Emerging Molecular Assays for Detection and Characterization of Respiratory Viruses

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- Padlock probes • Microarrays • Mass spectrometry

Rapid detection and identification of viral pathogens causing respiratory tract infections is critical for initiating antiviral therapy, avoiding unnecessary antimicrobial therapy, preventing nosocomial spread, decreasing the duration of hospitalization, and reducing management costs. Molecular assays, which provide high sensitivity and specificity, short test turnaround time, and automatic, high-throughput batch processing, have played critical roles in rapid detection, screening, and identification of emerging respiratory viral pathogens, such as severe acute respiratory syndrome coronavirus (SARS-CoV) and novel A/H1N1 influenza (Flu) virus.[1–3] The superiority of polymerase chain reaction (PCR), reverse transcription-PCR (RT-PCR), and other in vitro nucleic acid amplification assays over conventional methods for the diagnosis of respiratory viral infections has already been established.[4,5] This article describes several emerging molecular assays that have potential applications in the diagnosis and monitoring of respiratory viral infections.

DIRECT NUCLEIC ACID DETECTION BY QUANTUM DOTS BIOSENSORS

Biosensors offer the possibility of real-time monitoring, and the deployment of these devices in the field would provide a means for prompt etiologic diagnosis. All

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biosensors are essentially composed of a biologic recognition element or bioreceptor, which interacts with the analyte and responds in some manner that can be registered by a transducer. The bioreceptor is a crucial component, and its function is to impart selectivity so that the sensor responds only to a particular analyte or biomolecule of interest, hence avoiding interference from other substances. The transducer converts the microbial biorecognition event into an electrical signal detected using electrochemical, optical, or piezoelectric platforms.[6,7] A biosensor specifically targeting nucleic acids through hybridization is called a genosensor. Genosensors have been used to for direct, on-demand, and real-time detection and discrimination of microbial pathogens in clinical specimens. Malamud and colleagues[8] developed a group of genosensor-based assays to detect microbial pathogens in oral specimens for use in the diagnosis of multiple infectious diseases. A piezoelectric DNA biosensor to directly detect hepatitis B virus was developed based on the mass-transducing function of a quartz crystal microbalance and nucleic acid hybridization[9]; another hybridization-based amperometric biosensor, using osmium as an electrochemical indicator, was used for the detection and confirmation of virus-specific PCR products.[10] A generic semidisposable fluorescence biosensor was developed to directly detect dengue virus RNA.[11] A hybridization-based genosensor on gold film coupled with enzymatic electrochemical detection was designed to detect SARS-CoV RNA.[12]

Fluorescent semiconductor nanocrystals, known as quantum dots (Qdots), are colloidal particles consisting of a semiconductor core, a high band gap material shell, and typically an outer coating layer. The core-size–dependent photoluminescence with narrow emission bandwidths that span the visible spectrum and the broad adsorption spectra allow simultaneous excitation of mixed Qdot populations at a single wavelength. Qdots also exhibit several unique features: high quantum yield, high resistance to photodegradation, and better near-infrared emission.[13,14] The new generation of Qdots has far-reaching potential for the study of intracellular processes in broad fields, including diagnostics.[14] High-sensitivity bacterial detection using biotin-tagged phage and quantum-dot nanocomplexes has been described, which provides specific limits of detection at 10 bacterial cells/mL in 1 hour.[15] A bead-based microfluidic device was developed to achieve an ELISA with Qdots as the labeling fluorophore for virus detection.[16] Three groups have reported the use of Qdots conjugated to specific monoclonal antibodies to detect and identify the presence of respiratory syncytial virus (RSV) in a real-time manner, implying that Qdots may provide a method for early, rapid detection of RSV infections.[17–19] In addition to microbial pathogen antigen detection, positively charged compact Qdot-DNA complexes were described that can detect H5N1 Flu-A virus nucleic acids presented at concentrations as low as 200 nmol.[20] Simultaneous excitation of several emission-tunable Qdot populations can be combined with a pool of differentially labeled probes for multiplex target analysis.[21,22] Qdot-based techniques are under development to detect a panel of respiratory viruses, producing more efficient assays that require smaller quantities of target nucleic acids.

AMPLIFICATION METHODS AND PLATFORMS

Loop-Mediated Isothermal Amplification

First described by Notomi and colleagues[23] in 2000, loop-mediated isothermal amplification (LAMP) is a simple, rapid, and specific nucleic acid amplification method, which is characterized by the use of multiple primers specifically designed to recognize several distinct regions on the target gene. Amplification and detection of target genes can be completed in a single step, by incubating the mixture of samples, primers, DNA polymerase with strand displacement activity and substrates at a constant temperature. Because amplification is isothermal, LAMP does not require special reagents or sophisticated temperature control devices. Because the increase in turbidity of the reaction mixture according to the production of precipitate correlates with the amount of DNA synthesized, real-time monitoring of the LAMP reaction can be achieved by turbidity measurement.[24] With a detection limit of about one to two copies, LAMP is capable of detecting the presence of pathogenic agents earlier than PCR if the gene copy number is low.[25]

LAMP has successfully been applied to the rapid and real-time detection of several emerging and reemerging human pathogens, including West Nile virus, dengue virus, Japanese encephalitis virus, monkey pox virus, Rift Valley virus, SARS-CoV, Chikungunya virus, and noroviruses.[25–28] Poon and colleagues[29] described the use of an RT-LAMP to detect Flu-A viruses covering H1 to H3. Another similar RT-LAMP assay was described more recently that detects Flu-A virus H1 and H3 subtype strains and Flu-B virus strains.[30] At a limit of detection of 10 focus-forming units per mL, both assays can be completed within 3 hours, providing rapid and sensitive detection.[29,30] Two one-step RT-LAMP assays with analytical sensitivities of 0.01 to 0.1 plaque-forming units (pfu) per reaction were developed specifically for detection of highly pathogenic avian Flu-A (H5N1) viruses and validated using H5N1 viral strains isolated over the past 10 years and clinical specimens.[31–33] An RT-LAMP assay was reported to specifically detect the H9 subtype of avian Flu virus with a detection limit of 10 copies per reaction, 10-fold lower than that of RT-PCR.[34] In Japan, the LAMP assay was used to rapidly subtype Flu-A virus and confirm two cases of influenza in patients who had returned from Thailand.[30]

In addition to the detection and typing of Flu viruses, a subgroup-A/B–specific RT-LAMP assay was developed to amplify RSV to improve current diagnostic methods for RSV infections. The assay was validated using nasopharyngeal aspirates from children who had respiratory tract infections, and the results indicated that the RT-LAMP is more sensitive than viral isolation and antigen testing for RSV detection.[35,36] Several LAMP-based assays were reported for rapid detection of SARS-CoV with the advantages of rapid amplification, simple operation, and ease of detection.[28,37] LAMP-based assays have also been used to detect other respiratory viral pathogens, such as mumps,[38,39] measles,[40] and adenoviruses.[41] In comparison to conventional RT-PCR, RT-LAMP assays demonstrated 10- to 100-fold enhanced sensitivity, with a detection limit of 0.01 to 10 pfu of virus in most cases.

Multiplex Ligation-Dependent Probe Amplification

Recently established in The Netherlands, multiplex ligation-dependent probe amplification (MLPA) makes use of both ligation and PCR.[42] Inventively modified from previously described ligation-dependent PCR assays,[43,44] the MLPA platform features greatly reduced probe concentrations and longer hybridization periods to generate conditions compatible with multiplex analysis. Each MLPA probe consists of a pair of oligonucleotides subject to ligation when hybridized to a target sequence, analogous to a padlock probe (see later discussion). One oligonucleotide consists of a 5′ fluorescent label, a universal forward primer binding site, and a target-specific recognition sequence at the 3′ end, whereas the other oligonucleotide consists of a target-specific recognition sequence at the 5′ end, a nonspecific stretch of DNA of defined length (“stuffer” sequence), and a universal reverse primer binding site at the 3′ end. Each MLPA assay is divided into three basic steps: (1) annealing of probes to their target sequences, (2) ligation of the probes, and (3) PCR amplification of ligated probes using universal primers. Multiplexing is achieved by varying the length of stuffer sequence for each unique set of probes used in the assay. Amplification products are detected using high-resolution electrophoretic techniques, such as capillary electrophoresis, and it is claimed that this approach allows relative quantification.[42]

MLPA-based techniques have proved sufficiently sensitive, reproducible, and sequence specific for use in screening human DNA. Recent studies have use of the MLPA assay for the detection and identification of several pathogenic microorganisms, including rapid characterization of *Mycobacterium tuberculosis*,[45] and relative quantification of targeted bacterial species in oral microbiota.[46] Reijans and colleagues[47] described an MLPA technology–based RespiFinder assay to detect 15 respiratory viruses simultaneously in one reaction. In this case, the MLPA reaction was preceded by a preamplification step that ensured detection of both RNA and DNA viruses with the same specificity and sensitivity as individual monoplex real-time RT-PCR assays. The RespiFinder assay showed satisfactory specificity and perfect sensitivity for adenovirus, human metapneumovirus (hMPV), Flu-A, parainfluenza virus (PIV) types 1 and 3, rhinovirus (RhV), and RSV. Use of the RespiFinder assay resulted in a 24.5% increase in the diagnostic yield compared with cell culture. This assay is being extended to cover four additional bacterial pathogens that cause respiratory tract infections: *Mycoplasma pneumoniae*, *Chlamydophila pneumoniae*, *Legionella pneumophila*, and *Bordetella pertussis*.

Polymerase Chain Reaction Amplification Using Arbitrary Primers

PCR amplification techniques using arbitrary primers, including arbitrarily primed (AP) PCR,[48] sequence-independent single-primer amplification (SISPA),[49] and randomly amplified polymorphic DNA (RAPD),[50] are generally based on the PCR amplification of random DNA segments with short primers (usually a single 1 of 10 nucleotides) containing arbitrary nucleotide sequences. RAPD-based assays have increasingly been used to type microorganisms, especially during clinical outbreaks.[51] The RAPD-PCR technique seems to be practical and efficient for routine use in high-resolution viral diversity studies by providing assemblage comparisons through fingerprinting, probing, or sequence information.[52] Similar techniques have been used to characterize the polymerase gene and genomic termini of Nipah virus[53] and avian Flu virus genome sequences.[54]

On the other hand, AP-PCR and SISPA-based assays have mainly been used for the discovery and characterization of novel and noncultivatable viruses.[55] Because viral pathogens do not possess conserved, universal genes, such as 16S rRNA genes, SISPA was used in the early 1990s as a random PCR amplification strategy to amplify known and unknown viral genes, including those of hepatitis C virus, rotavirus, and norovirus.[56–58] The AP-PCR technique was used successfully to obtain sequence information on a novel hMPV after the virus was cultured.[59] Wang and colleagues[60,61] used a similar random amplification technique in conjunction with a long oligonucleotide pan-viral microarray to simultaneously screen and detect hundreds of viral pathogens. This system has successfully been used for the detection of a human PIV-4 strain associated with respiratory failure,[62] for identification of a novel gammaretrovirus in a patient who had prostate tumors,[63] for the diagnosis of a critical respiratory illness caused by hMPV,[64] and for the identification of cardioviruses related to Theiler murine encephalomyelitis virus in human infections.[65] Quan and colleagues[66] recently reported the use of a similar random amplification process followed by comprehensive microarray analysis (GreeneChipResp) to detect diverse respiratory viral pathogens and subtype Flu-A viruses.

A modified SISPA incorporating DNAse treatment has recently been used to discover, identify, and characterize several novel bovine and human viral pathogens directly from clinical samples.[67–70] The same technology has been used for the characterization of common epitopes in enterovirus (EnV),[71] identification of a novel human coronavirus,[72] detection of TT virus in stool samples collected during a gastroenteritis outbreak,[73] and discovery of novel unculturable viruses in specimens collected from patients presenting with fever of unknown origin.[74,75] Although PCR amplification using arbitrary primers has been an extremely powerful approach for screening and discovery of new or noncultivable viral pathogens directly from clinical specimens, subsequent identification and confirmation steps are hindered by a background of nonspecific random amplification products. Further development is thus required to optimize this technology for routine diagnostic use in molecular microbiology laboratories.

Target-Enriched Multiplexing Amplification

Multiplex PCR was developed to use numerous primers within a single reaction tube to amplify nucleic acid fragments from different targets. Multiple sets of high-concentration primers in the conventional multiplex reaction often favor primer-dimer formation, however, resulting in nonspecific amplification. To meet the challenges of conventional multiplex PCR, Han and colleagues[76] developed target-enriched multiplexing (TEM)-PCR technology, which uses nested gene-specific primers at extremely low concentrations to enrich specific targets during early PCR cycles and relies on universal forward and reverse "superprimers" at high, but unequal, concentrations to achieve exponential asymmetric target amplification. TEM-PCR amplification has been reported for the detection, typing, and semiquantification of 25 human papillomaviruses,[76] detection and differentiation of a panel of respiratory bacterial pathogens,[77–79] detection and differentiation of 24 antituberculosis drug resistance-related mutations,[80] determination of antibiotic resistance and detection of toxin-encoding genes in *Staphylococcus aureus*,[81] screening and differentiation of methicillin-resistant *S aureus* and vancomycin-resistant enterococci,[82] and characterization and typing of Flu-A, including H5N1.[83]

Using TEM technology, the ResPlex II assay was developed to detect Flu-A, Flu-B, PIV-1, PIV-2, PIV-3, PIV-4, RSV, hMPV, RhV, EnV, and SARS-CoV in a single reaction.[78,84,85] When monoplex RT PCR is used for pathogen detection, the clinician often does not consider the possible presence of other pathogens when given a positive result. The multiplex approach offered by the ResPlex II system enhances diagnosis through detection of respiratory viral etiologic agents in cases in which their presence was unsuspected and an appropriate test consequently was not ordered by the clinician.[85] A recent study by Brunstein and colleagues[84] revealed that, using the ResPlex II kit covering 12 viral pathogens, 2.5% of specimens were coinfected with two or three different viruses. (A low level of cross-reactivity between PIV-1 and PIV-3 was noticed using this assay.[85]) These coinfections are medically relevant, and effective treatment of severe respiratory tract infections will increasingly require diagnosis of all involved pathogens, as opposed to single-pathogen reporting.[84] The original ResPlex II system detects only RNA viruses, but adenoviruses, bocavirus, and four coronaviruses have been added to a recently released new version of ResPlex II. Preliminary data indicate that the overall sensitivity and specificity of ResPlex II v2.0 is comparable to that of the ResPlex II panel. A notable number of previously negative samples were found to be positive for one of the newly added bocavirus or coronavirus targets (John Brunstein, 2009; personal communication). A factor that could diminish the analytical and clinical performance of ResPlex II and ResPlex II v2.0 is the potential for false-positive results caused by carryover of PCR products using the Luminex platform.

AMPLIFICATION PRODUCT DETECTION AND IDENTIFICATION

Pyrosequencing

Direct amplicon sequencing provides simple, rapid, and accurate means of detection and identification of amplification products. The need for robust, high-throughput methods to replace the elegant Sanger method, which was described more than 30 years ago,[86] has led to the development of several new principles. Ronaghi and colleagues[87,88] described in 1998 a pyrosequencing technique, a non–gel-based real-time approach to sequencing DNA by monitoring DNA polymerase activity. Pyrosequencing is based on enzymatic inorganic pyrophosphate release by DNA polymerase. This reaction is stoichiometric; the amount of light produced is proportional to the number of pyrophosphate molecules generated and, hence, the number of incorporated nucleotides. Unincorporated nucleotides are degraded with apyrase before the next nucleotide is added. In this way, sequence information on an interrogated region is generated quantitatively in real time. Although basic approaches to performing pyrosequencing remain the same, numerous commercial systems have been used widely to rapidly identify infectious agents and screen for antimicrobial drug resistance.[89–91] Multiplexed pyrosequencing involving the simultaneous extension of several primers hybridized to one or more target DNA templates[92] has gained broad acceptance in the fields of cytogenetics, pharmacogenetics, and medical genetics.[71,93,94]

Most applications of pyrosequencing in the identification and characterization of respiratory viruses have focused on Flu-A. Based on pyrosequencing technology, a rapid and highly informative diagnostic assay was reported for the detection of H5N1 Flu viruses[95]; sequencing of critical regions within the H5 virus was developed as a screening method during high volumes of H5N1 activity.[95] A real-time RT-PCR pyrosequencing assay was developed that combines restriction enzyme digestion and direct sequencing to screen and verify H5 Flu infections in humans.[96] Another RT-PCR assay with subsequent pyrosequencing analysis allows for a rapid, high-throughput, and cost-effective screening of subtype A/H1N1, A/H3N2, and A/H5N1 viruses and can clearly discriminate wild-type from a mutant viruses.[97] A study reported by Bright and colleagues[98] showed an alarming increase in the incidence of amantadine- and rimantadine-resistant H3N2 Flu-A viruses worldwide when the pyrosequencing technique was configured to cover a 44–base pair region of the M2 protein-encoding gene. Pyrosequencing assay capabilities were expanded to screen for 52 amino acid changes defined as avian or human specific,[99] and pyrosequencing-based assays recently were designed for detection and surveillance of the most commonly reported mutations associated with resistance to neuraminidase inhibitors and the adamantanes.[100–106] The latter detects mutations associated with resistance directly in clinical specimens, thus reducing the time required for testing and avoiding selection of novel sequence variants by cell culture. In addition, pyrosequencing-based assays have been reported for the characterization, quantification, typing, subtyping, and drug-resistance profiling of other viruses.[107–112]

One unique feature of pyrosequencing is its theoretical adaptability to the analysis of any genetic marker, which allows for the detection of multiple known and unknown mutations in a single pyrosequencing reaction. Integration of high-throughput pyrosequencing with the Roche/454 instrument has become a powerful tool for whole genome sequencing without the need for additional equipment or molecular techniques other than standard PCR, Genome Sequencer FLX sample preparation, and the sequencing pipeline.[113] Pyrosequencing generates sequence content quantitatively, which has made pyrosequencing a primary choice for quantifying specific mutations (eg, detection of drug resistance–associated signatures) in mixed genomic populations. Because pyrosequencing byproducts inhibit the sequencing reaction, pyrosequencing read lengths are limited to less than 100 base pairs. Another drawback of pyrosequencing-based techniques includes secondary structure formation, which affects quality of the results, particularly with GC-rich targets. Additionally, it may be difficult to determine the precise number of nucleotides in a homopolymeric region based on peak heights.[87] It is expected that pyrosequencing-based diagnostic devices will soon become available for rapid characterization and typing of viral pathogens.

Padlock Probes

Padlock probes, originated by Nilsson and colleagues[114] in 1994, are linear oligonucleotides designed so that the two end segments, connected by a linker region, are both complementary to a target sequence. On hybridization to a target sequence, the two probe ends become juxtaposed and can be joined by a DNA ligase. Reacted probes can be detected by way of reporter molecules attached to the linker.[115] Alternatively, an amplified signal can be obtained from the circularized probes by rolling circle amplification. Padlock probes provide a means for detection and quantification of large numbers of DNA or RNA sequences and for highly multiplexed genetic studies.[116] The application of padlock probes for the detection of microbial pathogens is a recent trend in molecular diagnoses.[117]

The unique padlock probe design provides the benefit of speed and sensitivity derived from using a nucleic acid–based method, and the amount of information is greatly increased by extensive multiplexing. Indeed, this method was used to simultaneously detect and type 16 HA and 9 NA subtypes of avian Flu virus. The analysis is completed within approximately 4 hours and performed in a single reaction tube, which helps to decrease the risk for contamination, with just a few sequential additions of reagents before the readout is performed using an oligonucleotide array.[118] Padlock probes combined with back-end microarray technology have been developed to detect foot-and-mouth disease, vesicular stomatitis, and swine vesicular disease viruses.[119] Besides viral pathogens, padlock probe–based techniques have been rapidly extended in recent years to the identification and characterization of bacterial and fungal pathogens.[120–124] In addition to the applicability of padlock probes for direct target detection, a universal primer binding site can be introduced into the probe and used for MLPA (see previous discussion).

Microarrays

Applications of microarrays to detect and characterize respiratory viruses began with solid arrays. The first respiratory pan-viral microarray system was described in 2002, which incorporated 1600 unique 70-mer oligonucleotide probes covering approximately 140 viral genome sequences.[60–65] Resequencing microarrays were developed to use short oligonucleotides for the simultaneous identification of respiratory pathogens at both the species and strain level.[125–127] Another comprehensive and panmicrobial microarray, the GreeneChipResp system, was developed for the detection of respiratory viruses and subtype identification of Flu-A viruses.[66] Other recently developed solid microarray systems for detection and identification of a panel of respiratory viruses include the Infiniti analyzer, an integrated molecular diagnostic device incorporating microarray hybridization[128]; the electronic microarray-based Nanochip[85,129]; the TaqMan Low Density Array cards, which use real-time PCR assays for 13 viruses and 8 bacteria known to cause pneumonia (Dean Erdman, 2009; personal communication); and the FilmArray, which detects and differentiates 17 viral and 4 bacterial etiologies of respiratory tract infections (Mark Poritz, 2009; personal communication).

Suspension bead-based liquid xMAP microarrays have been developed by Luminex Corp, which are essentially three-dimensional arrays based on the use of microscopic polystyrene beads as the solid support and flow cytometry for bead and target detection.[130] Robust multiplexing detection is accomplished using different bead sets based on fluorescence. The system enables multiplexing of up to 100 analytes in a single reaction using small sample volumes.[131,132] Numerous studies have described the use of xMAP technology for the detection and differentiation of nucleic acid sequences of microbial pathogens, including enteric bacteria, viruses, mycobacteria, fungi, and protozoa.[76–82,133–136] A molecular typing method incorporating the suspension array was reported to characterize and type Flu-A viruses, including H5N1.[83] The Luminex suspension array has been incorporated into several commercial devices as the detection platform to support the laboratory differential diagnosis of common respiratory viral pathogens. These include the xTAG Respiratory Viral Panel from Luminex Molecular Diagnostics,[137–139] the ResPlex II assay from Qiagen,[78,84,85] and the MultiCode-PLx RVP assay from EraGen Biosciences.[140,141] The suspension array system exhibits rapid hybridization kinetics, flexibility in assay design and format, and relatively low costs, which have made it the most practical microarray platform for clinical diagnostic applications. Users should carefully determine the positive fluorescence threshold for each viral target in multiplexed, user-defined assays during validation.

Mass Spectrometry

Matrix-assisted laser desorption/ionization time-of-flight (MALDI-TOF) mass spectrometry (MS) is widely used as a powerful proteomic tool. Its rapidity and high resolution provide another powerful platform for the detection and characterization of nucleic acid amplification products. The technology is premised on the capacity of MALDI-TOF MS to discriminate individual PCR products contained in complex amplicon mixtures according to nucleotide base composition.[142] The deconvolution algorithm allows base composition of PCR products to be deduced from mass spectrometrically measured molecular weights and the complementary nature of DNA, leading to organism identification. Early studies successfully used this technique to directly detect amplification products from PCR[143] and ligase chain reaction (LCR).[144] Soon after, the MALDI-TOF MS platform was linked to PCR amplification for genotypic analysis of hepatitis C virus[145] and human papillomavirus.[146,147] Detection of human herpesviruses from clinical specimens was performed using MALDI-TOF MS following multiplex PCR amplification.[148] A MALDI-TOF MS-based genotyping assay has been described that monitors development of hepatitis B virus polymerase YMDD mutant genotypes during lamivudine treatment.[149,150]

An integrated system, the Ibis T5000 Biosensor, has been developed to couple broad-range nucleic acid amplification to high-performance electrospray ionization MS and base-composition analysis. The system enables the identification and quantification of a broad set of pathogens, including all known bacteria, all major groups of pathogenic fungi, and the major families of viruses that cause disease in humans and animals, along with the detection of virulence factors and antibiotic resistance markers.[151] The system has been used for rapid identification and strain typing of respiratory bacterial pathogens for epidemic surveillance,[152] identification and genotyping of *Acinetobacter baumannii* strains in an outbreak associated with war trauma,[153,154] determination of quinolone resistance in *Acinetobacter* species,[155,156] genotyping of *Campylobacter* species,[155,156] and rapid genotyping and clonal complex assignment of *Staphylococcus aureus* isolates.[157] We have used this system

Table 1
Comparison of commercially available multiplexed amplification and high-throughput systems for detection and identification of respiratory viruses

System	Company	Viruses/Genotypes Detected	Amplification Platform	Detection Platform	Characteristics
FimArray respiratory pathogen panel	Idaho Technology Inc (Salt Lake City, UT)	AdV, bocavirus, 4 CoV, Flu-A, Flu-B, hMPV, PIV-1, PIV-2, PIV-3, PIV-4, RSV, and RhV	Nested multiplex RT-PCR	Solid array analyzer	Integrated and closed system. Also covers 4 bacterial pathogens
Infiniti respiratory viral panel[128]	AutoGenomics, Inc (Carlsbad, CA)	Flu-A, Flu-B, PIV-1, PIV-2, PIV-3, PIV-4, RSV-A, RSV-B, hMPV-A, hMPV-B, RhV-A, RhV-B, EnV, CoV, and AdV	Multiplex PCR and RT-PCR	Infiniti solid array analyzer	Detection step by the Infiniti analyzer is completely automatic
Jaguar system	HandyLab, Inc (Detroit, MI)	Flu-A, Flu-B, and RSV A/B	Multiplex real-time RT-PCR	Melting temperature analysis	Completely closed and automatic. Universal system compatible with detection of other pathogens. Throughput of 1–24 specimens/run
MultiCode-PLx respiratory virus panel[140,141]	EraGen Biosciences (Madison, WI)	Flu-A, Flu-B, PIV-1, PIV-2, PIV-3, PIV-4, RSV, hMPV, RhV, AdV, and CoV	Multiplex PCR and RT-PCR	Luminex suspension array	Universal beads used for detection use EraCode sequences
NGEN Respiratory Virus (RVA) Analyte-specific reagent[85,129]	Nanogen (San Diego, CA)	Flu-A, Flu-B, PIV-1, PIV-2, PIV-3, and RSV	Multiplex RT-PCR	NanoChip (solid chip)	Discontinued in 2008. Probe labeling, target capture, and detection accomplished using electronic microarray technology

ProFLU+, ProPARAFLU+[161,162]	Prodesse, Inc (Waukesha, WI)	Flu-A, Flu-B, and RSV (ProFLU+); PIV-1, PIV-2, PIV-3, and PIV-4 (ProPARAFLU+)	Multiplex real-time RT-PCR	Melting temperature analysis	ProFLU+ FDA cleared. Limited multiplex formats (triplex)
ResPlex II[78,84,85]	Qiagen (Valencia, CA)	Flu-A, Flu-B, PIV-1, PIV-2, PIV-3, PIV-4, RSV-A, RSV-B, hMPV, RhV, EnV, and SARS-CoV	TEM-RT-PCR	Luminex suspension array	Unique Tem-PCR permits multiple target screening in single reaction without significant loss in sensitivity
Seeplex respiratory virus detection assay[163]	Seegene, Inc (Seoul, Korea)	AdV, hMPV, 2 CoV, PIV-1, PIV-2, PIV-3, Flu-A, Flu-B, RSV-A, RSV-B, and RhV	Two sets of multiplex RT-PCR	Gel electrophoresis	Dual priming oligonucleotide system
xTAG respiratory viral panel (RVP)[137–139]	Luminex Molecular Diagnostics (Toronto, Canada)	Flu-A, Flu-B, PIV-1, PIV-2, PIV-3, PIV-4, RSV-A, RSV-B, hMPV, AdV, EnV, CoV, and RhV	Multiplex PCR and RT-PCR	Luminex suspension array	FDA cleared. Target-specific primer extension used in combination with universal detection beads

Abbreviations: AdV, adenoviruses; CoV, coronaviruses; EnV, enteroviruses; Flu, influenza virus; hMPV, human metapneumovirus; PIV, parainfluenza virus; RhV, rhinoviruses; RSV, respiratory syncytial virus; TEM, target enriched multiplex.

to detect *Ehrlichia*, *Anaplasma*, and *Rickettsia* pathogens directly from blood specimens for diagnosis of tick-borne sepsis (manuscript in preparation). In the field of diagnostic virology, this strategy successfully led to the inclusion of SARS-CoV in the coronavirus family.[158] Furthermore, the Ibis T5000 Biosensor system has been used as a rapid and inexpensive tool for global surveillance of emerging Flu virus genotypes[159] and rapid detection and molecular serotyping of adenoviruses.[160] The system was able to detect and type all available Flu A genotypes, including recently emerged novel A/H1N1 (David Ecker, 2009; personal communication). The main advantages are high resolution, speed, and substantial degree of automation. The main disadvantages include the engineering difficulty of MS device miniaturization and need for continuous enrichment of databases with new genomic sequences.

MULTIPLEXING AMPLIFICATION AND HIGH-THROUGHPUT DETECTION SYSTEMS

Respiratory infections caused by a many bacterial, viral, and fungal pathogens often present with overlapping signs and symptoms nearly indistinguishable by clinical diagnosis. Molecular screening of at-risk populations for a group of possible viral pathogens is an exciting area of development in molecular microbiology. Several multiplexing amplification and high-throughput detection systems are commercially available for the detection and differentiation of a panel of respiratory viral pathogens. Examples include the FilmArray platform from Idaho Technology Inc; the Infiniti Respiratory Viral Panel from AutoGenomics, Inc.[128]; the Jaguar system from HandyLab, Inc.; the Multi-Code-PLx respiratory virus panel from EraGen Biosciences[140,141]; the NGEN Respiratory Virus ASR from Nanogen[85,129]; the proFLU+ and the proPARA-FLU+ from Prodesse, Inc.[161,162]; the ResPlex II assay from Qiagen[78,84,85]; the Seeplex respiratory virus detection assay from Seegene, Inc.[163]; and the xTAG Respiratory Viral Panel from Luminex Molecular Diagnostics.[137–139] Some of these systems cover all varieties of Flu A genotypes including recently emerged novel A/H1N1.[164]

A comparative summary of these devices is presented in **Table 1**. Relative simplicity, powerful multiplexing capabilities, and affordability for high-throughput detection make these platforms most attractive for screening and detection of a panel of respiratory viruses in clinical infectious disease diagnostics. Although not essential, the availability of Food and Drug Administration–cleared products is a critical step in getting these systems into less-experienced diagnostic microbiology laboratories.[4,5] Opening of postamplification tubes and subsequent pipetting steps in the workflow of suspension arrays increases the risk for intra- and inter-run contamination for some assays. Careful attention should be paid to contamination control measures and the re-establishment of dedicated postamplification laboratory space in the real-time PCR era. Simultaneous testing for all possible pathogens is an efficient means to obtain a conclusive result and improves etiologic diagnosis.[81,137,165] In addition, assaying for all potential pathogens may yield crucial information regarding coinfections or secondary infections.[84,166,167] One study from the Netherlands indicated that implementation of multiple molecular assays for the etiologic diagnosis of lower respiratory tract infections increased the diagnostic yield considerably, yet did not reduce antibiotic use or costs.[168] Clinical relevance and cost effectiveness of simultaneous multipathogen detection and identification strategies merit further investigation.

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