): 38-42
37. Brittain-Long R, Westin J, Olofsson S, et al. Prospective evaluation of a novel multiplex real-time PCR assay for detection of fifteen respiratory pathogens: duration of symptoms significantly affects detection rate. J Clin Virol 2010; 47 (3): 263-7
38. Li CC, Wang L, Eng HL, et al. Correlation of pandemic (H1N1) 2009 viral load with disease severity and prolonged viral shedding in children. Emerg Infect Dis 2010; 16 (8): 1265-72
39. Clinical and Laboratory Standards Institute. Laboratory design: approved guideline GP18-A2. 2nd ed. Wayne (PA): Clinical and Laboratory Standards Institute, 2007
40. Burd EM. Validation of laboratory-developed molecular assays for infectious diseases. Clin Microbiol Rev 2010; 23 (3): 550-76
41. Jennings L, Van Deerlin VM, Gulley ML. Recommended principles and practices for validating clinical molecular pathology tests. Arch Pathol Lab Med 2009; 133 (5): 743-55
42. Bolotin S, De Lima C, Choi KW, et al. Validation of the TaqMan influenza A detection kit and a rapid automated total nucleic acid extraction method to detect influenza A virus in nasopharyngeal specimens. Ann Clin Lab Sci 2009; 39 (2): 155-9
43. Mahony JB, Blackhouse G, Babwah J, et al. Cost analysis of multiplex PCR testing for diagnosing respiratory virus infections. J Clin Microbiol 2009; 47 (9): 2812-7
44. Lam WY, Yeung AC, Tang JW, et al. Rapid multiplex nested PCR for detection of respiratory viruses. J Clin Microbiol 2007; 45 (11): 3631-40
45. Grondahl B, Puppe W, Hoppe A, et al. Rapid identification of nine microorganisms causing acute respiratory tract infections by single-tube multiplex reverse transcription-PCR: feasibility study. J Clin Microbiol 1999; 37 (1): 1-7
46. Coiras MT, Aguilar JC, Garcia ML, et al. Simultaneous detection of fourteen respiratory viruses in clinical specimens by two multiplex reverse transcription nested-PCR assays. J Med Virol 2004; 72 (3): 484-95
47. Clinical and Laboratory Standards Institute. Verification and validation of multiplex nucleic acid assays: approved guideline MM17-A. Wayne (PA): Clinical and Laboratory Standards Institute, 2008
48. Procop GW. Molecular diagnostics for the detection and characterization of microbial pathogens. Clin Infect Dis 2007; 45 Suppl. 2: S99-111
49. Monis PT, Giglio S. Nucleic acid amplification-based techniques for pathogen detection and identification. Infect Genet Evol 2006; 6 (1): 2-12

© 2010 Adis Data Information BV. All rights reserved.

50. Gunson RN, Bennett S, Maclean A, et al. Using multiplex real time PCR in order to streamline a routine diagnostic service. J Clin Virol 2008; 43 (4): 372-5
51. Wang W, Ren P, Mardi S, et al. Design of multiplexed detection assays for identification of avian influenza a virus subtypes pathogenic to humans by SmartCycler real-time reverse transcription-PCR. J Clin Microbiol 2009; 47 (1): 86-92
52. Kim SR, Ki CS, Lee NY. Rapid detection and identification of 12 respiratory viruses using a dual priming oligonucleotide system-based multiplex PCR assay. J Virol Methods 2009; 156 (1-2): 111-6
53. Raymond F, Carbonneau J, Boucher N, et al. Comparison of automated microarray detection with real-time PCR assays for detection of respiratory viruses in specimens obtained from children. J Clin Microbiol 2009; 47 (3): 743-50
54. Mahony J, Chong S, Merante F, et al. Development of a respiratory virus panel test for detection of twenty human respiratory viruses by use of multiplex PCR and a fluid microbead-based assay. J Clin Microbiol 2007; 45 (9): 2965-70
55. Ecker DJ, Massire C, Blyn LB, et al. Molecular genotyping of microbes by multilocus PCR and mass spectrometry: a new tool for hospital infection control and public health surveillance. Methods Mol Biol 2009; 551: 71-87
56. Briese T, Palacios G, Kokoris M, et al. Diagnostic system for rapid and sensitive differential detection of pathogens. Emerg Infect Dis 2005; 11 (2): 310-3
57. Wu W, Tang YW. Emerging molecular assays for detection and characterization of respiratory viruses. Clin Lab Med 2009; 29 (4): 673-93
58. Bosch A, Guix S, Sano D, et al. New tools for the study and direct surveillance of viral pathogens in water. Curr Opin Biotechnol 2008; 19 (3): 295-301
59. Falchi A, Arena C, Andreoletti L, et al. Dual infections by influenza A/H3N2 and B viruses and by influenza A/H3N2 and A/H1N1 viruses during winter 2007, Corsica Island, France. J Clin Virol 2008; 41 (2): 148-51
60. Brunstein JD, Cline CL, McKinney S, et al. Evidence from multiplex molecular assays for complex multipathogen interactions in acute respiratory infections. J Clin Microbiol 2008; 46 (1): 97-102
61. Paranhos-Baccalà G, Komurian-Pradel F, Richard N, et al. Mixed respiratory virus infections. J Clin Virol 2008; 43 (4): 407-10
62. Peng D, Zhao D, Liu J, et al. Multipathogen infections in hospitalized children with acute respiratory infections. Virol J 2009; 6: 155
63. Christensen A, Nordbø SA, Krokstad S, et al. Human bocavirus commonly involved in multiple viral airway infections. J Clin Virol 2008; 41 (1): 34-7
64. Eshaghi A, Blair J, Burton L, et al. Characterization of an influenza A and influenza B co-infection of a patient in a long-term care facility with co-circulating influenza A and influenza B. Int J Infect Dis 2009; 13 (3): e127-8
65. Levett PN, Hansen T, Brandt K, et al. Application of multiplex PCR and fluid microbead-based assay to investigation of institutional outbreaks of viral respiratory disease [abstract]. Int J Antimicrob Agents 2009; 34 Suppl. 2: S6
66. Yacoub A, Kiss I, Zohari S, et al. The rapid molecular subtyping and pathotyping of avian influenza viruses. J Virol Methods 2009; 156 (1-2): 157-61
67. Bastien N, Antonishyn NA, Brandt K, et al. Human infection with a triple-reassortant swine influenza A (H1N1) virus containing the hemagglutinin and neuraminidase genes of seasonal influenza virus. J Infect Dis 2010; 201 (8): 1178-82
68. Lorusso A, Faaberg KS, Killian ML, et al. One-step real-time RT-PCR for pandemic influenza A virus (H1N1) 2009 matrix gene detection in swine samples. J Virol Methods 2010; 164 (1-2): 83-7
69. Crawford JM, Stallone R, Zhang F, et al. Laboratory surge response to pandemic (H1N1) 2009 outbreak, New York City metropolitan area, USA. Emerg Infect Dis 2010; 16 (1): 8-13
70. Espy MJ, Uhl JR, Sloan LM, et al. Real-time PCR in clinical microbiology: applications for routine laboratory testing. Clin Microbiol Rev 2006; 19 (1): 165-256
71. Persson K, Hamby K, Ugozzoli LA. Four-color multiplex reverse transcription polymerase chain reaction: overcoming its limitations. Anal Biochem 2005; 344 (1): 33-42
72. Drews SJ, Blair J, Lombos E, et al. Use of the Seeplex RV detection kit for surveillance of respiratory viral outbreaks in Toronto, Ontario, Canada. Ann Clin Lab Sci 2008; 38 (4): 376-9
73. Mahony JB, Hatchette T, Ojkic D, et al. Multiplex PCR tests sentinel the appearance of pandemic influenza viruses including H1N1 swine influenza. J Clin Virol 2009; 45 (3): 200-2
74. Notle FS, Marshall DJ, Rasberry C, et al. MultiCode-PLx system for multiplexed detection of seventeen respiratory viruses. J Clin Microbiol 2007; 45 (9): 2779-86
75. Ecker DJ, Sampath R, Massire C, et al. Ibis T5000: a universal biosensor approach for microbiology. Nat Rev Microbiol 2008; 6 (7): 553-8
76. Benson DA, Karsch-Mizrachi I, Lipman DJ, et al. GenBank. Nucleic Acids Res 2010; 38 (Database Issue): D46-51
77. Sampath R, Russell KL, Massire C, et al. Global surveillance of emerging influenza virus genotypes by mass spectrometry. PLoS One 2007; 2 (5): e489
78. Lamson D, Renwick N, Kapoor V, et al. MassTag polymerase-chain-reaction detection of respiratory pathogens, including a new rhinovirus genotype, that caused influenza-like illness in New York State during 2004-2005. J Infect Dis 2006; 194 (10): 1398-402
79. Welch DF, Ginocchio CC. Role of rapid immunochromatographic antigen testing in diagnosis of influenza A virus 2009 H1N1 infection. J Clin Microbiol 2010; 48 (1): 22-5

Correspondence: Dr. *Nick A. Antonishyn*, Saskatchewan Disease Control Laboratory, 5 Research Drive, Regina, Saskatchewan S4S 0A4, Canada.
E-mail: nantonishyn@health.gov.sk.ca

© 2010 Adis Data Information BV. All rights reserved.