JOURNAL OF CLINICAL MICROBIOLOGY, Nov. 2010, p. 3997–4002 Vol. 48, No. 11
0095-1137/10/$12.00 doi:10.1128/JCM.01118-10

Real-Time Detection of Influenza A, Influenza B, and Respiratory Syncytial Virus A and B in Respiratory Specimens by Use of Nanoparticle Probes▽†

Paul J. Jannetto,[1,2] Blake W. Buchan,[1] Kimberly A. Vaughan,[2] Joellen S. Ledford,[3] Dennis K. Anderson,[3] Donald C. Henley,[3] Neil B. Quigley,[3] and Nathan A. Ledeboer[1,2]*

Department of Pathology, Medical College of Wisconsin,[1] and Dynacare Laboratories,[2] 9200 West Wisconsin Ave., Milwaukee, Wisconsin 53226, and Molecular Pathology Laboratory Network, Inc., 250 East Broadway, Maryville, Tennessee 37804[3]

Received 2 June 2010/Returned for modification 22 July 2010/Accepted 28 August 2010

Seasonal epidemics of influenza and respiratory syncytial virus are responsible for significant morbidity and mortality worldwide. Infrequently, novel or reemergent strains of influenza A virus have caused rapid, severe global pandemics resulting in millions of fatalities. The ability to efficiently and accurately detect and differentiate respiratory viruses is paramount for effective treatment, infection control, and epidemiological surveillance. We evaluated the ability of two FDA-cleared nucleic acid-based tests, the semiautomated respiratory virus nucleic acid test (VRNAT) and the fully automated respiratory virus nucleic acid test *SP* (RVNAT$_{SP}$) (Nanosphere Inc., Northbrook, IL) to detect influenza A virus, influenza B virus, and respiratory syncytial virus A and B (RSV A/B) from clinical nasopharyngeal swab specimens. Detection of viral RNA in both tests is based on nucleic acid amplification followed by hybridization to capture probes immobilized on a glass slide. A novel technology utilizing gold nanoparticle-conjugated probes is utilized to detect the presence of captured target DNA. This microarray-based approach to detection has proven to be more sensitive than the traditional culture/direct fluorescent-antibody assay (DFA) method for detecting RSV and influenza viruses in clinical specimens, including the novel 2009 H1N1 strain. Specifically, we report 98.0% sensitivity and 96.5% specificity for the VRNAT compared to culture/DFA. Further, the VRNAT detected virus in an additional 58% of specimens that were culture negative. These data were confirmed using bidirectional sequencing. Evaluation of the fully automated RVNAT$_{SP}$, which is built on the same detection technology as the VRNAT but contains an updated processor enabling complete automation, revealed the two tests to be functionally equivalent. Thus, the RVNAT$_{SP}$ is a fully automated sample-to-result test capable of reliable detection of select respiratory viruses directly from clinical specimens in 3.5 h.

Influenza virus and respiratory syncytial virus (RSV) infect millions of individuals annually. Influenza virus is typically associated with infections of the upper respiratory tract and can cause mild to severe illness, with symptoms including abrupt onset of fever, malaise, severe myalgia, and a nonproductive cough. The bulk of illness is attributed seasonal epidemics of influenza virus types A and B, while type C is far less prevalent and rarely causes severe disease (9, 23). Type A influenza virus is often associated with more-severe symptoms, morbidity, and mortality owing to a greater genetic diversity typified by chromosomal reassortment between human and avian viruses. Such genetic reassortments are responsible for the emergence of novel subtypes of influenza A virus, including the infamous H1N1 Spanish flu virus of 1918 and the more recent 2009 H1N1 or "swine flu" virus (26).

RSV is capable of causing severe symptoms in infants, young children, and immunocompromised individuals (9, 24, 28) and is the leading cause of hospitalization in children under 5 years of age (24). Risk of developing severe symptoms is greatest in children less than 6 months of age, people with chronic lung disease, and the immunocompromised (7, 28). In individuals with these risk factors, RSV can migrate from its initial site of infection in the upper airway to the smaller bronchioles of the lower airway, where the pathogenic effects of viral infection, including airway inflammation and the resulting airway occlusion, are amplified (34). These effects can cause life-threatening bronchiolitis or pneumonia.

Patients infected with either influenza virus or RSV can present with similar early symptoms; however, the course of therapy and choice of antiviral agent are different for each virus. Therefore, rapid detection and differentiation of influenza virus and RSV as the etiologic agent in patients presenting with respiratory symptoms are paramount. Current detection methods for these viruses include viral culture, direct antigen tests, including enzyme immunoassays (EIA) and direct fluorescent-antibody assays (DFAs), and nucleic acid amplification tests (NAATs). Viral culture has high sensitivity; however, a major drawback is the labor-intensive nature of culture and the extended time to result, which can be up to 21 days for traditional tube culture (17). The extended time to result is problematic in outpatient settings, when results are returned only after discharge, and can be potentially serious in inpatient settings, where highly susceptible patients may be

* Corresponding author. Mailing address: Department of Pathology, Medical College of Wisconsin, 9200 West Wisconsin Ave., Milwaukee, WI 53226. Phone: (414) 805-7556. Fax: (414) 805-7560. E-mail: nledeboe@mcw.edu.

† Supplemental material for this article may be found at http://jcm.asm.org/.

▽ Published ahead of print on 8 September 2010.

exposed to infected patients awaiting the results of diagnostic testing. Antigen detection assays such as EIA and DFA offer a much more rapid result; however, they suffer from poor sensitivity, which limits their negative predictive value (8, 11, 25). The poor sensitivity of DFA and EIA rapid tests for detecting both seasonal and novel 2009 H1N1 influenza virus subtypes was highlighted by several studies during the recent outbreak of novel 2009 H1N1 influenza A virus. A comparison of several rapid antigen EIAs to reverse transcription-PCR (RT-PCR) revealed rapid EIA sensitivities of only 60 to 80% for detection of seasonal influenza virus strains (21). The sensitivity of EIAs was significantly worse when novel 2009 H1N1 strains were considered. Specifically, the sensitivities of BinaxNOW Influenza A&B (Inverness Medical, Waltham, MA), Rapid Detection Flu A+B (3M, St. Paul, MN), and QuickVue A+B (Quidel, San Diego, CA) are reported as 10 to 40%, 40 to 50%, and 50 to 70%, respectively (3, 8, 21, 35). These low sensitivities for detection of novel 2009 H1N1 were especially pronounced in specimens with a low viral load. These findings underscore the need for rapid, sensitive methods to detect viral pathogens from clinical specimens.

Assays based on nucleic acid amplification of virus-specific targets have the advantage of high sensitivity and specificity, which can be ≥99%, as well as the potential for turnaround times of ≤24 h (13, 19, 29). NAATs also have the added capability of detecting several viruses through the use of multiplexed PCR strategies. FDA-cleared assays, including ProFlu+ (Gen-Probe, San Diego, CA) and xTAG RVP (Luminex, Austin, TX), as well as analyte-specific reagents (ASRs) and laboratory-designed tests (LDTs), effectively use multiplexing to identify a number of respiratory viruses in clinical specimens (13). Despite these advantages, many of these NAATs require manual batch-based processing and extraction steps, which can be laborious and lead to increased time to result and possible specimen cross contamination. The availability of unique fluorescent markers for each target sequence also limits the number of specific pathogens that can be detected by some of these assays. Melt curve analysis can partially circumvent this limitation, although its use is generally restricted to discrimination of single nucleotide polymorphisms (SNPs), and probe design to encompass several targets and can present technical challenges.

Microarray-based detection of amplified nucleic acid targets offers several advantages over other liquid phase detection methods. The ability to detect a labeled probe spatially on a solid surface, rather than in a liquid phase, allows the use of a single fluorophore-, enzyme-, or nanoparticle-conjugated probe for detection. This design overcomes the multiplexing limitations presented by spectral overlaps between fluorescent probes in liquid phase detection systems. Nanoparticle technology can be applied to DNA detection through the coupling of target-specific thiolated oligonucleotide probes to gold nanoparticles. Detection of nanoparticle-conjugated probes relies on light scatter rather than fluorescence. The utility of this methodology for the detection of specific DNA sequences has been previously demonstrated (5) and is thoroughly reviewed elsewhere (32). Advantages of this detection method include an increase in sensitivity of up to 1,000-fold compared to fluorescence-based detection methods and a reduction in background noise (30), which can, in some cases, make detection possible without target amplification. This detection mechanism also requires comparatively simple excitation and detection optics.

Herein we describe and compare the clinical trials for two FDA-cleared microarray systems, the semiautomated respiratory virus nucleic acid test (VRNAT) and its successor, the fully automated respiratory virus nucleic acid test *SP* ($RVNAT_{SP}$) (Nanosphere, Northbrook, IL) for the detection of influenza A virus, influenza B virus, RSV A, and RSV B in clinical specimens. The analytical and clinical performance of the semiautomated Verigene RVNAT was directly compared to that of the fully automated $RVNAT_{SP}$ as well as to culture/DFA results. Finally, studies were conducted to evaluate the performance of the $RVNAT_{SP}$ for detection of the novel 2009 H1N1 influenza A virus subtype.

MATERIALS AND METHODS

Specimens. A total of 720 nasopharyngeal swabs were collected from two geographically diverse sites, one in the southern United States and another in the southeastern United States, during the 2007 to 2008 and 2008 to 2009 respiratory virus seasons. Subjects had a distribution of ages ranging from less than 1 year to over 65 years. All specimens were tested for influenza virus and RSV at the time of collection using culture or virus-specific DFA detection. Residual specimens were stored at the time of initial analysis at −70°C for later analysis using the VRNAT or $RVNAT_{SP}$ at one of three clinical test sites. This study has been reviewed and approved by each site's institutional review board.

Equipment and instrumentation. The $VRNAT_{SP}$ requires the use of the Verigene *SP* system. The Verigene *SP* comprises a single Verigene reader (approximately 16 in. by 12 in.), which has the capability to read and interpret the $VRNAT_{SP}$ microarray slides in <1 min. The reader can interface with up to 32 Verigene *SP* processors. Each processor is free standing (approximately 8 in. by 23 in.) and is capable of random-access, multifunctional test processing, including nucleic acid extraction, reverse transcription (if necessary), target amplification (if necessary), and DNA hybridization. The system can accommodate several different test cartridges for additional nucleic acid tests available from the manufacturer.

Manual extraction and amplification for the semiautomated VRNAT. Residual specimen samples were thawed to room temperature, and a 200-μl aliquot was removed. A 50-μl aliquot of MS2 phage was added to the clinical specimen as an internal control for extraction and PCR inhibition. Nucleic acid was extracted from the entire 250 μl using the NucliSENS EasyMAG (bioMerieux, Marcy l'Etoile, France) and was eluted into a final volume of 60 μl buffer. RT-PCR was carried out by adding 5 μl of the eluate to 20 μl of an enzyme premix containing *Tfi* SuperMix (Invitrogen, Carlsbad, CA), uracil deglycosylase (UDG), reverse transcriptase, and proprietary oligonucleotide primers specific for each viral target. Following amplification, 10 μl of the product was mixed with sample buffer and loaded into a disposable RV test cartridge. Processing and hybridization were carried out by the Verigene processor. Upon completion of processing, the RV test cartridge was removed from the processor and analyzed using the Verigene reader. Results were reported as "detected" or "not detected" for each of the four reportable agents detected by this assay (influenza A virus, influenza B virus, RSV A, and RSV B). The VRNAT is capable of discriminating RSV A and RSV B; however, the initial characterization by culture/DFA did not distinguish the two subtypes, so VRNAT results are reported as "RSV detected" when either subtype (RSV A or RSV B) is detected. A message of "no call" was reported by the reader in the event of assay failure, such as an internal control failure. Samples generating this message were rerun to obtain a valid result.

Specimen preparation and loading for the fully automated $RVNAT_{SP}$ assay. Single-use extraction and amplification trays containing all necessary reagents were loaded into the Verigene *SP* processor. Specimens were thawed, and a 200-μl aliquot was transferred to the specimen well in the extraction tray. A single-use RV test cartridge containing the slide array and hybridization reagents was loaded into the Verigene *SP* processor, and the assay was started. Upon completion of the test, the RV test cartridge was removed from the processor and the hybridization slide was inserted into the Verigene reader. Results were recorded as described above.

Analysis of discrepant specimens. Specimens producing discrepant results between culture/DFA and VRNAT were resolved using bidirectional sequencing

employing primer annealing sites that were different from that used in the VRNAT assay. Approximately 100 to 200 ng of purified DNA was sent to ACGT, Inc. (Wheeling, IL) for sequencing. All amplification reactions were performed on a Bio-Rad (Hercules, CA) C1000 thermal cycler or GeneAmp (Applied Biosystems, Carlsbad, CA) PCR System 9700 thermal cycler. Four positive controls [pGEM 3Zf(+) plasmid DNA with M13F (−21) primer] and four negative controls [nuclease-free water with M13F (−21) primer] were included in a 96-well plate and run concomitantly with the samples. The sequencing reaction products were purified with the Agencourt CleanSeq kit (Beckman Coulter, Danvers, MA) and analyzed by the ABI 3730 XL genetic analyzer (Applied Biosystems, Carlsbad, CA). The raw data from sequencing reactions were collected using ABI 3730 foundation data collection software and processed by InterPhace software (CodonCode, Dedham, MA) to generate a Phred quality score for each base in the sequence. The sequence data were used for analysis if the controls met the following criteria: none of the negative controls (0/4) have more than 100 consecutive bases with a score of Phred 30 (99.9% accuracy) or above, while 50% of the positive controls (2/4), at a minimum, have at least 200 bases with scores of Phred 30 (99.9% accuracy) or above. Sequencing data were assembled by Sequencher (version 4.2) software (GeneCodes, Ann Arbor, MI) to generate a single contig for each PCR product. A consensus sequence was produced from each alignment, which was then compared to those of the reference strains (see Table S1 in the supplemental material) for confirmation of virus identification.

Analytical specificity experiments. A total of 15 viral and 23 bacterial strains available from ATCC (see Table S2 in the supplemental material) were obtained, and stock dilution cultures were made. Bacterial CFU/ml was determined by serial dilution. The viral strains were quantified by assessing tissue culture infectivity to provide 50% tissue culture infective doses ($TCID_{50}$)/ml. The $TCID_{50}$/ml value was multiplied by a factor of 0.7 to obtain an approximate PFU/ml. Pure suspensions of each agent were extracted, amplified, and analyzed using the VRNAT, under conditions identical to those used for the analysis of clinical specimens.

Analytical sensitivity experiments. A stock culture containing a defined PFU/ml of each virus was diluted serially into universal transport media (Copan, Murrieta, CA). Dilutions of each agent were tested a minimum of three times using the VRNAT or RVNAT$_{SP}$ to establish a limit of detection (LoD). The presumptive LoD was then confirmed by an additional 20 replicate experiments. Achievement of ≥95% positive results was required to define the LoD for each agent.

Statistical analysis. The sensitivity, specificity, positive predictive value (PPV), and negative predictive value (NPV) were determined using standard methods (22). For calculations made before resolution of discrepant results, a true-positive/negative result was based solely on culture/DFA regardless of VRNAT result. Following discrepancy resolution, a true positive was defined by agreement between culture/DFA and VRNAT or by positivity in either assay, which was confirmed using bidirectional sequencing.

RESULTS

Analytical specificity/sensitivity and cross-reactivity with other infectious agents. To demonstrate the analytical specificity and sensitivity of the VRNAT, established titers of 11 different strains of influenza virus or RSV were serially diluted into viral transport medium and analyzed using the VRNAT. The limit of detection (LoD) for each strain, defined as the lowest viral concentration at which ≥95% of the replicates tested positive, was established and confirmed by not less than 20 replicate assays. Specifically, the LoD of influenza A and B virus strains tested ranged from 2 to 60 $TCID_{50}$/ml (Table 1). This is well below the established level of 10^3 to 10^7 $TCID_{50}$/ml of influenza A virus shed in the nasopharynx during active infections (36). Of special interest, the VRNAT was able to detect the novel 2009 H1N1 swine origin influenza A virus subtype at a similarly low LoD of 50 $TCID_{50}$/ml (Table 1). To assess the analytical specificity of the assay, a comprehensive panel of common respiratory viruses (15) and bacteria (23) was examined. Among the viruses tested were human adenovirus types 1 and 7, two types of human coronavirus, cytomegalovirus, an enterovirus (coxsackievirus B4), Epstein-Barr virus, human parainfluenza types 1, 2, 3, and 4a, measles virus, mumps virus, and human metapneumovirus. Included in the bacterial pathogens were common strains of *Acinetobacter*, *Bordetella*, *Chlamydophila*, *Corynebacterium*, *Escherichia coli*, *Haemophilus*, *Klebsiella*, *Lactobacillus*, *Legionella*, *Listeria*, *Moraxella*, *Neisseria*, *Proteus*, *Pseudomonas*, *Staphylococcus*, *Streptococcus*, *Mycoplasma*, and *Mycobacterium*. All strains were processed in pure culture by the VRNAT at concentrations ranging from 10^5 to 10^7 CFU/ml or PFU/ml (see Table S2 in the supplemental material). None of the tested microbes produced a positive result, demonstrating 100% specificity.

TABLE 1. Analytical sensitivities of VRNAT and RVNAT$_{SP}$ (limits of detection)

Virus strain	LoD of:	
	VRNAT	RVNAT$_{SP}$
Influenza A virus strains		
Influenza A/Wisconsin/67/05 (H3N2)	2 $TCID_{50}$[a]/ml	2 $TCID_{50}$/ml
Influenza A/New Caledonia/20/99 (H1N1)	50 $TCID_{50}$/ml	
Influenza A/Port Chalmers/1/73 (H3N2)	50 $TCID_{50}$/ml	
Influenza A/Wisconsin/629/2009 (2009 H1N1)	50 $TCID_{50}$/ml	
Influenza B virus strains		
Influenza B/Florida/04/2006	60 $TCID_{50}$/ml	50 $TCID_{50}$/ml
Influenza B/Lee/40	0.01 EID_{50}[b]/ml	
Influenza B/Hong Kong/5/72	0.05 EID_{50}/ml	
RSV A strains		
A2	10 $TCID_{50}$/ml	
Long	10 $TCID_{50}$/ml	10 $TCID_{50}$/ml
RSV B strains		
B-1 Wild Type (B WV/14617/85)	2 $TCID_{50}$/ml	2 $TCID_{50}$/ml
Wash/18537/62	0.5 $TCID_{50}$/ml	
9320	0.05 $TCID_{50}$/ml	

[a] $TCID_{50}$, 50% tissue culture infective dose.
[b] EID_{50}, 50% embryo infectious dose.

Comparison of VRNAT to culture/DFA methods for the detection of influenza A virus, influenza B virus, and RSV A and B. A total of 720 nasopharyngeal specimens were collected prospectively from multiple sites during the 2007 to 2008 and 2008 to 2009 respiratory virus seasons. The patient population was chosen to include a broad distribution of ages, containing 120 subjects 0 to 1 year of age, 229 between the ages of 1 and 5 years, 129 between the ages of 5 and 20 years, 204 between the ages of 20 and 65 years, and 38 over the age of 65 years. Patient specimens were assayed for the presence of influenza A virus, influenza B virus, RSV A, and RSV B using culture/DFA methods at the time of collection. Of the 720 specimens analyzed by culture/DFA, 123 (17%) were positive for influenza A virus by culture/DFA, 31 (4%) were positive for influenza B virus, 49 (7%) were positive for RSV, and 517 (72%) specimens were culture negative. When the same specimens were analyzed using the VRNAT, 181 (25%) were positive for influenza A virus, 40 (6%) were positive for influenza B virus, and 101 (14%) were positive for RSV A or B (Table 2). In

TABLE 2. Comparison of VRNAT to culture/DFA methods for influenza virus and RSV detection

Test method	No. of positive results[a]			
	Total	Influenza A virus	Influenza B virus	RSV
VRNAT	322 (119)	181 (58[b])	40 (9[c])	101 (52[d])
Culture/DFA	203	123	31	49

[a] Values in parentheses are numbers of additional positive results by VRNAT.
[b] All were positive by sequencing.
[c] Four were positive by sequencing.
[d] Forty-six were positive by sequencing.

comparison to culture/DFA, these results translate to VRNAT sensitivity and specificity of 96.6% and 93.6%, respectively (Table 3). Of the 133 discrepant specimens between the VRNAT and culture, bidirectional sequencing revealed that the VRNAT detected 108 additional positives (58 influenza A virus, 4 influenza B virus, and 46 RSV), all of which were negative by culture/DFA. Following discrepancy resolution, the VRNAT sensitivity for all agents was 98.0% and specificity was 96.5%. This also resulted in an overall increase in the PPV of the VRNAT from 60.9% to 91.6% (Table 3). Of note, since sequencing requires a significantly greater amount of amplified product than is needed for detection by the VRNAT, a few of the discrepant samples that were positive by VRNAT could not be confirmed by sequencing and may have been the result of low-level infections. Among the clinical specimens, there were also a number of dual infections comprising different influenza virus types or influenza virus-RSV coinfections. This included 3 influenza A virus-influenza B virus coinfections, 5 influenza A virus-RSV A or B coinfections, 4 influenza B virus-RSV A or B coinfections, and 3 RSV A-RSV B coinfections. The agents in these coinfections were reliably detected by the VRNAT, demonstrating its ability to simultaneously detect multiple pathogens from a single specimen.

TABLE 3. Clinical performance of VRNAT compared to culture/DFA methods[a]

Specimens considered and discord resolution status	Sensitivity	Specificity	PPV[b]	NPV[c]
All specimens				
Before discord resolution	96.6	93.6	60.9	99.6
After discord resolution	98.0	96.5	91.6	99.2
Influenza A virus positive				
Before discord resolution	99.2	90.1	67.4	99.8
After discord resolution	100.0	99.8	99.2	100.0
Influenza B virus positive				
Before discord resolution	96.8	98.5	75.0	99.9
After discord resolution	100.0	99.1	83.3	100.0
RSV positive				
Before discord resolution	89.8	91.5	43.6	99.2
After discord resolution	91.7	98.4	80.0	99.4

[a] A total of 720 nasopharyngeal swab specimens were analyzed using culture/DFA and VRNAT assays to detect influenza A and B virus and RSV. Discordant results were resolved using bidirectional sequencing.
[b] PPV, positive predictive value.
[c] NPV, negative predictive value.

TABLE 4. Comparison of manual VRNAT and automated $RVNAT_{SP}$ systems for detection of influenza A virus, influenza B virus, and RSV

$RVNAT_{SP}$ result	No. of specimens with VRNAT result of:		Total
	Positive	Negative	
Positive	191	0	191
Negative	4[a]	363	367
Total	195	363	558

[a] All 4 specimens that initially gave a negative result on the $RVNAT_{SP}$ were positive upon repeat assay.

Comparison of VRNAT to $RVNAT_{SP}$. In a follow-up study, we compared the performance of the fully automated $RVNAT_{SP}$ to that of the semiautomated VRNAT previously established in this study. To accomplish this, a total of 558 clinical specimens, including a distribution of culture/DFA-positive influenza A and B virus and RSV specimens, were analyzed using both the VRNAT and $RVNAT_{SP}$. Of the 558 specimens, 195 were positive for influenza A or B virus or RSV using the VRNAT and 191 were positive using the $RVNAT_{SP}$. This resulted in a concordance of 99.3% between the two assays (Table 4). All 4 specimens that were initially positive by VRNAT and negative by $RVNAT_{SP}$ were positive by the $RVNAT_{SP}$ following a repeat test. These results demonstrate the equivalence of the two systems for detection of influenza A virus, influenza B virus, and RSV A and B from clinical specimens.

Analytical sensitivity experiments were conducted independently using the $RVNAT_{SP}$. The experimental setup was identical to that used to establish the analytical sensitivity of the VRNAT (see results above and Materials and Methods), and experiments were conducted using one strain each of influenza A virus, influenza B virus, RSV A, and RSV B. Results indicated similarly high sensitivities for all agents tested, ranging from 2 to 50 $TCID_{50}$/ml (Table 1).

DISCUSSION

Rapid, accurate detection of respiratory viruses from clinical specimens such as nasopharyngeal swabs is critical to patient management and limiting the spread of infection. Methods to detect viral pathogens from the respiratory tract include laboratory culture in various susceptible cell lines, serologic detection of virus-specific antibodies, detection of viral antigens either directly from patient specimens (EIA) or in conjunction with cell culture (DFA), and molecular techniques involving nucleic acid amplification tests (NAATs) coupled with one of several detection methods. In the current study, we assessed the ability of two nucleic acid amplification assays, the semiautomated VRNAT and its fully automated successor, the $RVNAT_{SP}$, to detect influenza A virus, influenza B virus, RSV A, and RSV B from clinical specimens.

Our comparison of the manual VRNAT to culture/DFA for the detection of influenza A virus, influenza B virus, RSV A, and RSV B comprised 720 clinical specimens in the form of nasopharyngeal swabs. Importantly, the study included 242 adult subjects over the age of 20 years, a group for which

current data suggest EIAs demonstrate especially poor sensitivity (11, 27). Our data demonstrate the substantially greater clinical sensitivity of the VRNAT (98.7%) compared to the culture/DFA method. High specificity was also achieved in both clinical (99.3%) and analytical experiments using a wide variety of pathogenic and commensal organisms commonly isolated from the respiratory tract. Similarly, the analytical sensitivity of the VRNAT was examined using 4 different influenza A virus subtypes, including 2009 H1N1, 3 influenza B virus subtypes, 2 RSV A subtypes, and 3 RSV B subtypes. Results indicate that the sensitivity of detection is high enough to easily detect active influenza virus infection in adults and is similar to or greater than the sensitivities of other reported NAATs for respiratory virus detection (1, 2, 31).

Several multiplex liquid-phase NAATs for the detection of respiratory viruses are currently available and have been evaluated, including the Hexaplex and ProFlu-1 assays (Prodesse, Waukesha, WI), various ASR products, and several laboratory-developed assays (1, 6, 10, 12–15, 31). In contrast, there are few assays currently available that couple nucleic acid amplification with a microarray-based scheme for detection. Mchip and FluChip (19, 20, 33) are examples of such tests; however, these assays are available only as research use only (RUO) assays and are not widely used in clinical labs. The xTAG RVP (Luminex) is an FDA-cleared product capable of simultaneous detection of 10 different viruses, including two influenza A virus subtypes (16, 18). A major advantage of all of these NAAT methodologies, in addition to high sensitivity and specificity, is the potential for extremely rapid turnaround time. These assays all report times to result of approximately 5 h (xTAG RVP, Mchip) but require several manual or automated processing and extraction steps prior to starting the assay (4, 16), which can add up to 2 h to the total time to result and may discourage individual specimen processing at the time of receipt in favor of batch-based processing once or twice daily. Batched processing may also introduce variability in the quality and quantity of the extracted template and increases the potential for cross contamination or specimen misidentification.

A key advantage of the RVNAT$_{SP}$ is the fully automated sample-to-result capability, which includes extraction, amplification, and hybridization steps within an enclosed instrument. This automation enables a total time to result of 3.5 h, reduces the possibility for error or variation during manual nucleic acid extraction, and eliminates the need for batch processing. Such on-demand capability is especially valuable during seasonal influenza outbreaks, when test volume can rise rapidly and instruments are run at maximum capacity.

We have described and evaluated two nucleic acid amplification-based assays for the detection of influenza virus and RSV, which are also capable of accurately detecting the novel 2009 H1N1 strain of influenza A virus. A direct comparison of the VRNAT and RVNAT$_{SP}$ using 558 clinical specimens demonstrated full agreement and functional equivalence between the manual (VRNAT) and automated (RVNAT$_{SP}$) systems. Clinical trials are under way to validate an improved assay, the RVNAT$_{SP}$+, which can detect and discriminate influenza A virus H1, H3, and novel 2009 H1N1 subtypes, the H275Y oseltamivir resistance mutation, influenza B virus, RSV A, and RSV B.

ACKNOWLEDGMENTS

Materials and financial support for this study were provided by Nanosphere Inc. N. A. Ledeboer is a consultant to Nanosphere.

REFERENCES

1. **Beck, E. T., L. A. Jurgens, S. C. Kehl, M. E. Bose, T. Patitucci, E. LaGue, P. Darga, K. Wilkinson, L. M. Witt, J. Fan, J. He, S. Kumar, and K. J. Henrickson.** 2010. Development of a rapid automated influenza A, influenza B, and respiratory syncytial virus A/B multiplex real-time RT-PCR assay and its use during the 2009 H1N1 swine-origin influenza virus epidemic in Milwaukee, Wisconsin. J. Mol. Diagn. **12:**74–81.
2. **Choi, J. H., Y. S. Chung, K. S. Kim, W. J. Lee, I. Y. Chung, H. B. Oh, and C. Kang.** 2008. Development of real-time PCR assays for detection and quantification of human bocavirus. J. Clin. Virol. **42:**249–253.
3. **Crawford, J. M., R. Stallone, F. Zhang, M. Gerolimatos, D. D. Korologos, C. Sweetapple, M. de Geronimo, Y. Dlugacz, D. M. Armellino, and C. C. Ginocchio.** 2010. Laboratory surge response to pandemic (H1N1) 2009 outbreak, New York City metropolitan area, U. S. A. Emerg. Infect. Dis. **16:**8–13.
4. **Dawson, E. D., C. L. Moore, J. A. Smagala, D. M. Dankbar, M. Mehlmann, M. B. Townsend, C. B. Smith, N. J. Cox, R. D. Kuchta, and K. L. Rowlen.** 2006. MChip: a tool for influenza surveillance. Anal. Chem. **78:**7610–7615.
5. **Elghanian, R., J. J. Storhoff, R. C. Mucic, R. L. Letsinger, and C. A. Mirkin.** 1997. Selective colorimetric detection of polynucleotides based on the distance-dependent optical properties of gold nanoparticles. Science **277:**1078–1081.
6. **Erdman, D. D., G. A. Weinberg, K. M. Edwards, F. J. Walker, B. C. Anderson, J. Winter, M. Gonzalez, and L. J. Anderson.** 2003. GeneScan reverse transcription-PCR assay for detection of six common respiratory viruses in young children hospitalized with acute respiratory illness. J. Clin. Microbiol. **41:**4298–4303.
7. **Falsey, A. R., and E. E. Walsh.** 2000. Respiratory syncytial virus infection in adults. Clin. Microbiol. Rev. **13:**371–384.
8. **Ginocchio, C. C., F. Zhang, R. Manji, S. Arora, M. Bornfreund, L. Falk, M. Lotlikar, M. Kowerska, G. Becker, D. Korologos, M. de Geronimo, and J. M. Crawford.** 2009. Evaluation of multiple test methods for the detection of the novel 2009 influenza A (H1N1) during the New York City outbreak. J. Clin. Virol. **45:**191–195.
9. **Hay, A. J., V. Gregory, A. R. Douglas, and Y. P. Lin.** 2001. The evolution of human influenza viruses. Philos. Trans. R. Soc. Lond B Biol. Sci. **356:**1861–1870.
10. **Kehl, S. C., K. J. Henrickson, W. Hua, and J. Fan.** 2001. Evaluation of the Hexaplex assay for detection of respiratory viruses in children. J. Clin. Microbiol. **39:**1696–1701.
11. **Landry, M. L., and D. Ferguson.** 2003. Suboptimal detection of influenza virus in adults by the Directigen Flu A+B enzyme immunoassay and correlation of results with the number of antigen-positive cells detected by cytospin immunofluorescence. J. Clin. Microbiol. **41:**3407–3409.
12. **Lassauniere, R., T. Kresfelder, and M. Venter.** 2010. A novel multiplex real-time RT-PCR assay with FRET hybridization probes for the detection and quantitation of 13 respiratory viruses. J. Virol. Methods **165:**254–260.
13. **Legoff, J., R. Kara, F. Moulin, A. Si-Mohamed, A. Krivine, L. Belec, and P. Lebon.** 2008. Evaluation of the one-step multiplex real-time reverse transcription-PCR ProFlu-1 assay for detection of influenza A and influenza B viruses and respiratory syncytial viruses in children. J. Clin. Microbiol. **46:**789–791.
14. **Li, P. Q., J. Zhang, C. P. Muller, J. X. Chen, Z. F. Yang, R. Zhang, J. Li, and Y. S. He.** 2008. Development of a multiplex real-time polymerase chain reaction for the detection of influenza virus type A including H5 and H9 subtypes. Diagn. Microbiol. Infect. Dis. **61:**192–197.
15. **Liao, R. S., L. L. Tomalty, A. Majury, and D. E. Zoutman.** 2009. Comparison of viral isolation and multiplex real-time reverse transcription-PCR for confirmation of respiratory syncytial virus and influenza virus detection by antigen immunoassays. J. Clin. Microbiol. **47:**527–532.
16. **Mahony, J., S. Chong, F. Merante, S. Yaghoubian, T. Sinha, C. Lisle, and R. Janeczko.** 2007. 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. **45:**2965–2970.
17. **Mahony, J. B.** 2008. Detection of respiratory viruses by molecular methods. Clin. Microbiol. Rev. **21:**716–747.
18. **Mahony, J. B., T. Hatchette, D. Ojkic, S. J. Drews, J. Gubbay, D. E. Low, M. Petric, P. Tang, S. Chong, K. Luinstra, A. Petrich, and M. Smieja.** 2009. Multiplex PCR tests sentinel the appearance of pandemic influenza viruses including H1N1 swine influenza. J. Clin. Virol. **45:**200–202.
19. **Mehlmann, M., A. B. Bonner, J. V. Williams, D. M. Dankbar, C. L. Moore, R. D. Kuchta, A. B. Podsiad, J. D. Tamerius, E. D. Dawson, and K. L. Rowlen.** 2007. Comparison of the MChip to viral culture, reverse transcription-PCR, and the QuickVue influenza A+B test for rapid diagnosis of influenza. J. Clin. Microbiol. **45:**1234–1237.
20. **Moore, C. L., J. A. Smagala, C. B. Smith, E. D. Dawson, N. J. Cox, R. D. Kuchta, and K. L. Rowlen.** 2007. Evaluation of MChip with historic subtype

H1N1 influenza A viruses, including the 1918 "Spanish Flu" strain. J. Clin. Microbiol. **45:**3807–3810.
21. **Morbidity and Mortality Weekly Report.** 2009. Evaluation of rapid influenza diagnostic tests for detection of novel influenza A (H1N1) virus—United States, 2009. MMWR Morb. Mortal. Wkly. Rep. **58:**826–829.
22. **Motulsky, H.** 1995. Intuitive biostatistics. Oxford University Press, Inc., New York, NY.
23. **Neumann, G., and Y. Kawaoka.** 2006. Host range restriction and pathogenicity in the context of influenza pandemic. Emerg. Infect. Dis. **12:**881–886.
24. **Olson, M. R., and S. M. Varga.** 2008. Pulmonary immunity and immunopathology: lessons from respiratory syncytial virus. Expert Rev. Vaccines **7:**1239–1255.
25. **Rahman, M., B. A. Kieke, M. F. Vandermause, P. D. Mitchell, R. T. Greenlee, and E. A. Belongia.** 2007. Performance of Directigen flu A+B enzyme immunoassay and direct fluorescent assay for detection of influenza infection during the 2004–2005 season. Diagn. Microbiol. Infect. Dis. **58:**413–418.
26. **Schnitzler, S. U., and P. Schnitzler.** 2009. An update on swine-origin influenza virus A/H1N1: a review. Virus Genes **39:**279–292.
27. **Schultze, D., Y. Thomas, and W. Wunderli.** 2001. Evaluation of an optical immunoassay for the rapid detection of influenza A and B viral antigens. Eur. J. Clin. Microbiol. Infect. Dis. **20:**280–283.
28. **Simoes, E. A.** 2003. Environmental and demographic risk factors for respiratory syncytial virus lower respiratory tract disease. J. Pediatr. **143:**S118–S126.
29. **Steininger, C., M. Kundi, S. W. Aberle, J. H. Aberle, and T. Popow-Kraupp.** 2002. Effectiveness of reverse transcription-PCR, virus isolation, and enzyme-linked immunosorbent assay for diagnosis of influenza A virus infection in different age groups. J. Clin. Microbiol. **40:**2051–2056.
30. **Storhoff, J. J., S. S. Marla, P. Bao, S. Hagenow, H. Mehta, A. Lucas, V. Garimella, T. Patno, W. Buckingham, W. Cork, and U. R. Muller.** 2004. Gold nanoparticle-based detection of genomic DNA targets on microarrays using a novel optical detection system. Biosens. Bioelectron. **19:**875–883.
31. **Templeton, K. E., S. A. Scheltinga, M. F. Beersma, A. C. Kroes, and E. C. Claas.** 2004. Rapid and sensitive method using multiplex real-time PCR for diagnosis of infections by influenza a and influenza B viruses, respiratory syncytial virus, and parainfluenza viruses 1, 2, 3, and 4. J. Clin. Microbiol. **42:**1564–1569.
32. **Thaxton, C. S., D. G. Georganopoulou, and C. A. Mirkin.** 2006. Gold nanoparticle probes for the detection of nucleic acid targets. Clin. Chim. Acta **363:**120–126.
33. **Townsend, M. B., E. D. Dawson, M. Mehlmann, J. A. Smagala, D. M. Dankbar, C. L. Moore, C. B. Smith, N. J. Cox, R. D. Kuchta, and K. L. Rowlen.** 2006. Experimental evaluation of the FluChip diagnostic microarray for influenza virus surveillance. J. Clin. Microbiol. **44:**2863–2871.
34. **Tregoning, J. S., and J. Schwarze.** 2010. Respiratory viral infections in infants: causes, clinical symptoms, virology, and immunology. Clin. Microbiol. Rev. **23:**74–98.
35. **Vasoo, S., J. Stevens, and K. Singh.** 2009. Rapid antigen tests for diagnosis of pandemic (swine) influenza A/H1N1. Clin. Infect. Dis. **49:**1090–1093.
36. **Wright, M. W., and A. P. Kendal.** 2001. Orthomyxoviruses, p. 1533–1570. *In* D. M. Knipe and P. M. Howley (ed.), Fields virology. Lippincott, Williams & Wilkins, Philadelphia, PA.