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Comparison of Automated Microarray Detection with Real-Time PCR Assays for Detection of Respiratory Viruses in Specimens Obtained from Children▽

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Respiratory virus infections are a major health concern and represent the primary cause of testing consultation and hospitalization for young children. We developed and compared two assays that allow the detection of up to 23 different respiratory viruses that frequently infect children. The first method consisted of single TaqMan quantitative real-time PCR assays in a 96-well-plate format. The second consisted of a multiplex PCR followed by primer extension and microarray hybridization in an integrated molecular diagnostic device, the Infiniti analyzer. Both of our assays can detect adenoviruses of groups A, B, C, and E; coronaviruses HKU1, 229E, NL63, and OC43; enteroviruses A, B, C, and D; rhinoviruses of genotypes A and B; influenza viruses A and B; human metapneumoviruses (HMPV) A and B, human respiratory syncytial viruses (HRSV) A and B; and parainfluenza viruses of types 1, 2, and 3. These tests were used to identify viruses in 221 nasopharyngeal aspirates obtained from children hospitalized for respiratory tract infections. Respiratory viruses were detected with at least one of the two methods in 81.4% of the 221 specimens: 10.0% were positive for HRSV A, 38.0% for HRSV B, 13.1% for influenzavirus A, 8.6% for any coronaviruses, 13.1% for rhinoviruses or enteroviruses, 7.2% for adenoviruses, 4.1% for HMPV, and 1.5% for parainfluenzaviruses. Multiple viral infections were found in 13.1% of the specimens. The two methods yielded concordant results for 94.1% of specimens. These tests allowed a thorough etiological assessment of respiratory viruses infecting children in hospital settings and would assist public health interventions.

Respiratory tract infections are an important cause of hospitalization in children. Most of these infections are caused by RNA viruses that produce influenzalike symptoms of variable severity (18). Because of cost and technical limitations, virological testing is currently done sporadically and for a limited number of viruses at the clinician's request. The availability of a molecular diagnostic test that allows the detection of all respiratory tract infection-related viruses would permit better management of patients and possibly limit unnecessary use of antibiotics (2, 32, 36).

The most frequent virus detected in young children suffering from respiratory tract infections is the human respiratory syncytial virus (HRSV) (9, 11). HRSV is the causal agent in up to 70% of bronchiolitis episodes in infants and young children (29). Other well-known clinically relevant respiratory viruses include influenza virus, rhinovirus, enterovirus, coronavirus, parainfluenza viruses, and adenoviruses. Recently described respiratory pathogens include human metapneumovirus (HMPV) (5, 7, 14, 15); coronaviruses severe acute respiratory syndrome coronavirus, HKU1, and NL63 (10); and bocaviruses (17). When using conventional diagnostic methods, multiple virus infections are observed in 5% of respiratory tract infections (8), whereas codetection rates of 11 to 20% have been observed when using molecular methods (1, 3, 8).

Proper viral diagnosis has been shown to reduce the length of hospital stay (2, 36). The classic diagnostic methods for the detection of respiratory viruses consist of virus growth on cell culture and direct immunofluorescence assays (30). Although very specific, these methods lack sensitivity, are burdensome, require skilled personnel, and can take a few days, if not weeks, before generating results in the case of cell culture. Solid-phase immunoassays are often inexpensive and rapid, but they are limited to the detection of a single virus species and have reduced sensitivity and specificity compared to cell culture (30, 32). In addition, the development of immunological tests is limited for some viruses with many subtypes, for example, adenoviruses, enteroviruses, and rhinoviruses.

PCR has been used to amplify and detect many respiratory viruses (35). Conventional PCR or real-time PCR has the potential for high sensitivity and specificity compared to the sensitivities and specificities of previous methods (21, 22, 33, 35). PCR was initially limited by the number of species that could be detected and identified in a single test, often requiring multiple parallel reactions (3, 28). In the last years, numerous tests have been developed using single-tube multiplex PCR to detect many viruses in one assay (12, 13, 31). Single-tube or parallel multiplex PCR assays can be coupled to hybridization using nylon membrane DNA arrays (6), conventional microarrays (34), flow-thru DNA chips (19), semiconductor-based DNA microchips (24), or microspheres (25, 27). Several respi-

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ratory virus panels (RVP) using the Luminex technology have been commercialized, such as xTAG RVP from Luminex (20, 26), Multicode PLx RVP from Eragen (27), and Resplex II from Qiagen (23). So far, these tests have not been fully automated, which limits their use in most clinical laboratories (16).

In order to identify the etiology of respiratory tract infections, we developed a real-time PCR assay and a microarray assay detection system, allowing the diagnosis of 18 and 23 different respiratory virus types, respectively. The first method consists of single quantitative real-time PCR (qRT-PCR) TaqMan assays adapted to the 96-well plate format, each plate allowing the testing of four specimens, along with a series of positive and negative controls. The real-time PCR assay has been optimized to reduce hands-on time. The second method consists of a multiplex PCR test followed by primer extension and microarray hybridization. The microarray assay is automated with the Infiniti analyzer manufactured by AutoGenomics, Inc. (Carlsbad, CA). The Infiniti analyzer was 510K cleared by the FDA for several pharmacogenomic assays. After validation of both assays using laboratory strains of targeted viruses, we compared the performance of the two assays using specimens collected from children ≤3 years of age hospitalized for acute respiratory tract infections (ARTI).

MATERIALS AND METHODS

Specimen collection and preparation. This study was accepted by the ethics committee of the Centre Hospitalier Universitaire de Quebec. We tested nasopharyngeal aspirate (NPA) specimens from 221 children ≤3 years old who were hospitalized between November 2001 and April 2002 for an ARTI of ≤7 days as previously described (4, 5). NPAs (one per patient) were aliquotted and stored at −80°C. Clinical information and clinical laboratory results were prospectively collected after informed consent was obtained. Before nucleic acid extraction, NPAs were thawed on ice and 0.5 μl of hepatitis C genotype 1a armored RNA (Ambion Diagnostics, CA) was added to 200 μl of each NPA as an internal control. Nucleic acid extraction was performed with a Qiamp viral RNA mini kit (Qiagen, Mississauga, Ontario, Canada) using the protocol suggested by the manufacturer, except for the final elution volume, which was 40 μl. Reverse transcription was done using a Superscript II reverse transcriptase kit (Invitrogen, Carlsbad, CA). The reaction mixture was composed of 1 μl of 50 ng/μl random primers (Amersham, Piscataway, NJ), 1 μl of 10 μM deoxynucleoside triphosphates (dNTPs), and 10 μl of extracted RNA. The mixture was incubated at 65°C for 5 min and then put on ice. The following reagents were then added to the solution: 4 μl of 5× first-strand buffer (Invitrogen), 2 μl of 0.1 M dithiothreitol (Invitrogen), and 1 μl of 40 U/μl RNAsin (Promega, Madison, WI). The solution was incubated at room temperature for 2 min, and then 200 units of Superscript II (Invitrogen) were added. The solution was incubated at room temperature for 10 min, then at 42°C for 50 min, and finally at 70°C for 15 min. The cDNA was kept at −20°C. The reverse-transcripted samples were then tested using both the microarray assay and the qRT-PCR assay (Fig. 1).

Clone generation and sensitivity studies. Laboratory strains or clinical specimens were used to generate the clones for the sensitivity analyses. Amplicons generated using qRT-PCR primers or multiplex PCR primers were cloned in the pCR II (Invitrogen) or pGEM-T Easy (Promega) vectors. The insert sequence was verified by DNA sequencing. Prior to sensitivity studies, amplicons were digested with EcoRV (pCR II) or NdeI (pGEM-T Easy) for 120 to 180 min at 37°C. They were then purified using Qiagen PCR purification kits and quantified with a NanoDrop (Thermo Scientific, Wilmington, DE). Serial dilutions were performed in water to obtain a range of concentrations between 100,000 and 0.1 copies/μl.

Both the cycle thresholds for the real-time PCR assay and the signal thresholds for the microarray assay were determined by analyzing the results obtained with laboratory strains and the results of the sensitivity study. Then, thresholds were adjusted according to the background signal of several negative specimens and the signals obtained with several laboratory strains and clinical specimens.

Primers and probes. The PCR primers for the real-time PCR assay and for the multiplex PCR targeted the same gene, except for the HMPV and adenovirus primers. However, even when targeting the same gene, both assays had distinct primer sets. All primers for qRT-PCR were obtained from Invitrogen Canada. TaqMan probes with minor groove binding quenchers were obtained from Applied Biosystems (Streetsville, Ontario, Canada). The sequences of primers and probes used for the qRT-PCR assay are shown in Table 1. The multiplex PCR primer mix contains all PCR primers at final concentrations ranging between 50 and 200 nM depending on the targeted virus. The primers used for primer extension were composed of a proprietary tag sequence followed by a virus-specific detection sequence. The primer concentration in the primer extension mix ranged from 250 to 500 nM, depending on the virus targeted. The multiplex PCR primer mix and primer extension mix were obtained from AutoGenomics, Inc.

qRT-PCR assay. All reactions were performed in a 96-well plate using TaqMan universal PCR master mix (Applied Biosystems) in an ABI 7500 apparatus (Applied Biosystems). PCR primers were used at a 200 nM concentration, and TaqMan probes were used at a total probe concentration per well of 250 nM. Each 96-well PCR plate allows for the testing of four specimens, positive controls consisting of cloned amplicons of specific viruses, and negative controls consisting of water for each virus. For each specimen tested, 14 wells of the plate were used. One microliter of specimen cDNA or plasmid control was added to each well of the plate. The PCR program consisted of the following steps: 2 min at 50°C and 10 min at 95°C, followed by 50 cycles of 15 s at 95°C, 15 s at 55°C, and 40 s at 60°C. A specimen was considered positive for a virus if its cycle threshold was lower than a predefined value (Table 2).

Infiniti microarray assay. First, reverse-transcribed samples were amplified using a highly multiplexed PCR. Then, amplicons were cleaned by enzymatic reactions and were then subjected to primer extension within the Infiniti analyzer. The assay relies on a proprietary tag system in which amplicons are tagged at the primer extension step. Amplicons are also labeled with fluorescent nucleotides at the primer extension step. Hybridization of the tags to the antitags immobilized on the microarray allows the specific identification of targets. Each antitag hybridizes to three replicates on the microarray. Microarray washing and scanning were done within the Infiniti analyzer without human intervention. A summary of the microarray assay protocol is shown in Fig. 1.

Multiplex PCR was performed in a T1plus thermocycler (Biometra; Montreal Biotech, Montreal, Canada). The multiplex PCR primer mix was composed of 46 primers at concentrations ranging from 50 nM to 200 nM. The amplification solution was composed of 10× buffer, 0.2 μM dNTPs, 1.5 mM $MgCl_2$, multiplex PCR primer mix, 0.5 units of platinum *Taq* DNA polymerase (Invitrogen Canada), and 2.5 μl of cDNA in a final volume of 20 μl. The PCR program consisted of the following steps: 60 s at 94°C, followed by 39 cycles of 30 s at 94°C, 30 s at 55°C, and 60 s at 72°C. The reaction mixture was then incubated at 72°C for 3 min. Then, 3 units of shrimp alkaline phosphatase (Clontech, Mountain View, CA), 7.5 units of exonuclease (Clontech), and 0.25 μl of 50× titanium DNA polymerase (Clontech) were added to the solution, which was incubated at 37°C for 50 min and at 94°C for 20 min. This step allows for the degradation of remaining dNTPs and PCR primers that were not used in the multiplex PCR. The subsequent steps were automated by the Infiniti analyzer (AutoGenomics, Inc.). The primer extension solution, comprising 34 tagged detection primers (AutoGenomics, Inc.), was then added to the solution. The primer extension reaction consisted of the following steps: 60 s at 94°C, followed by 39 cycles of 15 s at 94°C and 15 s at 50°C. Primer extension was done in the presence of Cy5-dCTP. Following the primer extension reaction, 80 μl of hybridization solution (AutoGenomics, Inc.) was added to each reaction mixture. The total volume of 120 μl was then hybridized to a DNA microarray (AutoGenomics, Inc.) for 90 min at 42°C at high humidity. The tags on the extension primers hybridize to corresponding probes on the microarray. After hybridization, each chip was washed five times with 300 μl of 1× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate). The chips were dried and scanned using a confocal scanner. A specimen was considered positive for a virus if the ratio between the signal for a virus and the background signal was above a defined threshold (Table 2) after background correction.

RESULTS

Sensitivities of real-time PCR assay and microarray assay. The capacity of both methods to detect each targeted virus was initially validated by using laboratory isolates and clinical specimens. The technical specificity was assessed by using laboratory strains and clinical specimens validated by using culture or sequencing (Table 3). No false positives were observed, and all described specimens were positive by both assays. Then, sen-

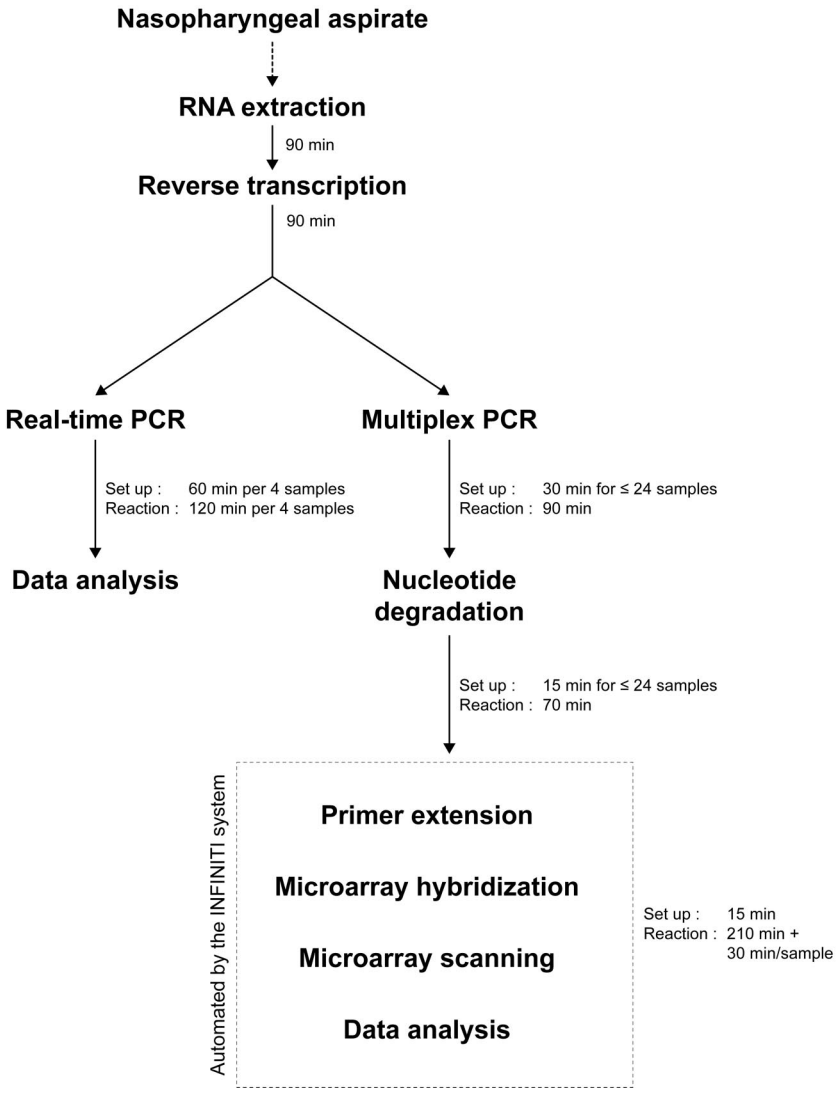

FIG. 1. Flowchart comparing the protocols for the real-time PCR assay and for the microarray assay. RNA extraction and reverse transcription steps are common to both methods. The real-time PCR assay has only one setup step, while the microarray assay has three. However, the time required to perform the real-time PCR assay increased for each four samples tested. The time required for the automated microarray assay is increased for each 24 samples. Overall, the qRT-PCR assay requires 60 min of setup time and a total of 120 min of reaction time for 4 specimens, while the microarray assay requires 60 min of setup time and 17 h of reaction time for 24 specimens.

sitivity assays were conducted using cloned amplicons (Table 2). The sensitivities of each single TaqMan qRT-PCR assay ranged from 5 to 250 copies of target DNA per reaction, depending on the virus targeted. The sensitivity of the Infiniti microarray assay ranged from 10 copies of target DNA for HRSV B and HMPV A to 2,500 copies for parainfluenza viruses type 2 and 3 and coronavirus 229E and 5,000 copies for parainfluenza virus type 1. In most cases, the qRT-PCR assay was more sensitive than the microarray assay. However, similar sensitivities of the two assays were obtained for HRSV B and an increased sensitivity was obtained with the microarray assay for HMPV A.

Retrospective study results. The study included 221 specimens collected during the 2001-to-2002 winter season from children ≤3 years old hospitalized for ARTI. All specimens were positive by both methods for the armored-RNA internal control. Specimens were considered positive for one virus if they were positive by one or both methods. Of the 221 specimens, 81.4% of the specimens were positive for at least one virus. Furthermore, 68.3% of the specimens were positive for one virus, 12.2% were positive for two viruses, and 0.9% were positive for three viruses. Among coinfections, 41.4% involved adenoviruses ($P < 0.001$; Fisher's exact test) and 34.5% involved picornaviruses ($P < 0.05$; Fisher's exact test).

Table 4 shows the sample positivity for each virus as detected by the two methods. We considered a specimen positive for a virus if it was positive with either method. The most frequently detected virus was HRSV, including 38% type B and 10% type A. Influenza virus A and picornaviruses (rhinoviruses or enteroviruses) were both detected in 13.1% of specimens. Adenoviruses, coronaviruses, HMPV, and parainfluenza viruses were detected in 7.2%, 8.6%, 4.1%, and 1.5% of specimens, respectively.

TABLE 1. Sequences of primers and probes for the qRT-PCR assay

Well	Virus	PCR primer sequence(s)	Type	TaqMan sequence
1	Adenovirus	CCCTCACADATTTGCATTTCCCA GGTAATTTATGGACCCACTGGCTG ATTTATGGCCCCACCGGATG ATTTACGGTCCCACYGGGTG ATTTACGGGCCCACCGGC	A, B, C	6FAM-CAGATGGGGGRATCATGT-MGBNFQ 6FAM-CGAGGGTGGAATCATGT-MGBNFQ
2	Adenovirus	AGGATGCATGCGRGGGGA GCMTTCCCTTCCAAGTTGCAT	E	6FAM-AARTTCCCCAAGTGCAC-MGBNFQ
3	Armored RNA	CACTCCCCTGTGAGGAACTACTG AGGCTGCACGACACTCATACTAAC		6FAM-TTCACGCAGAAAGC-MGBNFQ
4	Coronavirus	TTGAAGGCTCAGGAAGGTCTGCT TGCTTAGTKACTTGCTGAGGTTTAG	HKU1 OC43	6FAM-TAAAACAAGATTAGCGATCTC-MGBNFQ VIC-CAGAACAAGACTAGCAATT-MGBNFQ
5	Coronavirus	TAGTCTTAYACACAATGGTARGCCAGTG TGGCTCTTCCATTGTTGGCKCG	229E NL63	6FAM-AATGCGATCTTTGATTACTCCA-MGBNFQ VIC-TATGCGATCTTTAAGTACTCCA-MGBNFQ
6	Enterovirus, rhinovirus	AGCCTGCGTGGCKGCC GAAACACGGACACCCAAAGTAGT TGGCTGCGYTGGCGG	Enterovirus A, B, C, D Rhinovirus A, B	VIC-ATTAGCCGCATTCAGG-MGBNFQ 6FAM-GTTAGCCRCATTCAGG-MGBNFQ
7	HMPV	GGCTCCATGCAAATATGAAGTG CATCAGCTCTATCAGTGTTCCTTAAAA	A	6FAM-CTAACGAGTGTGCGCAAG-MGBNFQ
8	HMPV	GGCTCCATGCAAATATGAAGTG CATCAGCCTTATCWGTGTTTCTTAAAA	B	6FAM-CTAACGAGTGTGCGCAAG-MGBNFQ
9	HRSV	CCATTATGCCTAGACCTGCTGCA CCAGCAGCATTGCCTAATACTACACT GCAGCATTGCCTAATACTACACTGGA GAATGCCTATGGTKCAGGGCAAGT	A B	VIC-TGCTTCTCCACCCAAT-MGBNFQ 6FAM-AGCTTCTCCTCCCAACT-MGBNFQ
10	Influenza virus	GGCGCTGCAGTCAAAGGART GGTGCTGCAGTCAAAGGART CCCGRCTYTCTCTCACTTGATC	A	6FAM-CAGAATGATCAAACGGG-MGBNFQ 6FAM-CAGAATGGTCAAACGGG-MGBNFQ 6FAM-AGGATGATCAAACGTGG-MGBNFQ
11	Influenza virus	GGTGTTGCAATCAAAGGAGGTG GGTGTTGCGATCAAAGGAGGTG TGGRTTYCTACTTCCGATCACTTGATC	B	6FAM-CCATTCGATTTATAGGAAGAG-MGBNFQ
12	Parainfluenza virus	ACAGGAATTGGCTCAGATATGYG GACTTCCCTATATCTGCACATCCTTGAGTG	1	6FAM-ACCATGCAGACGGC-MGBNFQ
13	Parainfluenza virus	GCTCTTGCAGCATTTTCTGGGGA GCTCCCTGCTGTTTCCTTGC	2	6FAM-CCAGAAATTAAAAGCTCTC-MGBNFQ
14	Parainfluenza virus	ACAGATGTATATCAACTGTGTTCRACTCC TTGGATGTTCAAGACCTCCATAYCCG	3	6FAM-TGATGAAAGATCAGATTATG-MGBNFQ

For some viruses, the type was also identified. Due to the methodological design, the identification of virus types for adenoviruses and picornaviruses was only possible with the microarray assay. Of the nine specimens that were positive for adenoviruses by the microarray, three were adenovirus type B, five were adenovirus type C, and one was positive for both type B and C. Of the 29 specimens that were positive for respiratory picornaviruses, 16 were positive for rhinovirus type A, one was positive for enterovirus type B, and 11 were untyped picornaviruses. HRSV types were identified by both methods for all HRSV-positive specimens, which included 22 type A and 84 type B. HMPV types were also identified by both methods for all positive specimens, including five type A (including one microarray false negative), and four type B. Coronaviruses HKU1, NL63, and OC43 were identified by qRT-PCR in nine, seven, and three specimens, respectively.

Comparison of real-time PCR assay with microarray assay. Overall, 79.6% of the 221 specimens were positive for at least one virus with both techniques, 18.5% were negative for all viruses by both methods, and 1.8% (4/221) were positive for at least one virus by qRT-PCR only (Table 5). No viruses were detected with the microarray method only. The results with both methods were compared for each specimen, and a concordance in the diagnosis was observed for 94.1% of the specimens. When specific diagnosis for each virus was considered, a perfect concordance between the two methods was observed for HRSV types A and B, parainfluenza viruses, HMPV type B, and coronavirus OC43. Of the 5.9% (13/221) of specimens with discordant results (all positive with qRT-PCR only), there were seven specimens of adenoviruses, two of coronavirus NL63, one of coronavirus HKU1, one of HMPV A, one of influenza virus A, and one of picornavirus (Table 6). In most

TABLE 2. Sensitivity of qRT-PCR and microarray assay for each virus

Virus	Gene(s)[a]	qRT-PCR assay		Microarray assay	
		Threshold[b]	Sensitivity (copy no.)	Threshold[c]	Sensitivity (copy no.)
Adenovirus	IVA2/L4100kd	40	10	1.5	1,000 (group B)/250 (group C)
Coronavirus 229E	Nucleocapsid	45	50	3	2,500
Coronavirus HKU1	Nucleocapsid	40	10	1.5	500
Coronavirus NL63	Nucleocapsid	40	100	1.5	500
Coronavirus OC43	Nucleocapsid	45	50	2	250
Enterovirus	5′ untranslated region	40	5	1/1.5	250
Rhinovirus	5′ untranslated region	40	250	1/1.5	1,000
Influenza virus A	Nucleocapsid	40	50	1.5	100
Influenza virus B	Nucleocapsid	42	10	1.5	100
HMPV A	Matrix/F	40	50	1	10
HMPV B	Matrix/F	40	50	3	250
HRSV A	Nucleocapsid	45	250	1	50
HRSV B	Nucleocapsid	45	10	1	10
Parainfluenza virus 1	Hemagglutinin-neuraminidase	43	50	2	5,000
Parainfluenza virus 2	Fusion	40	5	2	2,500
Parainfluenza virus 3	Hemagglutinin-neuraminidase	44	50	2	2,500

[a] A slash shows target genes that differ in qRT-PCR/microarray assays.
[b] Maximum cycle threshold for positivity.
[c] Minimum ratio for positivity. A slash shows threshold for *Picornaviridae* detection/threshold for species-specific detection.

cases, no signal was observed with the microarray assay for discordant specimens. However, discordant coronavirus HKU1, influenza virus A, and picornavirus specimens gave equivocal ($0 <$ ratio $<$ threshold) results with the microarray assay and had high cycle thresholds of 38.0, 39.9, and 38.9, respectively, with the qRT-PCR assay. Discordant specimens were tested by DNA sequencing, as shown in Table 6. Seven discordant specimens were confirmed by DNA sequencing, while we were unable to confirm five specimens positive for adenovirus with the qRT-PCR assay. One specimen, positive for coronavirus NL63 with the qRT-PCR assay and giving an equivocal coronavirus 229E signal with the microarray assay, was shown to be positive for coronavirus 229E by DNA sequencing, suggesting possible specificity issues with the qRT-PCR assay. We observed no false positives with the microarray assay compared to the results from the qRT-PCR assay and DNA sequencing.

TABLE 3. Specimens tested in the analytical specificity study

Virus	No. of specimens
Adenovirus B	1
Adenovirus C	1
Adenovirus E	1
Coronavirus 229E	1
Coronavirus NL63	1
Coronavirus OC43	1
Enterovirus B	2
HMPV	1
HRSV A	1
HRSV B	5
Influenza virus A	3
Influenza virus B	4
Parainfluenza virus 1	4
Parainfluenza virus 2	4
Parainfluenza virus 3	4
Parainfluenza virus 4	5
Rhinovirus A	2

From a technical viewpoint, the microarray assay required 77 events of manual pipetting for 24 specimens, while qRT-PCR required at least 288 manual pipetting events for only 4 specimens and appropriate controls. Figure 1 shows a flow-

TABLE 4. Sample positivity by qRT-PCR and microarray for each virus

Virus	No. (%) of specimens (n = 221) positive by:	
	qRT-PCR[a]	Microarray
Adenovirus	16 (7.2)	
Adenovirus A	NA	0 (0.0)
Adenovirus B	NA	4 (1.8)
Adenovirus C	NA	6 (2.7)
Adenovirus E	NA	0 (0.0)
Coronavirus 229E	0 (0.0)	0 (0.0)
Coronavirus HKU1	9 (4.1)	8 (3.6)
Coronavirus NL63	7 (3.2)	5 (2.3)
Coronavirus OC43	3 (1.4)	3 (1.4)
Influenza virus A	29 (13.1)	28 (12.7)
Influenza virus B	0 (0.0)	0 (0.0)
HMPV A	5 (2.3)	4 (1.8)
HMPV B	4 (1.8)	4 (1.8)
Human parainfluenza virus 1	1 (0.5)	1 (0.5)
Human parainfluenza virus 2	1 (0.5)	1 (0.5)
Human parainfluenza virus 3	1 (0.5)	1 (0.5)
HRSV A	22 (10.0)	22 (10.0)
HRSV B	84 (38.0)	84 (38.0)
Picornavirus (rhinovirus or enterovirus)	29 (13.1)	28 (12.7)[b]
Rhinovirus A	NA	16 (7.2)
Rhinovirus B	NA	0 (0.0)
Enterovirus A	NA	0 (0.0)
Enterovirus B	NA	1 (0.5)
Enterovirus C	NA	0 (0.0)
Enterovirus D	NA	0 (0.0)

[a] Genotyping of adenoviruses and picornaviruses (rhinoviruses and enteroviruses) was not done with the qRT-PCR method. NA, not applicable.
[b] An independent probe targeting all picornaviruses (enteroviruses and rhinoviruses) was used. A specimen can be positive for this probe without being positive for a type-specific enterovirus or rhinovirus probe.

TABLE 5. Comparison of qRT-PCR and microarray results for 221 specimens

Virus	No. of specimens with indicated qRT-PCR/microarray results[b]				Microarray[c]	
	Pos/pos	Pos/neg	Neg/pos	Neg/neg	Sensitivity	Specificity
Adenovirus[a]	9	7	0	205	0.563	1.000
Coronavirus 229E	0	0	0	221	NA	1.000
Coronavirus HKU1	8	1	0	212	0.889	1.000
Coronavirus NL63	5	2	0	214	0.714	1.000
Coronavirus OC43	3	0	0	218	1.000	1.000
Enterovirus/rhinovirus	28	1	0	192	0.966	1.000
Influenza virus A	28	1	0	192	0.966	1.000
Influenza virus B	0	0	0	221	NA	1.000
HMPV A	4	1	0	216	0.800	1.000
HMPV B	4	0	0	217	1.000	1.000
HRSV A	22	0	0	199	1.000	1.000
HRSV B	84	0	0	137	1.000	1.000
Parainfluenza virus 1	1	0	0	220	1.000	1.000
Parainfluenza virus 2	1	0	0	220	1.000	1.000
Parainfluenza virus 3	1	0	0	220	1.000	1.000
Any virus	176	4	0	41	0.978	1.000

[a] Results of qRT-PCR and microarray assays were significantly different only for adenovirus. ($P < 0.05$; McNemar's test).
[b] Pos, positive; neg, negative.
[c] NA, not applicable.

chart comparing the steps and timelines for the real-time PCR assay and for the microarray system. Excluding RNA extraction and reverse transcription, the automated microarray assay required at most 60 min of setup time for up to 24 samples, while the real-time PCR assay required the same time for a 96-well plate preparation to test only 4 specimens.

DISCUSSION

The comparison of the microarray assay automated by the Infiniti analyzer with the single qRT-PCR assays showed that both techniques were useful to detect and identify a panel of respiratory viruses in clinical specimens, present either as single agents or as part of a coinfection. Overall, the single qRT-PCR assays were associated with better analytical sensitivity than the multiplex PCR followed by microarray detection. However, the results suggest that the 46-primer multiplex PCR assay should, in more than 94% of cases, give results similar to those obtained with the qRT-PCR assay when using nasopharygeal aspirates from children.

Because the sensitivity of the qRT-PCR assay is usually higher than that of the microarray assay, discordant specimens could generally be explained by differences in detection limits for the two assays (Tables 2 and 6). This seems particularly true for adenoviruses, for which the real-time PCR assay could

TABLE 6. Signal, internal control, and DNA sequence validation results for specimens with discordant results in qRT-PCR and microarray assays

Specimen	qRT-PCR assay			Microarray assay			Validation sequencing result
	Viruses detected[a]	Signal (C_T)[b] of:		Viruses detected	Signal (ratio)[c] of:		
		Discordant virus	Internal control		Discordant virus	Internal control	
032	**Coronavirus HKU1**, HRSV B	38.04	35.03	HRSV B	0.92	3.51	Coronavirus HKU1
034	**Influenza virus A**	39.88	32.09	Negative	0.71	10.82	Influenza virus A
049	**Adenovirus**, influenza virus A, HRSV A	35.54	32.28	Influenza virus A, HRSV A	0	8.08	Adenovirus C
069	**Coronavirus NL63**, influenza virus A	39.27	30.8	Influenza virus A	2.44[d]	9	Coronavirus 229E
075	**Adenovirus**, HRSV B	37.97	31.16	HRSV B	0	11.33	Negative
081	**Adenovirus**	32.49	33.34	Negative	0	2.56	Negative
092	**Adenovirus**, influenza virus A	35.35	32.19	Influenza virus A	0	2.49	Negative
121	**HMPV A**	38.44	34.54	Negative	0	2.88	HMPV A
156	**Coronavirus NL63**, HRSV B	35.7	34.56	HRSV B	0	11.56	Coronavirus NL63
173	**Adenovirus**, rhinovirus	34.66	33.04	Rhinovirus A	0	8.53	Adenovirus C
182	**Adenovirus**, HRSV B	38.9	32.48	HRSV B	0.31	10.34	Negative
186	**Adenovirus**, rhinovirus	35.31	32.44	Rhinovirus A	0.33	8.86	Negative
218	**Rhinovirus/enterovirus**	38.9	37.68	Negative	0.78	9.71	Rhinovirus A

[a] Viruses with discordant results are shown in boldface.
[b] C_T, cycle threshold.
[c] Ratio of results for virus or internal control/background.
[d] An equivocal signal was seen for coronavirus 229E.

detect as few as 10 copies of the target sequence, while the microarray assay detected only 250 copies of adenovirus C and 1,000 copies of adenovirus B. Moreover, the mean cycle threshold of the discordant adenovirus samples (35.7) was higher than that of the concordant adenovirus samples (31.9) ($P = 0.08$; Student's t test). In all seven discordant adenovirus cases, no significant signal (<0.5) was observed on the microarray assay. Only two of the seven discordant adenovirus specimens were confirmed by DNA sequencing. Thus, it is unclear if these specimens are false positives of the qRT-PCR assay or if the viral load is too low to allow DNA sequencing. Differences in primer sequences may explain some discrepancies, either because of sequence variations or different PCR efficiencies. A low sensitivity for adenovirus detection was also described for other multiplex RVP assays (20, 25).

It is of note that 69.4% (9/13) of the discordant specimens had more than one virus detected by qRT-PCR ($P < 0.001$; Fisher's exact test). However, this analysis could be biased by the high rate of discordant adenoviruses that were part of coinfections (6/7). The rate of multiple infection was not different for discordant and concordant adenovirus-positive specimens ($P = 0.57$; Fisher's exact test). Still, multiple viruses within a specimen could potentially reduce the sensitivity of the microarray assay for some viruses, but further studies would be required to confirm this hypothesis.

The current version of the qRT-PCR 96-well-plate assay, although optimized to reduce pipetting steps, is labor intensive, time consuming, and has low throughput, allowing the testing of only four clinical samples per 96-well plate. Contrarily, the microarray assay, when automated by using the Infiniti analyzer, requires fewer human interventions and allows the testing of up to 24 samples per run. Due to its automation, this assay is also potentially less susceptible to manipulation errors and to cross-contamination than plate-based qRT-PCR tests. Notably, up to 5 h of hands-on time can be saved by using the automated microarray assay. The reduction in hands-on time with the microarray assay could be a financial advantage of this technique. Samples tested using the qRT-PCR assay could have results available on the same day, while samples tested using the microarray assay will have results available on the next day. Although results on the same day would be the ideal scenario, ease of implementation and a higher throughput may still be important factors in choosing a molecular diagnostic assay, especially in high-volume laboratories.

As new highly multiplexed molecular diagnostic devices are developed, it will be important to compare these techniques in order to establish their relative sensitivities, specificities, and ease of implementation in a clinical setting. Comparison of the automated microarray assay with commercially available RVP, such as xTAG RVP from Luminex (20, 26), Multicode PLx RVP from Eragen (27), and Resplex II from Qiagen (23), is an essential step in assessing the quality and clinical usefulness of the next generation of respiratory virus diagnostic methods. The viruses included in each panel may vary from product to product, according to assay design and approval by regulatory institutions. Also, threshold levels should be validated in each laboratory when implementing a new multiplex molecular assay, because they may vary according to sample preparation methods and other internal issues. The ease of implementation of these techniques in a clinical setting is a critical factor in the selection of a diagnostic assay (16). Currently, because of its automation on the Infiniti analyzer, the microarray assay described herein is the most adaptable system for clinical laboratories. Moreover, the ease of use of this assay could still be improved by performing one-step reverse transcription and by reducing the time dedicated to or completely removing the nucleotide degradation step. These improvements would further reduce the pipetting events required to test 24 specimens to only three steps per specimen. On the other hand, as for other multiplex assays, the workload of a clinical laboratory is an important criterion when selecting a diagnostic test for respiratory viruses.

While the precise identification of all viruses is not a high priority for clinicians at the moment, the multiplex assays should become increasingly helpful for epidemiological studies, to assess clinical outcome according to virus type or multiple infections, to understand the role of emerging viruses, and to limit the use of antibiotics. Future therapeutic modalities for many respiratory viruses should also increase the usefulness of these assays, in particular for immunocompromised patients and in urgent or intensive care settings. As respiratory viruses become a greater concern worldwide, such a tool will have increased usefulness for diagnosis and adequate use of antibiotics and antiviral agents.

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ERRATUM

Comparison of Automated Microarray Detection with Real-Time PCR Assays for Detection of Respiratory Viruses in Specimens Obtained from Children

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Volume 47, no. 3, p. 743–750, 2009. Page 746, Table 1, "TaqMan sequence" column, rows for wells 7 and 8: "6FAM-CTAA CGAGTGTGCGCAAG-MGBNFQ" should read "6FAM-ACAATTACTGGAGTTGGC-MGBNFQ."