SUPPLEMENT ARTICLE

Multiplex PCR and Emerging Technologies for the Detection of Respiratory Pathogens

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Molecular methods are becoming more widely used for the detection of respiratory pathogens, in part because of their superior sensitivity, relatively rapid turnaround time, and ability to identify pathogens that are slow growing or difficult to culture. The recent novel H1N1 influenza A pandemic served to underscore how quickly new molecular tests can become available for clinical use. Over the years PCR has been the dominant amplification method. Recently, modifications of this technology have emerged, some of which allow for the rapid detection of multiple pathogens in a single test. This review will focus on emerging multiplex molecular technologies and their clinical utility for the detection of respiratory pathogens.

The recent 2009 H1N1 influenza A pandemic has heighten our awareness of the need for improved diagnostic tests for respiratory pathogens. Because there is such overlap in the clinical presentation of various respiratory infections, it is not possible to reliably predict the pathogen based on clinical signs and symptoms. Moreover, rapid diagnostic tests has been shown to reduce the length of hospital stay and the costs for testing for those with viral respiratory tract infections [1, 2]. Here we will discuss emerging multiplex technologies, which can detect or quantify multiple respiratory pathogens in a single test. This is not an exhaustive discussion of emerging technologies but rather an overview of 6 technologies that are being applied to respiratory pathogens (Table 1.). Note that none of tests described herein have been cleared by the US Food and Drug Administration, and all are currently available only for research use. A brief overview of each technology will be presented along with any published data on respiratory virus testing. Each test should be viewed in the context of clinical utility. For example, is the method applicable for near-patient testing or more suited for testing in the clinical laboratory? What is the turnaround time for a result, and how will this affect utility for outpatient and/or inpatient diagnostic testing? Does the test identify all needed pathogens, or does it identify more or less pathogens than would be needed in a certain clinical setting? Finally will the result alter clinical management, that is, will it lead to better-directed (or reduced) use of antiviral therapy or antibiotics?

EMERGING TECHNOLOGIES

RespPlex Technology

The RespPlex technology (QIAplex Technology; Qiagen) involves nucleic acid purification and multiplex amplification by polymerase chain reaction (PCR), followed by detection using a liquid-phase bead-based array technology (Luminex xMAP). The technology allows for a high degree of multiplexing, with the detection of $\geqslant$15 pathogens from a single test. The RespPlex I test is designed to detect bacterial respiratory pathogens, whereas the RespPlex II test detects respiratory viruses. For each pathogen in the multiplex PCR, nested gene-specific primers are designed and

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Table 1. Summary of Emerging Multiplex Technologies for the Diagnosis of Respiratory Pathogens[a]

Characteristic	Test System					
	RespPlex	Infiniti	Jaguar	FilmArray	STAR	PLEX-ID
Pathogens detected	Viruses and bacteria	Viruses	Viruses	Viruses and bacteria	Viruses	Viruses and bacteria
Degree of multiplexity, no. of targets	>15	>15	2–6	>15	>15	>15
Complexity	High	High	Low	Low	High	High
Fully integrated system (all steps)	No	No	Yes	Yes	No	No
Testing location	Laboratory	Laboratory	Near-patient facility and/or laboratory	Near-patient facility and/or laboratory	Laboratory	Laboratory
Time required for result, h	5-6	6.5–10	1.5–2	1	5-6	6-8
Throughput	Moderate to high	Moderate to high	Moderate	Low	Moderate to high	Moderate to high
Carryover contamination risk	Moderate	Moderate	Low	Low	Low	Low
Quantification	No	No	No	No	Yes	No
Pathogen discovery	No	No	No	No	No	Yes

[a] These data reflect the state of technology as of October 2009; manufacturers may alter their test systems in the future.

included in the reaction [3]. These target specific primers are added at very low concentrations and are used to enrich the targets during the first few cycles of PCR. The inner gene-specific primers have tag sequences that are recognized by a universal set of primers, called SuperPrimers. The SuperPrimers are added at a higher concentration than the gene-specific primers, which allows for the exponential amplification of all targets present in the sample. The reverse SuperPrimer is labeled with biotin and is added at a higher concentration that the forward SuperPrimer; the asymmetric PCR yields more reverse strands for detection and eliminates the need to denature the PCR products before hybridization with probe. The labeled PCR products are detected using a capture probe specific for each target; these probes are covalently coupled to a color-coded bead, and each specific target corresponds to a unique bead color. The biotin-labeled amplified products are then detected by the addition of streptavidin-phycoerythrin. Detection occurs in the Luminex instrument, each unique colored bead is recognized by the laser, and the presence or absence of the phycoerythrin-labeled product determines whether that gene-specific target is present in the sample. The unique design of the ResPlex II system allows for sensitive, specific, and reproducible amplification of multiple targets.

The RespPlex system has been used to identify viral and bacterial pathogens in patients with symptoms of acute respiratory infection [4, 5]. The tests used in one study [4] were designed to detect 21 pathogens, including common viral and bacterial pathogens. Of the 1742 respiratory samples tested, 67% had $\geq$1 pathogen detected, including 40% with 1 pathogen, 21% with 2, and 6% with $\geq$3. For the viruses that were also detected by direct immunofluorescence testing [direct fluorescent antibody DFA] (influenza A and B, parainfluenza 1–3, respiratory syncytial virus [RSV]), the RespPlex test had a sensitivity of 84%–100% compared with DFA. For adenovirus, the ResPlex test had a sensitivity of <10% compared with DFA, which led to the reconfiguration of the adenovirus component of the test. Another study showed similar results [5]; the sensitivity of the RespPlex II test varied from 72% to 100% when compared with culture and monoplex real-time PCR assays, with a very high specificity (>99%). Although the sensitivity for some pathogens needs to be improved, overall the RespPlex tests have potential as a diagnostic tool. The ability to detect multiple pathogens in a single test is an important diagnostic advance and provides an approach to study the outcomes and clinical significance of coinfections.

The RespPlex system has also been used to detect H5N1 influenza A virus. The assay was designed to target H1, H2, H3, H5, H7, H9, N1, and N2 genes from influenza A virus as well as the NS genes from influenza A and influenza B viruses. This test allowed for the detection and genotyping of influenza A and B viruses. A total of 217 clinical samples were tested, including 14 samples with human H5N1 infection, and results were compared with those of viral culture and conventional and real-time reverse-transcription (RT) PCR. The RespPlex test had a clinical sensitivity of 93% and a specificity of 100% [3], providing a rapid method for identifying and subtyping influenza viruses.

The RespPlex I test, which detects 6 bacterial pathogens (*Streptococcus pneumoniae, Neisseria meningitidis, Haemophilus influenzae, Legionella pneumophila, Mycoplasma pneumoniae,* and *Chlamydophila pneumoniae*), was used to test 26 formalin-fixed, paraffin-embedded tissue samples from fatal cases of pneumonia or meningitis/meningococcemia that were confirmed by immunohistochemistry and organism-specific real-time PCR. Overall, the RespPlex I test identified 20 of 23 cases

with a single pathogen and 1 of 3 with multiple pathogens [6]. Multiplex tests have value in respiratory specimens and formalin-fixed, paraffin-embedded tissue when there is a broad differential and a limited volume of specimen.

The advantage of the ResPlex II test is that it can identify a large number of pathogens in a single test. However, this is a complex test with multiple steps, requiring batching of specimens with a turn-around time for results of 5–6 h. This is not practical for near-patient testing; the test needs to be performed in a clinical laboratory. One of the most significant limitations of this technology is the need for postamplification manipulation of samples, which greatly increases the risk of carryover contamination, and could lead to false-positive results.

Infiniti System

The Infiniti system (AutoGenomics) couples multiplex PCR with an automated microarray hybridization using the Infiniti analyzer. After nucleic acid extraction, a multiplex conventional RT-PCR reaction is performed with primers specific for >20 respiratory viruses. After amplification, the amplicons are purified with an enzymatic step to remove unincorporated nucleotides and primers and are then subjected to a proprietary primer extension step within the Infiniti analyzer. This step attaches a specific tag on each amplicon and also incorporates fluorescent-labeled nucleotides (Cy5-dCTP). The tagged and labeled amplicons are then hybridized to anti-tags immobilized on the microarray allowing specific identification of targets. Three replicates of each anti-tag on the microarray provide redundancy. The primer extension and, tagging, hybridization, scanning, and data analysis steps are automated and performed within the Infiniti analyzer.

A recent study by Raymond et al [7] compared the microarray test with single-target quantitative real-time PCR assays for 23 respiratory viruses that commonly infect children. Nasopharyngeal aspirates were collected from children ⩽3 years of age hospitalized for acute respiratory tract infections. Overall, 79.6% of specimens were positive for ⩾1 virus by both real-time and the microarray tests, 18.5% were negative for all viruses by both methods, 1.8% of the samples (4 of 221) were positive only with the real-time tests, and no viruses were detected only with the microarray test. The most common viruses detected were RSV, influenza A, coronaviruses, and rhinoviruses or enteroviruses; multiple viruses were found in 13.1% of the specimens. When results were compared between the 2 methods for each specimen, there was a 94.1% concordance. The sensitivity of the microarray test was ⩾90% for all of the viruses except adenoviruses and coronavirus NL63. Overall, the single real-time PCR tests were more sensitive than the microarray test, although the latter required fewer manual steps; for 24 specimens, 5 h less hands-on time is required, and throughput is higher than for the performance of the individual real-time tests. The microarray test requires several steps before loading of the Infiniti analyzer and is designed for batching specimens. The nucleic acid extraction, RT-PCR, and enzyme digestion steps take ~3 h; once the Infiniti analyzer is loaded, the first result is available in ~3.5 h, and each subsequent result requires an additional 20 min. Based on the complexity, batching, and time required for results, this technology is designed for the clinical laboratory and not for near-patient testing.

Jaguar System

The Jaguar system (Handy Lab, recently acquired by BD Diagnostics-GeneOhm) couples an automated nucleic acid extraction method with real-time PCR technology for the detection of multiple respiratory pathogens. After the clinical specimen is pipetted into the specimen tube, the reagents and disposables for the nucleic acid extraction and amplification reactions are loaded onto the instrument. Nucleic acid extraction is performed in individual reagent cartridges which are loaded into the Jaguar instrument along with the amplification cartridge. The remainder of the test is completely automated. The proprietary extraction method allows for the rapid and efficient purification and concentration of nucleic acid from a relatively large specimen volume (500 μL to 1.0 mL) into an elution volume of 10 μL , which is then used to resuspend the amplification reagents. The amplification step is performed in a cartridge that is about 7 × 11 cm and 2 mm thick, each cartridge can accommodate 12 reactions, each with a reaction volume of 4 μL. This small volume allows for a very rapid cycling time hence the entire test is completed in ~90–110 min. Because the system uses real-time PCR, the multiplexing capabilities are limited to 2–6 targets. However, the Jaguar system has several advantages: it is automated and very simple to use, essentially requiring a single pipette step, and because it uses real-time PCR there is no postamplification manipulation of the specimen, eliminating the risk of carryover contamination. The ease of use and rapid time to result would allow this system to be placed in a rapid-response laboratory in an emergency department to provide near-patient testing, with results available quickly enough to affect clinical management.

A multiplex assay for the detection of influenza A, influenza B, and RSV from nasopharyngeal specimens has been developed on the Jaguar system. The sensitivities for the detection of influenza A, influenza B, and RSV were 100%, 90%, and 100%, respectively, compared with tissue culture results [8]. The overall specificity was 99%. During a 10-day period, 1232 clinical samples were tested, and 134 were positive for influenza A. The Jaguar assay demonstrated 100% positive agreement when compared with laboratory-developed molecular tests; 2 false-positive findings were noted, for a 99.8% negative agreement. Of the 134 specimens, 126 were subtyped and found to be 2009 H1N1; all of these were detected by the Jaguar test, although the

assay as currently designed does not distinguish between influenza A subtypes. The Jaguar system required 20 min of technical and 3.5 h to test 24 specimens.

FilmArray Technology

The FilmArray system (Idaho Technologies) integrates nucleic acid extraction, nested PCR, detection, and data analysis in a single-use pouch. The system is automated and enables the detection of numerous viral and bacterial respiratory pathogens in a single test. All of the needed reagents for the assay are enclosed in the single-use pouch; after addition of water (to hydrate the lyophilized reagents) and the respiratory specimen, the pouch is loaded into the FilmArray instrument, and the rest of the test is completely automated. After extraction and purification of nucleic acid, a nested PCR reaction is performed within the pouch. The first-step PCR is a multiplexed reaction containing primers for all of the viral and bacterial targets, the amplicons from the first PCR are then diluted, and a second round of PCR reactions is performed in a multiwell array, each well containing a single primer set targeting a specific pathogen.

Redundancy is built into the array, with each target is amplified in 3 separate wells. Using amplification and melt curve analysis the FilmArray software generates a result for each target. The system is very robust, detecting a low concentration of pathogen in the presence of a high concentration of a second pathogen, with results available in 1 h. Although nested PCR is used, the system is closed, so there is no risk of carryover contamination. Preliminary studies show that the FilmArray system detects more viral pathogens and more coinfections than DFA testing. A respiratory panel is under development that detects adenoviruses, bocaviruses, conornaviruses, influenza A and B, influenza A subtypes (novel H12009, H1, H3), metapneumonvirus, parainfluenza viruses 1–4, RSV, and rhinoviruses, as well as *Bordetella pertussis*, *C. pneumoniae*, and *M. pneumoniae*. The ability to detect multiple pathogens within 1 h, with a simple set-up and fully automated system, raises the possibility of near-patient testing with the FilmArray technology, in the emergency department or an outpatient clinic. The current instrumentation is limited to a single test per run, so multiple instruments may be needed, depending on the required throughput.

Scalable Target Analysis Routine (STAR) Technology

Scalable Target Analysis Routine (STAR) technology (PrimeraDx) integrates conventional PCR with capillary electrophoresis, providing the ability to detect a large number of pathogens (>20) in a single reaction; it also has the unique feature of providing quantitative data. STAR is similar in principle to real-time PCR but uses amplicon size instead of fluorescent probes to identify targets. After nucleic acid extraction, a multiplex PCR reaction is set up with unique primers for each specific target. The thermal cycler for the amplification reaction and capillary electrophoresis device are contained in the ICEPlex instrument. During the amplification reaction a very small sample (nanoliter) of the master mix is subjected to capillary electrophoresis; with sequential sampling (every 2 cycles, starting at cycle 19) and size separation, the signal for the amplicon increases. The accumulation of the amplicon is plotted over time, generating an amplification curve, and a cycle threshold is determined for each target. Each different target can be identified based on the unique size of the amplicon. Quantification of the targets is accomplished by adding 3 standards to the master mix, each at a different concentration and with a unique amplicon size. A standard curve is generated by plotting the cycle threshold values for the standards against their known concentration, and this curve is used to determine the concentration of the targets. All of the amplification, capillary electrophoresis, and data analysis steps are automated with the ICEPlex instrument.

STAR technology can demonstrate a large number of pathogens in a single reaction, and the ability to quantify pathogens may prove very important in distinguishing colonization from invasive disease for pathogens such are *S. pneumoniae*. Because the assay requires ~5 h to complete, testing is performed in a clinical laboratory and samples are batched; this technology is therefore not designed for near-patient testing. The instrument is a closed system, so there is minimal risk of carryover contamination. Currently, a test for the quantification of cytomegalovirus, Epstein-Barr virus, BK virus, and human herpesviruses 6 and 7 in transplant recipients has been developed and is being evaluated; a test for respiratory pathogens is also planned.

PLEX-ID Technology

PLEX-ID (Abbott Molecular) is a unique system that combines PCR with electrospray ionization mass spectrometry (ESI-MS) to detect a broad array of pathogens including bacteria, viruses, fungi, and parasites. After nucleic acid is extracted and purified from the clinical specimen, multiple PCR reactions are performed in 96-well microtiter plates that use primers targeting highly conserved regions of the microbial genome that flank variable regions. These types of primers have been also used for sequence-based pathogen identification. The plates are loaded into the PLEX-ID analyzer, which automates the desalting step, ESI-MS, determination of base composition, and data analysis. The amplified mixture is injected into an electrospray ionization time-of-flight mass spectrometer, where the mass of each amplicon is measured. From the mass of the amplicon it is possible to determine the nucleotide base composition (ie, the number of A, G, C, and T nucleotides for that DNA molecule). By requiring the base composition for the forward and reverse strands to be complementary, the number of possibilities is reduced to a single unambiguous solution. The organism(s) in the sample are determined by comparing the base composition of the observed amplicons to a database comprising known sequence data from

a wide array of pathogens. Compared with culture or nucleic acid probe assays, which are limited to the detection of only those microbe(s) that grow or are targeted by the assay primer sequences, this broad-based approach enables the identification of known, emerging, or unanticipated pathogens.

The PLEX-ID system has been used to identify viral respiratory pathogens from clinical specimens with results compared with an expanded reference standard, including a combination of antigen detection, culture, and PCR methods. The PLEX-ID system performed well with a sensitivity of 95% for the detection of influenza A and B and 88% for RSV. Its sensitivity for the detection of adenoviruses, parainfluenza viruses, and coronaviruses was 100%, although the number of specimens with these pathogens was low [9]. The PCR/ESI technology has been used to examine samples obtained from military recruits during outbreaks of respiratory disease and identified a variety of bacterial pathogens including *H. influenzae, N. meningitidis,* and *Streptococcus pyogenes* [10].

This unique technology can detect a wide array of pathogens from clinical samples and is well suited for pathogen discovery and evaluation of emerging pathogens. For example, the PLEX-ID system was able to determine the mixed lineage (swine, avian, human) of the novel H1N1 influenza A virus. PCR/ESI technology was also able to identify and distinguish between a diverse collection of coronavirus species that included the acute respiratory syndrome–associated coronavirus [11]. Testing can be completed in 6–8 h; the system is designed for use in clinical laboratories, and it can be adapted for high-throughput testing, although the high cost of the instrumentation has to date limited its use to research studies.

SUMMARY

There are a wide array of emerging technologies for the detection and quantification of respiratory pathogens directly from clinical specimens. Some of these technologies have potential for high-throughput testing, and others will allow rapid near-patient testing. More studies are needed to fully elucidate their performance characteristics and determine their ideal clinical application. The goal is to identify the tests that will improve diagnostic testing for respiratory pathogens, affect patient management decisions, and lead to improved patient outcomes, more appropriate use of antivirals and antibiotics, and/or more cost-effective delivery of care.

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