JOURNAL OF CLINICAL MICROBIOLOGY, Nov. 2007, p. 3631–3640 Vol. 45, No. 11
0095-1137/07/$08.00+0 doi:10.1128/JCM.00280-07

Rapid Multiplex Nested PCR for Detection of Respiratory Viruses▽

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Received 4 February 2007/Returned for modification 11 May 2007/Accepted 19 August 2007

Respiratory tract infections can be caused by a heterogeneous group of viruses and bacteria that produce similar clinical presentations. Specific diagnosis therefore relies on laboratory investigation. This study developed and evaluated five groups of multiplex nested PCR assays that could simultaneously detect 21 different respiratory pathogens: influenza A virus (H1N1, H3N2, and H5N1); influenza B virus; parainfluenza virus types 1, 2, 3, 4a, and 4b; respiratory syncytial virus A and B; human rhinoviruses; human enteroviruses; human coronaviruses OC43 and 229E; severe acute respiratory syndrome coronavirus; human metapneumoviruses; *Mycoplasma pneumoniae*; *Chlamydophila pneumoniae*; *Legionella pneumophila*; and adenoviruses (A to F). These multiplex nested PCRs adopted fast PCR technology. The high speed of fast PCR (within 35 min) greatly improved the efficiency of these assays. The results show that these multiplex nested PCR assays are specific and more sensitive (100- to 1,000-fold) than conventional methods. Among the 303 clinical specimens tested, the multiplex nested PCR achieved an overall positive rate of 48.5% (95% confidence interval [CI], 42.9 to 54.1%), which was significantly higher than that of virus isolation (20.1% [95% CI, 15.6 to 24.6%]) and that of direct detection by immunofluorescence assay (13.5% [95% CI, 9.7 to 17.4%]). The improved sensitivity was partly due to the higher sensitivity of multiplex nested PCR than that of conventional methods in detecting cultivatable viruses. Moreover, the ability of the multiplex nested PCR to detect noncultivatable viruses, particularly rhinoviruses, coronavirus OC43, and metapneumoviruses, contributed a major gain (15.6%) in the overall positive rate. In conclusion, rapid multiplex nested PCR assays can improve the diagnostic yield for respiratory infections to allow prompt interventive actions to be taken.

Respiratory tract infection is a major cause of hospitalization. The 2003 outbreaks of severe acute respiratory syndrome (SARS) and the more recent human avian H5N1 influenza virus cases underscore the importance of a rapid and accurate laboratory diagnosis to investigate infections associated with severe individual or public health consequences (1).

Respiratory tract infections in humans can be a result of infection caused by a heterogeneous group of viruses and bacteria that produce similar clinical presentations (10, 18, 30). Specific diagnosis therefore relies almost entirely on laboratory investigation. Rapid antigen detection assays are now widely used in routine diagnostic laboratories, but these assays have been shown to be inferior in sensitivity and specificity to assays based on PCR (5, 12), which can also be designed to screen for a wider range of pathogens. Numerous studies have developed and evaluated multiplex nested PCR, reverse transcription (RT)-PCR, or real-time PCR for the detection of respiratory viruses (4, 11, 21, 24, 27). However, these studies are still limited by their turnaround time and/or the range of viruses being detected.

This study developed and evaluated five groups of multiplex nested PCR assays that included an RT step where necessary. These assays could simultaneously detect 21 different respiratory pathogens including influenza virus group A (FluA) (subtypes H1, H3, and H5), influenza virus group B (FluB), parainfluenza virus type 1 (PIV-1), PIV-2, PIV-3, PIV-4a, PIV-4b, human respiratory syncytial viruses (hRSV), all serotypes of human adenoviruses (ADVs) (A to F), human metapneumoviruses (hMPV), human coronaviruses (HCoVs) (HCoV-229E, HCoV-OC43, and SARS coronavirus [SARS-CoV]), all serotypes of human enteroviruses (hEVs), and all serotypes of human rhinoviruses (hRVs) as well as the fastidious respiratory bacteria *Chlamydophila pneumoniae*, *Mycoplasma pneumoniae*, and *Legionella pneumophila*.

MATERIALS AND METHODS

Primer design and preparation. The primers used in this study were either modified from previously published primer sequences (2–4, 7–9, 11, 17, 19, 23, 24, 26, 28, 29, 31–34) or designed from consensus genome regions obtained from GenBank (http://www.ncbi.nlm.nih.gov/). Typically, sequences of 10 to 20 representative strains of each pathogen were downloaded. The sequences were aligned using Clustal X (http://bips.u-strasbg.fr/en/Documentation/ClustalX/) (25). The program GeneTool Lite 1.0 (BioTools Inc., Edmonton, Alberta, Canada) was used to predict the compatibility of primer pairs and to estimate the optimal annealing temperatures. Primer pairs were selected to ensure that the sizes of the amplicons of different pathogens could be easily differentiated by agarose gel electrophoresis. The primers used were 20 to 30 bp in length and had G+C contents less than or equal to 70%, thus having an annealing temperature of 50 to 66°C.

Multiplex PCR primer grouping. Five groups of multiplex nested PCR assays, targeting 21 respiratory viruses and bacteria, were developed. Each multiplex nested PCR detected four to five viruses and/or bacteria: group 1 was comprised of FluA and FluB group-specific and subtype H1-, H3-, and H5-specific primers. Group 2 was comprised of PIV-1, PIV-2, PIV-3, PIV-4a, and PIV-4b. Group 3 was comprised of hRSV A and B, hRV, and hEV. Group 4 was comprised of HCoV (HCoV-OC43, HCoV-229E, and SARS-CoV) and hMPV. Group 5 was comprised of *Mycoplasma pneumoniae*, *Chlamydophila pneumoniae*, *Legionella*

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▽ Published ahead of print on 5 September 2007.

TABLE 1. Primers used in the first round of multiplex nested PCR

Organism	Primer	Sequence (5′→3′)[a]	Primer concn (μM)	Product length (bp)	Target gene[b]	Source or reference
Group 1						
Influenza A virus	FluA-OF3	TYGAGGCTCTCATGGARTGGCTAAAG	0.052	412	Matrix	This study
	FluA-OR3	GCTGGCCARMACCATTCTGTTYTCAT	0.052			
Influenza A virus H1	H1-OF1	CCCAGGRTATTTCKCCGAYTATGAGG	0.104	760	Hemagglutinin	This study
	H1-OR1	TACCATTCCAGTCCACCCCCCTTCA	0.104			
Influenza A virus H3	H3-OF1	ATGGGACCTTTTTRTYGAACGCAGCA	0.052	519	Hemagglutinin	This study
	H3-OR1	CCCCKAGGAGCAATTAGATTCCCTGT	0.052			
Influenza A virus H5	H5N1-VIET-1B	ATCAAACAGATTAGTCCTTGCG	0.208	265	Hemagglutinin	This study
	H5N1-VIET-2B	GGCCTCAAACTGAGTGTTCATT	0.208			
Influenza B virus	FluB-OF3	AGGAAGRGCAATGGCAGAYAGAGG	0.834	883	Nucleoprotein	This study
	FluB-OR2	TGCTGTGTCCCTCCCAAAGAAGAAA	0.834			
Group 2						
PIV-1	PIV-1-OF1	TCTGGATCCACCACAATTTCAG	0.933	848	Hemagglutinin-neuraminidase	This study
	PIV-1-OR1	WACCAGTTGCAGTCTKGGTTTC	0.933			
PIV-2	PIV2-F1	CTTGCAGCATTTTCTGGGGAACTCC	0.0373	716	Hemagglutinin-neuraminidase	This study
	PIV2-OR1	GCATCATCATCCTGGGAGCCTCTGT	0.0373			
PIV-3	PIV-3-OF1	GATTTTTGGAGATGCACGTCTG	0.187	1,118	Hemagglutinin-neuraminidase	This study
	PIV-3-OR1	GAGAGTGTTYTGTTTCGGATGG	0.187			
PIV-4	PIV-4AB-IF1	AYGGATGCATTCGAATTCCATCATTC	0.093	432	Hemagglutinin-neuraminidase	This study
	PIV-4AB-OR1	TCCRTRAGRCCYCCATACAARGG	0.093			
Group 3						
hRSV A	RSV-A-OF2	CAGCTCCGTTATCACATCTCTAGG AGCC	0.278	576	Fusion protein	This study
	RSV-A-OR2	TGGGTTGTCTATGAGCAGATAKKAA ACCA	0.278			
hRSV B	RSV-B-OF2	CGGGCCAGAAGAGAAGCACCAC AGTA	0.069	673	Fusion protein	This study
	RSV-B-OR2	TGATCCTTCTTTGATGTTGGTGGTGC	0.069			
hRV	OL26-MOD-RV	CACTTCTGTTTCCCCGGAGCGAG	0.139	388	5′ UTR	14
	RV-OR2	GAAACACGGACACCCAAAGTAGT CGGT	0.139			
hEV	EV-OF3	CTGCGYTGGCGGCCYMCC	0.139	481	5′ UTR	14
	EV-OR2	CCGGATGGCCAATCCAATAACTATA TGGT	0.139			
Group 4						
HCoV-OC43	HCoVOC43-OF3	CGGTTACACTGTTCAGCCAATYGCA	0.313	793	Spike protein	This study
	HCoVOC43-OR3	CCAACCCAAAAATGCTTGTGGTYG	0.313			
SARS-CoV	COR1	CACCGTTTCTACAGGTTAGCTAACGA	0.313	310	Polymerase	32
	COR2	AAATGTTTACGCAGGTAAGCGTAAAA	0.313			
HCoV-229E	COR229E-IF2	TCACCCATTTGAAGAATTGGAATT TTGG	0.313	566	Matrix	This study
	COR229E-IR2	TCGTACGTAGAAAACCCAGCCTGTGC	0.313			
hMPV	Meta-M-OF2	CAATATGGTTCCCTTTGTTTCAGGCCA	0.313	462	Matrix	This study
	Meta-M-OR2	TGGTCTGCTTCACTGCTTATWGCA GCTT	0.313			
Group5						
M. pneumoniae	Mpneumoniae-OF2	GACCATTCCACCCAGCCCCAGC	0.089	343	Cytadhesin P1 gene	17
	Mpneumoniae-OR2	GTTCAGCGAGTGGGGTGCGTACAATA	0.089			
C. pneumoniae	Chlamy-pneum-OF4	TGCGCTACTTGGTGCGACGCTA	0.179	571	Outer membrane protein A	17
	Chlamy-penum-OR4	CGCCTTTATAGCCCTTGGGTTTRTTT	0.179			
L. pneumophila	Legionella-OF1	CGCTCAATTGGCTTTAACCGAACAG	0.054	425	Macrophage infectivity potentiator (“*mip*”)	This study
	Legionella-OR1	CGCTRCGTGGRCCATATGCARGAC	0.054			
ADV	ADVAtoF-OF3	TACATGCACATCKCSGGVCAGGA	0.179	983	Hexogene	This study
	ADVAtoF-OR3	CCRGCCARHACHCCCATRTTDCCHGT	0.179			

[a] Degenerate primer abbreviations are as follows: M, A/C; R, A/G; W, A/T; S, C/G; Y, C/T; K, G/T; V, A/C/G; H, A/C/T; D, A/G/T; N, A/C/G/T.
[b] UTR, untranslated region.

pneumophila, and ADV. The sequences and amplicon sizes of the outer and inner sets of primers are given in Tables 1 and 2.

Nucleic acid extraction and cDNA synthesis. Total RNA and DNA were extracted together by using the QIAamp MinElute Virus Spin kit (QIAGEN, Valencia, CA), and 60 μl of nucleic acids was eluted according to the manufacturer's recommendations.

The RNA template (8 μl) was mixed with 1 μl of random primers (2.5 ng/μl) and 1 μl of deoxynucleoside triphosphates (0.5 mM each) in a final volume of 10 μl for incubation at 65°C for 5 min. The solution was equilibrated at 4°C and completed with 2 units of RNaseOUT, 4 μl of 5× first-strand buffer, 0.5 mM dithiothreitol, and 10 U Superscript III reverse transcriptase (Invitrogen, Carlsbad, CA) in a final volume of 20 μl. RT was performed for 50 min at 50°C and then stopped by heating for 15 min at 70°C. The resulting cDNA products were used immediately for PCR.

Fast PCR conditions. In order to provide the shortest possible turnaround time, the recently available “fast” thermal cycler (fast PCR machine; Applied Biosystems, Foster City, CA) was used. When coupled with the DNA polymerase contained in the fast PCR master mix (GeneAmp; Applied Biosystems, Foster City, CA), a 35-cycle PCR assay could be completed within 35 min, compared to the ~180 min that would normally be required for standard thermocyclers. All multiplex nested PCR assays were optimized to fulfill the manufacturer's recommendation that two-step cycling with annealing at 64°C was used. Both the

TABLE 2. Primers used in the second round of multiplex nested PCR

Organism	Primer	Sequence (5′→3′)[a]	Primer concn (μM)	Product length (bp)	Target gene[b]	Source or reference
Group 1						
Influenza A virus	FluARe-F1	AAGACCAATCCTGTCACCTCTGA	0.103	104	Matrix	30
	FluARe-R1	CAAAGCGTCTACGCTGCAGTCC	0.103			
Influenza A virus H1	H1-IF2	TCGCCGACTATGAGGAACTGAGGGA	0.021	431	Hemagglutinin	This study
	H1-IR2	TTGTATCCCCGGGTTCCAGCAGAGT	0.021			
Influenza A virus H3	H3-IF2	CCCTTATGATGTGCCGGATTATGCC	0.205	259	Hemagglutinin	This study
	H3-IR2	GGTGGTGAACCCCCCAAATGTACAA	0.205			
Influenza A virus H5	H5N1-VIET-1A	TGCGACTGGRCTCAGAAATA	0.512	172	Hemagglutinin	This study
	H5N1-VIET-4B	GGATTCTTTGTCTGCAGCGT	0.512			
Influenza B virus	FLUB-IF3	AAAACAARTGCTCTGCRCCYCAAC	0.41	516	Nucleoprotein	This study
	FLUB-IR3	CRTCTCCACCTACTTCRTTYCCCCC	0.41			
Group 2						
PIV-1	PIV-1-IF1	AATTGGTGATGCAATATATGCKT ATTC	0.61	600	Hemagglutinin-neuraminidase	This study
	PIV-1-IR1	TCGACAACAATYTTTGGCCTATC	0.61			
PIV-2	PIV2-F2	AGGACAGCAGAGGACCTCGGCATG	0.0305	343	Hemagglutinin-neuraminidase	This study
	PIV2-R2	ACCTGATGTTCTTTGCGGTATGGGG	0.0305			
PIV-3	PIV-3-IF1	CAACTGTGTTCRACTCCCAAAG	0.457	717	Hemagglutinin-neuraminidase	This study
	PIV-3-IR1	TGGGTTYACTCTCGATTTTTGY	0.457			
PIV-4	PIV-4AB-IF2	GACGGATGYYTRCKGWATTGTGT	0.153	231	Hemagglutinin-neuraminidase	This study
	PIV-4AB-IR2	CCRTRAGRCCYCCATACAARGGAA	0.153			
Group 3						
hRSV A	RSVA-IF2	TGACCCATTAGTGTTCCCCTCTGAT GAAT	0.278	228	Fusion protein	This study
	RSVA-IR2	CTTCTGGCCTTRCAGTATARGAG CAGT	0.278			
hRSV B	RSV-B-IF1	GTCGCATCTCCAACATTGRAAC	0.069	336	Fusion protein	This study
	RSV-B-IR1	TGGTGCATAGAGGTGATGTGTG	0.069			
hRV	RV-OF2	CACTTCTGTTTCCCCGGAGCGAGG	0.139	283	5′ UTR	14
	JWA-1B-MOD-RV	CCGCATTCAGGGGCCGGAG	0.139			
hEV	EV-IF3	CCTCCGGCCCCTGAATGCG	0.139	106	5′ UTR	3, 14
	EV-IR3	CCAAAGTAGTCGGTTCCGCYRCRGA	0.139			
Group 4						
HCoV-OC43	HCoVOC43-IF2	CKGTGCCCTCTCCATTAAATTGGG	0.25	635	Spike protein	This study
	HCoVOC43-IR2	GACCCGAACAGTGCTCACCTATGCC	0.25			
SARS-CoV	COR3	AGTGAGATGGTCATGTGTGG	0.5	210	Polymerase	32
	COR4	CACTCATAGAGCCTGTGTTG	0.5			
HCoV-229E	COR229E-IF3	TTGGGATTCTAATTGGGCCTTTGTTGC	0.25	361	Matrix	This study
	COR229E-IR3	GCTCGGCACGGCAACTGTCATGTAT	0.25			
hMPV	Meta-M-IF2	CCCTTTGTTTCAGGCCAAYACACCACC	0.25	431	Matrix protein	2
	Meta-M-IR2	GCAGCTTCAACAGTRGCTGATTCAC TCTC	0.25			
Group 5						
M. pneumoniae	Mpneumoniae-OF1	AGGGGGTTCTTCAGGCTCAGGTCAA	0.094	160	Cytadhesin P1 gene	17
	Mpnuemoniae-OR1	CCCCACCACATCATTCCCCGTATTA	0.094			
C. pneumoniae	Chlamy-pneum-IF6	RCCTACWGGATCCGCTRCTGCRAA	0.313	317	Outer membrane protein A	17
	Chlamy-pneum-IR6	GCRCCTACGCTCCAAGMRAAAGWRG	0.313			
L. pneumophila	Legionella-IF1	TGAAAACAAAAACAAGCCAGGC GTTG	0.063	232	Macrophage infectivity potentiator ("*mip*")	This study
	Legionella-IR1	TGGCATCAATTGYAAAGCYTCTGTCC	0.063			
ADV	ADVAtoF-IF3	TGGCYWSCACNTWCTTTGACATYMG	0.782	463	Hexogene	This study
	ADVAtoF-IR3	GCRWAWGAHCCRTARCAKGGYT DCAT	0.782			

[a] Degenerate primer abbreviations are as follows: M, A/C; R, A/G; W, A/T; S, C/G; Y, C/T; K, G/T; V, A/C/G; H, A/C/T; D, A/G/T; N, A/C/G/T.
[b] UTR, untranslated region.

first and second round of PCRs were conducted in a 20-μl reaction mixture. Two microliters of the cDNA preparation was used as the template for the first round of PCR for groups 1 to 4, whereas 8 μl of the extracted preparation was used for group 5, which was comprised of bacteria and a DNA virus. In the second round of PCR, a 0.2-μl aliquot of the first-round PCR product was used as a template. The final concentration of each primer present in the reaction mixture is shown in Tables 1 and 2. The cycling conditions for the first and second round of PCRs were an initial denaturation step at 95°C for 10 s and then 30 cycles of denaturation at 95°C for 1 s and annealing/extension at 64°C for 40 s, followed by a final extension step at 72°C for 10 s. The cycling conditions were the same for groups 1 to 4, whereas 35 cycles of denaturation at 95°C for 5 s was used instead for group 5.

The PCR products were identified by electrophoresis in 2% agarose gels and stained by ethidium bromide.

Preparation of controls. Cultured stocks of the target pathogens were used as positive controls for the corresponding sets of the multiplex nested PCR assays. For noncultivatable pathogens, clinical specimens known to contain the target agents were used. For enteroviruses, the primers were designed to detect all serotypes of enteroviruses. In this study, the most commonly encountered serotypes including coxsackievirus serotypes A9, B1, B2, B3, and B5; echovirus serotypes 3, 7, 11, and 30; enterovirus serotype 71; and poliovirus type 1 (vaccine strain) were selected for the evaluation process.

Prevention of PCR contamination. Precautions were taken to prevent cross-contamination. The preparation of reagents, processing of samples, and nested

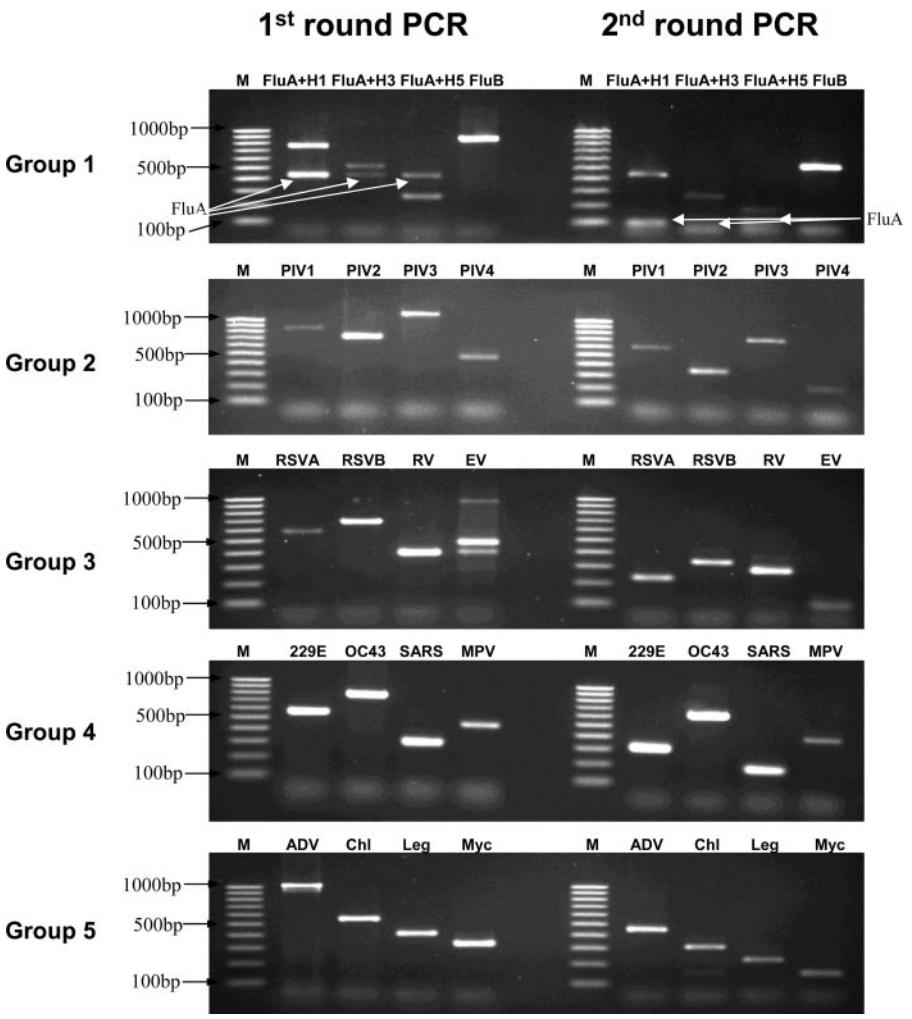

FIG. 1. Agarose gel (2%) electrophoresis showing the first and second rounds of multiplex nested PCR products. M, marker (100-bp ladder); RSV, hRSV; RV, hRV; EV, hEV; MPV, hMPV; Chl, *C. pneumoniae*; Leg, *L. pneumophila*; Myc, *M. pneumoniae*.

PCR assays were carried out in separate rooms away from the area where amplified products were analyzed. Filtered pipette tips were used throughout the experiments.

Specificity of the assay. The ability of the multiplex nested PCR assays to detect the presence of more than one pathogen in the same specimen was assessed by use of simulated specimens spiked with two or more pathogens.

After the initial primer selection and optimization using known positive samples, the specificity of each multiplex nested PCR assay was further evaluated by running the assays on 50 clinical specimens known to contain respiratory pathogens other than the intended targets. This was to reconfirm that the primer sets did not produce false-positive or nonspecific results.

Sensitivity of the assay. For viruses with an RNA genome, the assay sensitivity was determined by using synthetic RNA standards. Synthetic RNA target standards were generated using T7 polymerase (Ambion, Austin, TX) with primers incorporating a T7 promoter sequence. The copy number of synthetic RNA molecules was determined by UV spectrometry and serially diluted in 2.5 μg/ml yeast tRNA (Ambion). Eight microliters of diluted RNA was reversely transcribed with random hexamers using Superscript III reverse transcriptase (Invitrogen) according to the above-mentioned protocol, and the output cDNA was used as a template for the multiplex nested PCR assays. For DNA pathogens, specific cDNA targets were also quantified by UV spectrometry. The DNA targets with known copy numbers were then serially diluted to serve as templates for the multiplex nested PCR assays.

Furthermore, for cultivable viruses, the method of limiting dilution was used to compare the analytical sensitivity of the multiplex nested PCR assays with that of virus isolation, and in the case of bacterial pathogens, the detection limit of the multiplex nested PCR assays was expressed as CFU/milliliter.

Evaluation of clinical specimens. A total of 303 nasopharyngeal aspirate (NPA) specimens were collected for this study. These NPA samples were taken from patients who were admitted to the Prince of Wales Hospital for suspected respiratory tract infections. The specimens were kept at 4 to 10°C during transportation and temporary storage and were processed on the same day of collection. Two hundred thirty-five specimens were obtained from pediatric patients aged 1 month to 5 years (mean, 2 years of age). The other 68 specimens were obtained from elderly patients aged 65 to 107 years (mean, 65 years of age). Upon receipt, the specimens were separated into two halves, with one half submitted to routine virus isolation and antigen detection by immunofluorescence assay (IFA) and the other half used for the multiplex nested PCR.

Direct immunofluorescent test (IFA). Specimens were tested for influenza A virus, influenza B virus, PIV (types 1 to 3), respiratory syncytial virus, and ADV by use of a direct immunofluorescence test to screen for the presence of respiratory viruses. Briefly, the respiratory specimens were washed with phosphate-buffered saline. One drop of cell suspension was coated onto a 12-well slide and allowed to dry. The slide was fixed in 100% acetone and incubated with virus-specific mouse fluorescein isothiocyanate-conjugated monoclonal antibodies (Chemicon, Temecula, CA) for 30 min at 37°C. Slides were washed with phosphate-buffered saline and read using a fluorescence microscope. The presence of bright green fluorescence within intact cells was considered to be a positive result. The results were confirmed by two experienced technicians.

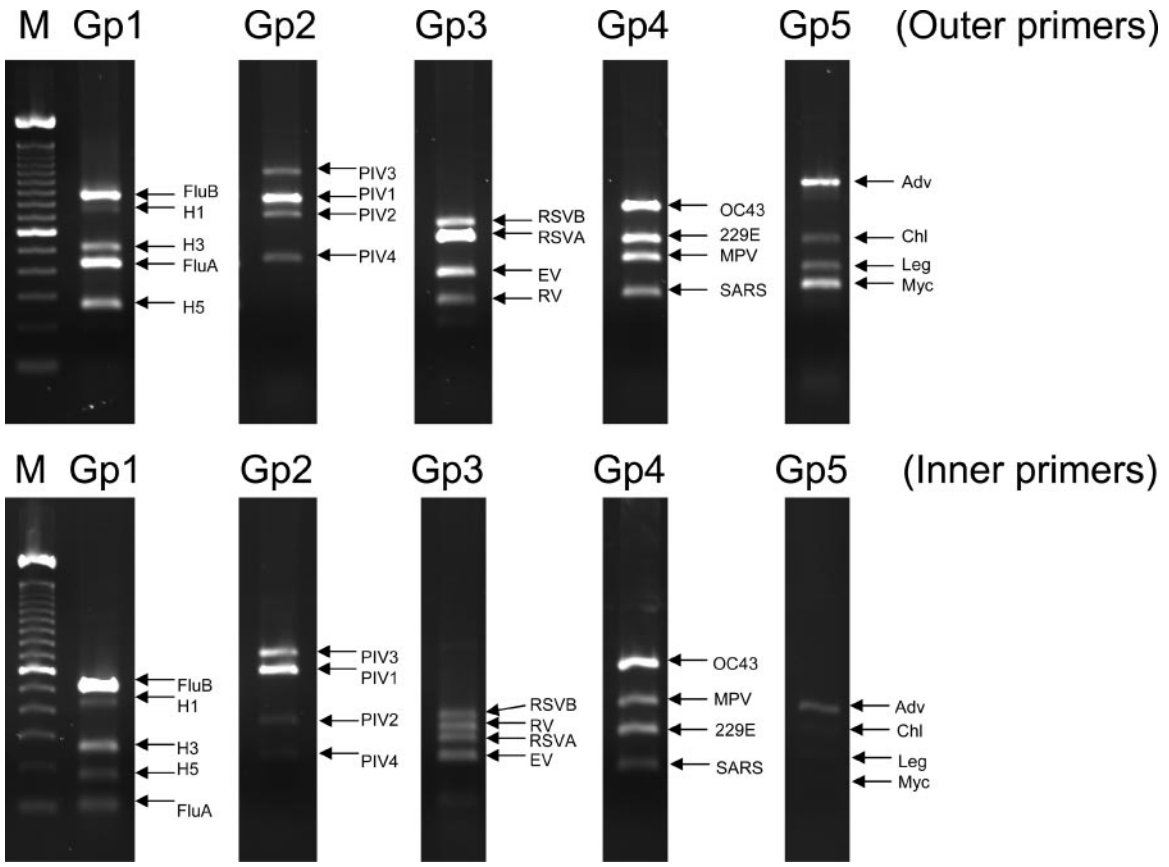

FIG. 2. Agarose gel (2%) electrophoresis showing the first and second rounds of the group 1 to group 5 (Gp1 to Gp5) multiplex nested PCR products using a mixture of pathogens as a template. M, marker (100-bp ladder); RSV, hRSV; RV, hRV; EV, hEV; MPV, hMPV; Chl, *C. pneumoniae*; Leg, *L. pneumophila*; Myc, *M. pneumoniae*.

Virus isolation. A 200-μl aliquot of specimen was inoculated onto HEp-2, MDCK, and LLC-MK_2 cell monolayers. After 1 h of adsorption at 37°C, maintenance medium was added, and the tubes were incubated at 37°C for HEp-2 cells and at 33°C for MDCK and LLC-MK_2 cells. The HEp-2 tubes were incubated for 14 days and examined daily for a viral cytopathic effect. The hemadsorption assay was performed on day 10 for MDCK and LLC-MK_2 cells. The positive growth of viruses was confirmed by IFA using virus-specific antibodies. All specimens were tested for FluA, FluB, PIV (types 1 to 3), hRSV, and ADV using IFA as previously described (13). Cells were collected and stained by use of standard methods (16). The results were confirmed by two experienced technicians.

RESULTS

All five multiplex nested PCR assays produced amplification products with the expected sizes, which were clearly distinguishable by agarose gel electrophoresis (Fig. 1). The ability of the assays to detect multiple infections is shown in Fig. 2. It was found that multiple infections did not reduce the sensitivity of the assays. Testing of the 50 clinical specimens known to contain pathogens did not reveal any nonspecific cross-amplification.

The analyses of analytic sensitivity showed that the multiplex nested PCR assays were highly sensitive, with a low detection limit of less than 10 copies of the target nucleic acids, with the exception of enteroviruses that could still reach a detection limit of 10^4 copies of nucleic acids (Fig. 3 and Table 3).

The sensitivity of multiplex nested PCR assays to detect cultivable viruses was found to be 100- to 1,000-fold more sensitive than virus isolation by cell culture. The detection limit of group 5 multiplex nested PCR for *Legionella pneumophila* and *Mycoplasma pneumoniae* was 1,000 to 10,000 CFU/milliliter.

A total of 303 NPA specimens were tested using virus isolation, IFA, and multiplex nested PCR. Altogether, 61 specimens were positive by virus isolation, and 41 specimens were positive by IFA. All these isolation- or IFA-positive specimens were also found to be positive by multiplex nested PCR, with the same corresponding viruses detected (Table 4). The overall positive rate as determined by multiplex nested PCR was 48.5% (95% confidence interval [CI], 42.9 to 54.1%), which was significantly higher than those of virus isolation (20.1% [95% CI, 15.6 to 24.6%]) and IFA (13.5% [95% CI, 9.7 to 17.4%]). The positive rates for each pathogen with respect to detection method are shown in Table 4.

A subgroup analysis was performed on viruses that were detected by virus isolation. All these cultivatable viruses showed a higher positive rate by multiplex nested PCR than by virus isolation, except for ADV. However, the differences were not statistically significant (the positive rate [95% CI] for FluA by PCR versus isolation was 6.3% [3.5 to 9.0%] versus 5.0% [2.5 to 7.4%], respectively; the positive rate for FluB by PCR versus isolation was 3.3% [1.3 to 5.3%] versus 3.0% [1.1 to

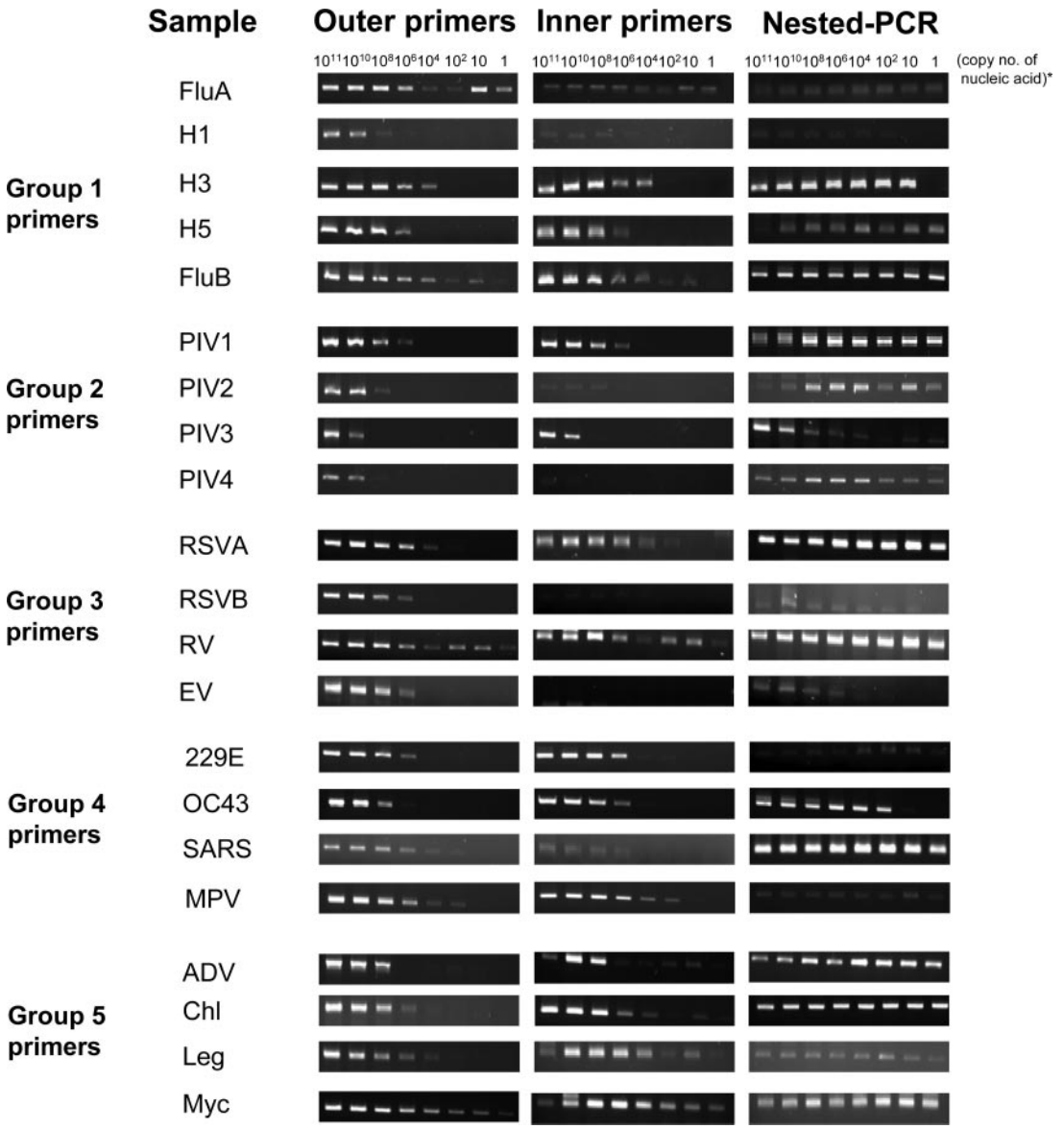

FIG. 3. Agarose gel (2%) electrophoresis showing the performance of the five outer and inner primer groups as well as nested PCR of the multiplex assays. A single template was added to each corresponding group of primers to test the sensitivity of the assay in detecting that pathogen. For the nested PCR limit detection test, the PCR products from the first-round PCR are used as a template for the second-round PCR (copy number of nucleic acids − copy number of RNA or DNA template in the original samples). RSV, hRSV; RV, hRV; EV, hEV; MPV, hMPV; Chl, *C. pneumoniae*; Leg, *L. pneumophila*; Myc, *M. pneumoniae*.

4.9%], respectively; the positive rate for PIV-1 by PCR versus isolation was 6.3% [3.5 to 9.0%] versus 4.6% [2.3 to 7.0%], respectively; the positive rate for PIV-2 by PCR versus isolation was 2.0% [0.4 to 3.5%] versus 0.3% [0 to 1.0%], respectively; the positive rate for PIV-3 by PCR versus isolation was 1.0% [0 to 2.1%] versus 0.7% [0 to 1.6%], respectively; the positive rate for hRSV by PCR versus isolation was 2.7% [0.8 to 4.4%] versus 1.7% [0.2 to 3.1%], respectively; the positive rate for hEV by PCR versus isolation was 1.0% [0 to 2.1%] versus 0% [0%], respectively; and the positive rate for ADV by PCR versus isolation was 5.0% [2.5 to 7.4%] versus 5.0% [2.5 to 7.4%], respectively). Compared to virus isolation, the overall gain in the positive rate for this group of cultivatable viruses as achieved by multiplex nested PCR was an increase from 20.1% to 29.7%.

When the group of viruses that can be diagnosed by direct detection using IFA was compared, the positive rate obtained by multiplex nested PCR was higher than that of IFA for all the viruses. However, again, the differences were not statistically significant. The positive rate (95% CI) for FluA by PCR versus IFA was 6.3% (3.5 to 9.0%) versus 3.6% (1.5 to 5.7%), respectively; that for FluB by PCR versus IFA was 3.3% (1.3 to 5.3%) versus 2.3% (0.6 to 4.0%), respectively; that for PIV-1 by PCR versus IFA was 6.3% (3.5 to 9.0%) versus 3.3% (1.3 to 5.3%), respectively; that for PIV-2 by PCR versus IFA was 2.0% (0.4 to 3.5%) versus 0.3% (0 to 1.0%), respectively; that

TABLE 3. Performance of individual and nested primer pairs in the corresponding multiplex nested PCR assay group

Organism	Detection limit (copy no. of nucleic acids)		
	Outer primer[a]	Inner primer[a]	Nested PCR[b]
Group 1			
Influenza A virus	1	1	1
Influenza A virus H1	10^6	10^6	10^2
Influenza A virus H3	10^3	10^4	10
Influenza A virus H5	10^6	10^6	1
Influenza B virus	1	10	1
Group 2			
PIV-1	10^6	10^6	1
PIV-2	10^8	10^6	1
PIV-3	10^{10}	10^6	1
PIV-4a,b	10^8	10^{10}	1
Group 3			
hRSV A	10^2	10^2	1
hRSV B	10^4	10^6	10
hRV	1	1	1
hEV	10^6	10^8	10^4
Group 4			
HCoV-OC43	10^6	10^4	10
SARS-CoV	10^2	10^6	1
HCoV-229E	10^6	10^2	1
hMPV	10^2	10^2	1
Group 5			
M. pneumoniae	1	1	1
C. pneumoniae	10^4	1	1
L. pneumophila	10^2	1	1
ADV (A to F)	10^8	10^{10}	1

[a] The detection limits of outer and inner primers were tested in the form of multiplex primer mix.
[b] The PCR products from the first-round PCR are used as a template for the second-round PCR in the nested PCR limit detection test.

for PIV-3 by PCR versus IFA was 1.0% (0 to 2.1%) versus 0.3% (0 to 1.0%), respectively; that for hRSV by PCR versus IFA was 2.7% (0.8 to 4.4%) versus 2.0% (0.4 to 3.5%), respectively; that for hEV by PCR versus IFA was 1.0% (0 to 2.1%) versus 0%, respectively; and that for ADV by PCR versus IFA was 5.0% (2.5 to 7.4%) versus 1.7% (0.2 to 3.1%), respectively. The overall gain in the positive rate achieved by multiplex nested PCR for this group of viruses was an increase from 13.5% to 27.7%.

Of the 21 pathogens included in the study, 7 were not detectable by isolation or IFA. Within this group, three were commonly found in our study samples, including rhinovirus, with a positive rate of 5.3%, HCoV-OC43 (5.3%), and hMPV (5.0%). Overall, these three viruses contributed 34.0% of the PCR-positive cases.

Multiple respiratory viruses were observed in 7 of the 303 (2.3%) specimens (Table 4). None of these coinfections were detected by virus isolation or IFA, as the majority of them contained a noncultivatable organism. These cases contributed 4.8% of PCR-positive cases.

Another advantage of this multiplex nested PCR was that it could be used to subtype pathogens in the same testing cycle. For the 19 FluA cases detected in multiplex nested PCR, 17 were H1 infections, and 2 were H3 infections. For the eight cases of hRSV identified, five were hRSV A infections and three were hRSV B infections. These subtyping results could be obtained directly by agarose gel electrophoresis without further testing.

DISCUSSION

Respiratory tract infection accounts for a majority of the admissions in acute care hospitals. While it has long been recognized that viruses contribute to a significant proportion of these cases, the urgency for laboratory diagnosis remains paradoxically low in most settings. One of the main reasons is the long turnaround time of conventional virus detection methods and their inability to detect fastidious viruses. The lack of specific treatment for most viral infections is another practical consideration when prioritizing laboratory resources. The outbreak of SARS and the threat of avian influenza virus reawakened the need for rapid diagnosis to enable the prompt and accurate diagnosis of index cases.

In this study, we sought to develop and evaluate multiplex nested PCR assays for the rapid and accurate diagnosis of respiratory tract infections. The rationale for primer groupings was as follows. Firstly, RNA pathogens and DNA pathogens were separated, i.e., groups 1 to 4 for RNA pathogens and group 5 for DNA pathogens. Secondly, pathogens of the same or similar family were grouped together, for example, FluA (H1 to H5) and FluB were grouped together into group 1. PIVs were grouped into group 2. In this way, each family member amplified within the same PCR could be easily differentiated. Thirdly, PCR product size was another factor affecting multiplex grouping. For example, the primers designed for hRSVA, hRSVB, hEV, and hRV were compatible to form a multiplex. Fourthly, only four pathogens were included in each group because, on one hand, the size of the PCR products being amplified would be very suitable for visual differentiation on agarose gel and, on the other hand, the amplification efficiency for each PCR would not be jeopardized too much by multiplexing. For example, if too many pathogens were included in a single multiplex reaction, in order to have sufficient visual differentiation of PCR products on an agarose gel, some of the PCR products would need to be very large, and that might lower the sensitivity of the pathogens being amplified.

Molecular techniques have increased the speed and sensitivity with which such pathogens can be detected and allow laboratories to identify organisms that do not grow or grow slowly in conventional viral culture. However, the gain in analytical sensitivity may not necessarily be reflected in clinical situations. For instance, in settings where clinical specimens are collected and maintained in good quality, the amount of virus present may well be enough for detection by the "less sensitive" conventional methods (culture and IFA). In fact, our data are in line with this. When cultivatable viruses were compared, despite the finding that a higher sensitivity for multiplex nested PCR was observed, the differences were not statistically significant. In particular, we observed the same positive rate for ADV using both PCR and conventional virus isolation methods. In a previous study, a discrepancy between direct detection and RT-PCR for ADV was also reported (19). Although the lack of a statistically significant improvement in a positive detection rate could be due to a low general preva-

TABLE 4. Performance of multiplex nested PCR assays compared to conventional methods

Organism	No. (%) of positive specimens (n = 303)		
	Multiplex nested PCR	Virus isolation	IFA
Any infection	147 (48.5)	61 (20.1)	41 (13.5)
Single infection	140 (46.2)	61 (20.1)	41 (13.5)
Influenza A virus	19 (6.3)	15 (5.0)	11 (3.6)
Influenza A virus H1	17 (5.6)	—[a]	—[a]
Influenza A virus H3	2 (0.7)	—[a]	—[a]
Influenza A virus H5	0	—[a]	—[a]
Influenza B virus	10 (3.3)	9 (3.0)	7 (2.3)
PIV-1	19 (6.3)	14 (4.6)	10 (3.3)
PIV-2	6 (2.0)	1 (0.3)	1 (0.3)
PIV-3	3 (1.0)	2 (0.7)	1 (0.3)
PIV-4	2 (0.7)	—[a]	—[a]
Respiratory syncytial virus	8 (2.7)	5 (1.7)	6 (1.9)
Respiratory syncytial virus group A	5 (1.7)	—[a]	—[a]
Respiratory syncytial virus group B	3 (1.0)	—[a]	—[a]
Rhinovirus	16 (5.3)	—[a]	—[a]
Enteroviruses	3 (1.0)	0	0
HCoV-OC43	16 (5.3)	—[a]	—[a]
HCoV-229E	3 (1.0)	—[a]	—[a]
SARS-CoV	0	0	0
hMPV	15 (5.0)	—[a]	—[a]
M. pneumoniae	5 (1.7)	—[a]	—[a]
L. pneumophila	0	—[a]	—[a]
C. pneumoniae	0	—[a]	—[a]
ADVs	15 (5.0)	15 (5.0)	5 (1.7)
Coinfection	7 (2.3)	0	0
Influenza A virus and *M. pneumoniae*	1 (0.3)	—[a]	—[a]
Influenza A virus H1 and *C. pneumoniae*	1 (0.3)	—[a]	—[a]
Influenza A virus H3 and HCoV-229E	1 (0.3)	—[a]	—[a]
Influenza A virus H3 and PIV-2	1 (0.3)	—[b]	—[b]
hMPV and *M. pneumoniae*	2 (0.7)	—[a]	—[a]
hMPV and PIV-4	1 (0.3)	—[a]	—[a]

[a] Organisms not isolated/differentiated by virus isolation.
[b] PIV-2 was isolated.

lence of the individual organism, the overall results indicate that the main impact of this multiplex nested PCR was its broader spectrum of detection. Expanding the detection spectrum has also been the main focus of previous studies, and as many as nine different respiratory pathogens have been targeted (11, 15, 22, 27). In the current study, we included 21 respiratory pathogens and provided the widest spectrum ever reported. We found that the gain in the overall positive detection rate from clinical specimens was attributed mainly to the inclusion of hRV, HCoV-OC43, and hMPV detection. All these viruses are not detectable by conventional cell culture isolation or direct detection using IFA. The improvement in the diagnostic yield by adding hRV was also reported previously by Gruteke et al. (14). Given that these "trivial" respiratory viruses can cause severe illnesses (6, 20), they should be included in the development of multiplex assays. Another advantage of multiplex nested PCR as demonstrated in this study is the ability to detect coinfections, although the overall improvement in the positive rate was not substantial due to the relatively few instances of coinfection in our study cohort.

While multiplex nested PCR assays may be more economical due to the fact that multiple pathogens can be detected in a single assay without a proportional increase in reagent costs, they have their drawbacks. First, their detection sensitivities are often lower than those of equivalent monoplex PCR assays. In this study, only about 30% of the positive specimens showed a positive result from the first round of PCR. This finding indicates the need for a nested PCR, which may be associated with a higher risk of cross-contamination. Second, the presence of several pairs of primers in a PCR increases the probabilities of mispairing and nonspecific amplification, particularly the formation of primer-dimers.

In group 1 multiplex nested PCR, we incorporated specific primers for influenza A virus subtypes H1, H3, and H5. This is important in the context where rapid differentiation between H5 and non-H5 influenza virus is necessary. The group 1 multiplex nested PCR assay was also comprised of primer pairs targeting the consensus region of influenza A virus. This would allow the detection of non-H1/H3/H5 subtypes, which may occasionally cause human infections (e.g., H7 and H9 influenza viruses).

A nucleic acid extraction kit that can extract both viral DNA and RNA simultaneously was used in our study. This can minimize the amount of samples required for the detection of both DNA and RNA viruses. At the same time, this can minimize the time, labor, and materials involved in nucleic acid extraction.

Also, a newly available fast thermal cycler was used in our

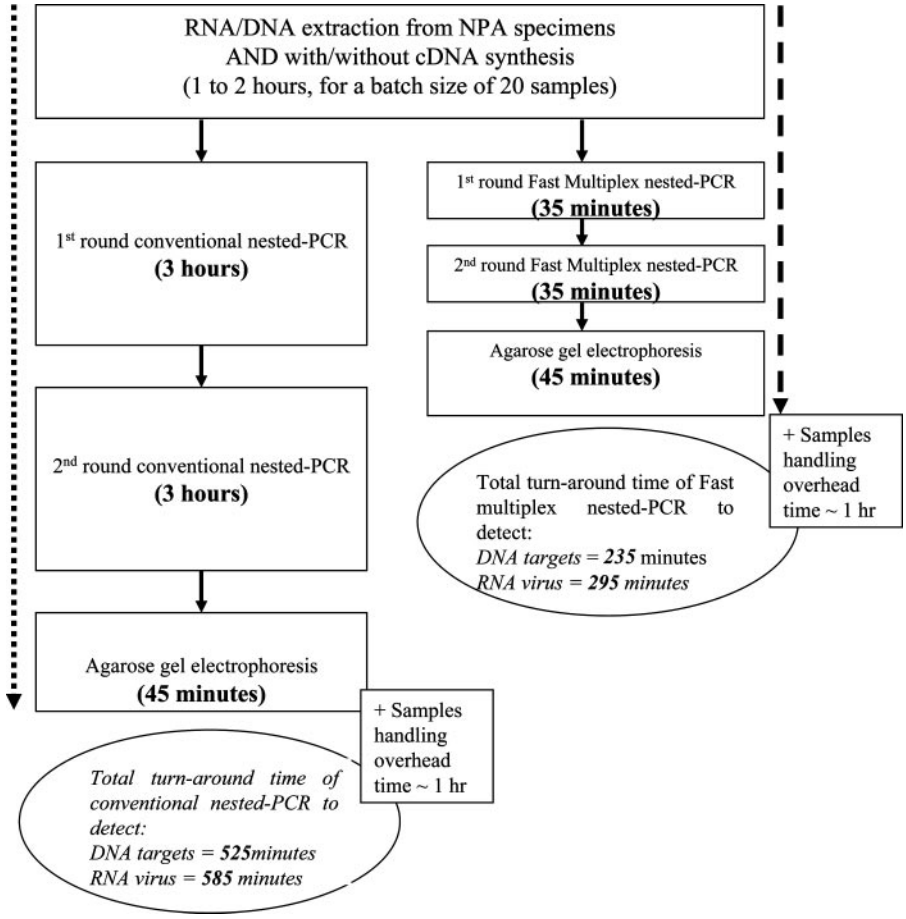

FIG. 4. Estimated turnaround times for the fast multiplex nested PCR protocol and conventional nested PCR protocol.

study, which allowed rapid cycling, shortening the time required to complete the PCR. The thermal cycler's patented sample temperature control provided a quick and uniform thermal response. Therefore, the cycling times for the first- and second-round PCRs were considerably reduced. Furthermore, the GeneAmp fast PCR master mix allows a two-step PCR (same temperature holding for the annealing and extension steps) instead of the more conventional three-step PCR. With the use of this fast PCR system, the time required for a single round of PCR was reduced from 3 h to 35 min, i.e., saving a total of about 300 min in a nested PCR assay (Fig. 4). Therefore, the whole testing process can be completed within 1 day. This rapid turnaround not only is critical in urgent outbreak investigations but may potentially decrease the overall costs for the hospital, as has been shown in previous studies (1, 30). However, one disadvantage of using this fast PCR system is that primers used in standard multiplex PCR assays need to be redesigned with higher annealing temperatures.

The multiplex nested PCR assays developed in this study improved the diagnostic yield in terms of the overall sensitivity as well as the spectrum of coverage for respiratory infections. Furthermore, the assay provided a rapid turnaround time, with results being available within the same day of specimen collection. The overall cost reduction may justify the routine use of these broader-spectrum, rapid molecular diagnostic assays.

ACKNOWLEDGMENTS

This study was supported by the Research Fund for the Control of Infectious Diseases from the Health, Welfare, and Food Bureau of the Hong Kong Special Administrative Region Government (project no. 01030782).

Part of the work was performed at the Lo Kwee Cheong Research Laboratory.

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