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Simultaneous Detection of Fourteen Respiratory Viruses in Clinical Specimens by Two Multiplex Reverse Transcription Nested-PCR Assays

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There is a need for rapid, sensitive, and accurate diagnosis of lower respiratory tract infections in children, elderly, and immunocompromised patients, who are susceptible to serious complications. The multiplex RT-nested PCR assay has been used widely for simultaneous detection of non-related viruses involved in infectious diseases because of its high specificity and sensitivity. A new multiplex RT-PCR assay is described in this report. This approach includes nested primer sets targeted to conserve regions of human parainfluenza virus haemagglutinin, human coronavirus spike protein, and human enterovirus and rhinovirus polyprotein genes. It permits rapid, sensitive, and simultaneous detection and typing of the four types of parainfluenza viruses (1, 2, 3, 4AB), human coronavirus 229E and OC43, and the generic detection of enteroviruses and rhinoviruses. The testing of 201 clinical specimens with this multiplex assay along with other one formerly described by our group to simultaneously detect and type the influenza viruses, respiratory syncytial viruses, and a generic detection of all serotypes of adenovirus, covers the detection of most viruses causing respiratory infectious disease in humans. The results obtained were compared with conventional viral culture, immunofluorescence assay, and a third multiplex RT-PCR assay for all human parainfluenza viruses types described previously. In conclusion, both multiplex RT-PCR assays provide a system capable of detecting and identifying simultaneously 14 different respiratory viruses in clinical specimens with high sensitivity and specificity, being useful for routine diagnosis and survey of these viruses within the population. ***J. Med. Virol. 72:484–495, 2004.***

KEY WORDS: multiplex RT-PCR assay; diagnosis of respiratory infection; parainfluenza virus; human coronavirus; enterovirus; rhinovirus

INTRODUCTION

The clinical presentation of the respiratory infections caused by different viral pathogens can be very similar, making etiological diagnosis difficult. Rapid virological methods should permit specific and sensitive diagnosis at an early stage of the illness not only to assist the clinician in making therapeutic decisions, but also to prevent nosocomial infections, and to alert the onset of epidemics. The use of standard diagnostic methods such as the viral isolation in cell culture or the antigen detection assays can result in long delays before final results are available. Molecular techniques have enabled major advances in the speed and sensitivity of the laboratory diagnosis of viral respiratory infections. Within this group of assays, the polymerase chain reaction (PCR) is used most commonly, although there are several other methods that must be explored in order to improve their applicability across the range of viral pathogens [Myint, 2002]. For many RNA viruses, including most of respiratory viruses, the multiplex reverse transcription (RT)-PCR assay-based diagnosis has been shown to be rapid, sensitive, and specific [Gilbert et al., 1996; Coiras et al., 2003]. Moreover, this assay allows the coamplification of more than one target RNA sequence in a small quantity of specimen, thus

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providing useful information about the significance of mixed infections in the prognostic and recrudescence of the respiratory disease. The multiplex RT-PCR system can be incorporated to the diagnosis routine increasing the throughput of specimens and reducing analytical costs associated with screening for multiple organisms [Gilbert et al., 1996].

In a previous study [Coiras et al., 2003], a multiplex RT-nested PCR assay was described to detect simultaneously and identify influenza A, B, and C viruses, respiratory syncytial viruses (RSV) types A and B, and a generic detection of all adenovirus serotypes. All these viruses are of primary importance since infections produced by them range from mild respiratory illness to fatal pneumonia, and cause considerable morbidity and excess deaths in children, elderly people, and in immunocompromised individuals throughout the world [Fleming and Cross, 1993; Arola et al., 1995; Dowell et al., 1996]. This assay has been integrated into the clinical and virological surveillance and it proved to be a useful rapid and sensitive diagnostic method.

Nevertheless, many other viral respiratory pathogens such as the human parainfluenza viruses types 1, 2, and 3 and the rhinoviruses, are among the causing agents of most common infectious diseases world-wide, especially in children under 6 years of age. These viruses, as happens with RSV and influenza A and B viruses, cause a range of respiratory illnesses from mild upper respiratory tract symptoms to bronchiolitis, exacerbation of asthma, and primary pneumonia with associated death [McMillan et al., 1993; Nicholson et al., 1993; Apalsch et al., 1995; Arruda et al., 1997]. On the other hand, the human coronaviruses have also a world-wide distribution and infect all age groups [Gwaltney, 1980; Hruskova et al., 1990]. Indeed, coronaviruses are thought to be responsible for approximately 10% of common colds, as well as lower respiratory tract infections in infants and exacerbation of asthma [McIntosh et al., 1970; Johnston et al., 1995]. In addition, it is important to define the role of enteroviruses as etiological agents in childhood obstructive bronchiolitis [Andréoletti et al., 2000], usually related with enteric diseases [Casas et al., 1999; Trallero et al., 2000].

Consequently, a second multiplex RT-nested PCR assay has been developed and standardised to simultaneously detect and identify the following viruses: human parainfluenza viruses types 1, 2, 3, and 4AB, the coronaviruses involved in respiratory infections in humans (types 229E and OC43), and a generic detection of human enteroviruses and rhinoviruses. The application of both multiplex assays to the analysis of the nucleic acid extract from a clinical specimen can therefore be used for simultaneous detection of the most common viral respiratory pathogens. The simultaneous use of both diagnostic tools will not only facilitate the undertaking of prophylactic measures but it will also provide early information on circulating epidemic respiratory viruses and will be useful to check the efficacy of future vaccines.

MATERIALS AND METHODS

Clinical Specimens

A total of 201 clinical specimens were used to validate the multiplex RT-nested PCR assay. These specimens were received for virological study at the Respiratory Virus laboratory, in the National Centre for Microbiology (CNM, Instituto de Salud Carlos III, Majadahonda, Madrid, Spain) and were as follows: nasopharyngeal aspirates from 147 children presenting acute respiratory syndromes as bronchiolitis, bronchial asthma, and pneumonia, or an influenza-like illness, were submitted from the Severo Ochoa Hospital, in Madrid, Spain; nasal washes from 32 immunosupressed patients by HIV, cancer, or organ transplantation were received for respiratory infection diagnosis purposes; and 22 nose and throat swabs from individuals presenting influenza-like illnesses were taken by physicians from the Comunidad de Madrid, Spain.

One hundred and eighty-five specimens of the total of 201 described above were selected because they were negative for influenza, RSV, and adenovirus detection using the multiplex RT-nested PCR assay previously described by Coiras et al. [2003]. Sixteen specimens were positive with the former multiplex assay and were used to check the specificity of the assay here described.

Viruses and Controls Preparation

Prototype strains of human parainfluenza viruses types 1, 2, 3, 4A, and 4B, coronaviruses, enteroviruses, and rhinoviruses, as indicated in Table I, were used as positive controls. Reference strains of enteroviruses and rhinoviruses were obtained from the American Type Culture Collection (ATCC, Manassas, VA).

To assess the sensitivity of each PCR run, additional controls were prepared in two dilutions that contained 10 and 100 molecules of cloned amplified product of each type of human parainfluenza viruses (1, 2, 3, 4A), coronavirus 229E, echovirus 30, and rhinovirus serotype 14. The pGEM-T Vector System I (Promega,

TABLE I. Prototype Strains of Human Parainfluenza Viruses, Coronaviruses, Enteroviruses, and Rhinoviruses Used as Positive Controls in the Multiplex RT-Nested PCR Assay

Viruses	Type	Strain	Viral culture
Parainfluenza	1	C-35	NCI-H292[a]
Parainfluenza	2	Greer	NCI-H292
Parainfluenza	3	C-243	NCI-H292
Parainfluenza	4A	M-25	NCI-H292
Parainfluenza	4B	19.153	NCI-H292
Coronavirus	229E	229E	HEF[b]
Coronavirus	OC43	OC43	—
Enterovirus	Echovirus 30	Bastianni	HEF
Rhinovirus	2	HGP	HEF
Rhinovirus	14	1059	HEF
Rhinovirus	89	41467 Gallo	HEF

[a]NCI-H292: human lung mucoepidermoid carcinoma cell line.
[b]HEF: human embryonic lung fibroblast cell line.

Madison, WI) and TOPO TA Cloning System (Invitrogen Corporation, San Diego, CA) were used for the cloning of PCR products. Transformation of each ligated product PCR-pGEM-T vector and -pCR2.1-TOPO vector was performed using JM109 high-efficiency competent cells and transforming one shot competent cells provided by the manufacturers, respectively. Selection of transformants was made on LB/ampicillin plates. The number of copies per tube was adjusted after measurement of the OD_{260} from plasmid DNA purified with Wizard Plus SV Miniprep DNA Purification System (Promega). Negative controls with RNAse-free sterile water (Sigma, St. Louis, MO) were included in each batch of test samples for checking up carryover contamination.

An internal control described by Coiras et al. [2003] was used for checking the extraction process, the amplification efficiency, and the presence of inhibitors in the clinical specimens. To control the retrotranscription reaction limit dilutions of RNA from human parainfluenza viruses types 1, 2, 3, and 4A, human echovirus 30, human rhinovirus 14, and human coronavirus 229E are included in each batch of test samples to detect any failure in the transcription reaction.

Primer Design and Preparation

Primers for human parainfluenza viruses were designed in a conserved region of the haemagglutinin genes; primers for coronaviruses were designed also in a conserved region of the spike protein genes; and primers for rhinoviruses and enteroviruses were designed in polyprotein gene, between 5′ non-coding region (5′-NCR) and VP4/VP2 regions. In all cases, a computer-assisted analysis of the sequences available in the public databases with MACAW 2.0.5 program (Multiple Alignment Construction and Analysis Workbench, NCBI, Bethesda, MA) was used. The GenBank accession numbers of the viral sequences used in primer design are shown in Table II. A total of 11 primers were used in the first amplification. Individual viruses (human parainfluenza viruses 1, 2, 3, 4A, and 4B, coronaviruses 229E and OC43), and the picornavirus group, as well as the internal control, were identified by a subsequent nested PCR using 12 primers. The criteria used in the design of the oligonucleotides were the range of sizes of the amplicons produced and the similarity of reaction kinetics. The sequences and properties of all primers are shown in Table III. Primers used to amplify the internal control were described in Coiras et al. [2003].

Nucleic Acid Extraction and Multiplex RT-Nested PCR Assay

Nucleic acids from either virus present in clinical specimens or in infected cell cultures were extracted from 200 μl of the sample using the guanidinium thiocyanate extraction method previously described by Casas et al. [1995]. Negative controls were treated following the same procedure. The lysis buffer included 100 molecules of the cloned amplified product of the internal control. After processing, the dried pellet was resuspended in 15 μl of RNAse-free sterile water (Sigma).

TABLE II. Representative Virus Sequences From the GenBank Database Used in Primer Design for the Multiplex RT-PCR and Nested PCR

Virus	Gene	Accession number
Parainfluenza 1	Haemagglutinin	AF016280
	Haemagglutinin	U70948
	Haemagglutinin	U70947
	Haemagglutinin	U70946
Parainfluenza 2	Haemagglutinin	AF039937
	Haemagglutinin	D00865
	Haemagglutinin	AF213352
	Haemagglutinin	X57559
Parainfluenza 3	Haemagglutinin	Z26523
	Haemagglutinin	M21649
	Haemagglutinin	M18761
	Haemagglutinin	M17641
Parainfluenza 4a	Haemagglutinin	M34033
	Haemagglutinin	E02727
Parainfluenza 4b	Haemagglutinin	AB006958
Coronavirus 229E	Spike protein	Y10052
	Spike protein	Y10051
Coronavirus OC43	Spike protein	Z21849
	Spike protein	Z32769
Enterovirus	5′ NCR-VP4/VP2	U22521
	5′ NCR-VP4/VP2	D00627
	5′ NCR-VP4/VP2	V01149
Rhinovirus	5′ NCR-VP4/VP2	X02316
	5′ NCR-VP4/VP2	X01087
	5′ NCR-VP4/VP2	M16248

A single step combined RT-PCR amplification reaction, henceforth called multiplex assay 2, was performed using the Promega Access RT-PCR system kit (Promega), which consisted in a PCR mixture containing 2 mM $MgSO_4$, 300 μM each of dATP, dGTP, dCTP, and dTTP, 20 pmol of specific primers for human parainfluenza viruses and coronaviruses, 10 pmol of specific primers for enteroviruses and rhinoviruses, 10 μl of 5× reaction buffer, 5 U of AMV reverse transcriptase (RT), and 5 U of *Tfl* DNA polymerase. A 5 μl aliquot of RNA/DNA extracts was added to a final volume of 50 μl. Amplifications were carried out into thin-walled reaction tubes (Sorenson, BioScience, UT) in a PTC-200 (Peltier Thermal Cycler, MJ Research, Watertown, MA). Samples were subjected to an initial cycle of 48°C for 45 min, and 94°C for 3 min. Cycling conditions of the PCR were 45 cycles: 94°C for 30 sec; 55°C for 1.5 min; 72°C for 1 min, and a final incubation of 72°C for 10 min.

The following nested PCR mixture of the multiplex assay 2 contained 2 mM $MgCl_2$ (Perkin Elmer, Branchburg, NJ), 200 μM each of dATP, dGTP, dCTP, and dTTP (Amersham Pharmacia Biotech, Piscataway, NJ), 20 pmol of specific primers for human parainfluenza viruses and coronaviruses, 10 pmol of specific primers for enteroviruses and rhinoviruses, 60 mM Tris-HCl (pH 8.5), 15 mM $(NH_4)_2SO_4$, and 1.25 U AmpliTaq DNA Polymerase (Perkin Elmer). A 2 μl aliquot from the first reaction was added to a final volume of 50 μl. Before PCR, samples were heated to 95°C for 4 min. Cycling

TABLE III. Properties of Primers for Human Parainfluenza Viruses (Parainf.), Coronaviruses, Enteroviruses (Enterov.), and Rhinoviruses (Rhinov.) Used in the First Round Multiplex RT-PCR and in the Following Nested PCR

Amplification steps and primer[a]	Sequence (5′ → 3′)	Gene	Gene position	Melting temp (°C)	G + C content (%)	Amplicon size (bp)
RT-PCR[a]						
1-PIV13	AGGWTGYSMRGATATAGGRAARTCATA	HA	Parainf. 1 (641-667) Parainf. 3 (635-661)	52–60	30–48	Parainf. 1 (635) Parainf. 3 (635)
2-PIV13	CTWGTATATATRTAGATCTTKTTRCCTAGT	HA	Parainf. 1 (1277-1248) Parainf. 3 (1270-1241)	52–56	23–33	
1-PIV2	TAATTCCTCTTAAAATTGACAGTATCGA	HA	Parainf. 2 (259-286)	53	29	Parainf. 2 (683) Parainf. 4AB (1070)
1-PIV4	ATCCAGARRGACGTCACATCAACTCAT	5′NCR-HA	Parainf. 4 (107-81)[c]	57–60	41–48	
2-PIV24	TRAGRCCMCCATAYAMRGGAAATA	HA	Parainf. 2 (942-919) Parainf. 4 (963-940)	49–59	29–54	
1-HcoV	TGTGCCATAGARGAYWTACTTTTT	SP	229E (2068-2090) OC43 (2727-2750)	49–52	29–38	229E (851) OC43 (806)
2-HcoV	AACCGCTTKYACCAKCAAYGCACA	SP	229E (2919-2896) OC43 (3533-3511)	54–61	42–58	
1-EV/RV	CTCCGGCCCCTGAATRYGGCTAA	5′NCR-VP4/VP2	Enterov. 445-467[d]	59–62	57–65	Enterov. (755) Rhinov. (639)
2-EV/RV	TCIGGIARYTTCCASYACCAICC	5′NCR-VP4/VP2	Rhinov. 1200-1178	53–64	43–68	
Nested[b]						
3-PIV13	ACGACAAYAGGAARTCATGYTCT	HA	Parainf. 1 (754-776) Parainf. 3 (748-770)	50–55	35–48	Parainf. 1 (439) Parainf. 3 (390)
4-PIV1	GACAACAATCTTTGGCCTATCAGATA	HA	Parainf. 1 (1193-1168)	55	38	
4-PIV3	GAGTTGACCATCCTYCTRTCTGAAAAC	HA	Parainf. 3 (1138-1112)	57–60	41–48	
3-PIV24	CYMAYGGRTGYAYTMGAATWCCATCATT	HA	Parainf. 2 (487-514) Parainf. 4 (509-536)	53–63	29–54	Parainf. 2 (297) Parainf. 4AB (174)
4-PIV2	GCTAGATCAGTTGTGGCATAATCT	HA	Parainf. 2 784-761	54	42	
4-PIV4	TGACTATRCTCGACYTTRAAATAAGG	HA	Parainf. 4 683-358	52–56	31–42	
3-HcoV	TTGTGCGCAATGTTATAAWGGYAT	SP	229E (2174–2197) OC43 (2831-2854)	51–52	33–38	229E (630) OC43 (587)
4-HcoV	GATAATRTGAGTRCCATTWCCACA	SP	229E (2804–2781) OC43 (3418–3696)	51–54	32–42	
3-EV/RV	ACCRASTACTTTGGGTRWCCGTG	5′ NCR-VP4/VP2	Enterov. 536–559[c]	55–59	48–57	Enterov. (226) Rhinov. (110)
4-EV/RV	CTGTGTTGAWACYTGAGCICCCA	5′ NCR-VP4/VP2	Rhinov. 762–743	55–59	48–57	

[a]1, forward; 2, reverse in first-round RT-PCR.
[b]3, forward; 4, reverse in nested PCR.
[c]Primer located upstream from coding region for haemagglutinin gene.
[d]Gene position referred to Poliovirus 1 strain Sabin (Accession no. V01150). Note: All rhinoviruses have a deletion of approximately 116 bp as regards enteroviruses.

conditions were 35 cycles: 94°C for 30 sec; 55°C for 1 min; 72°C for 30 sec, and a final incubation of 72°C for 10 min.

Other 5 μl aliquot of nucleic acid extracts was also added to a final volume of 50 μl of a RT-PCR reaction mixture to perform the multiplex RT-nested PCR assay described previously by Coiras et al. [2003], henceforth called multiplex assay 1. Thermal cycling conditions to perform this multiplex assay have been adapted to be used at the same time with the multiplex assay 2.

A 2 μl aliquot from the first reaction of multiplex assay 1 was added to a final volume of 50 μl of the nested PCR. Cycling conditions were identical to that of the multiplex assay 2 described above to perform both analysis at the same time in one thermalcycler.

Nested PCR products were analysed by electrophoresis on 3.5% Seakem agarose (FMC, Rockland, ME) gel containing 5 μg/ml of ethidium bromide in 1× Tris-borate buffer. The sizes of PCR products were 837 bp for internal control, 630 bp for coronavirus 229E, 587 bp for coronavirus OC43, 439 bp for parainfluenza virus type 1, 390 bp for parainfluenza virus type 3, 297 bp for parainfluenza virus type 2, 226 bp for enteroviruses (on average), 174 bp for parainfluenza virus type 4, and 110 bp for rhinoviruses (on average) (Table III, Fig. 1). Due to the high variability of the gene region amplified with nested primers from the enterovirus and rhinovirus genomes not all the fragment obtained has the same length. Regarding both groups of viruses, the nested fragment length could be from 200 to 232 bp for the enteroviruses and from 100 to 120 bp for the rhinoviruses.

The sizes of PCR products obtained with multiplex assay 1 were described previously in Coiras et al. [2003].

Confirmation of PCR Results

All samples were aliquoted at reception and those not used in the assays were stored at −70°C for later confirmation of PCR results. Positive results were considered valid when the PCR results matched in two different aliquots analysed in consecutive days. In addition, positive results for parainfluenza viruses were confirmed with the multiplex RT-PCR assay described previously by Aguilar et al. [2000], cell culture in human lung mucoepidermoid carcinoma (NCI-H292) cell line, and indirect immunofluorescence assay (IFA). Positive results for enteroviruses were confirmed with isolation in human embryonic fibroblasts (HEF) cell line and IFA; positive results for coronaviruses and rhinoviruses were confirmed with DNA sequencing. When discordant results between these assays were obtained, other aliquot of the same respiratory sample was analysed.

Prevention of PCR Contamination

Because of the high sensitivity of nested PCR, precautions must be taken to prevent contamination of reaction tubes with a product amplified previously, or target DNA from other specimens and controls. The preparation of reagents, processing of samples, aliquots of the respiratory specimens, and nested PCR assays were carried out in safety cabinets located in separate laboratories, all away from the area where amplified products were analysed. Each cabinet was equipped with an independent batch of reagents, micropipette sets, sterile reagent tubes, and filtered pipette tips.

Virus Isolation

All specimens were collected in 3 ml virus transport medium (MEM, Gibco BRL, Life Technologies, Paisley, Scotland; penicillin 200 U/ml, and streptomycin 200 μg/ml, BioWhittaker, Walkersville, MA; mycostatin 200 U/ml, Sigma, St. Louis, MI; bovine serum albumin 0.25%, Merck, Darmstadt, Germany).

Semi-confluent monolayers of NCI-H292 cells, Madin–Darby canine kidney (MDCK) cells, human laryngeal epidermoid carcinoma (HEp-2), and HEF cell line were used for primary viral isolation of human parainfluenza viruses, influenza viruses, and RSV, enteroviruses, rhinoviruses, and some coronaviruses. The monolayers were inoculated with 200 μl of homogenised samples and the adsorption was enhanced by centrifugation at 3,000 rpm for 30 min–1 hr. HEp-2 and HEF cell lines were maintained in Eagle's minimal essential medium supplemented with 2% fetal calf

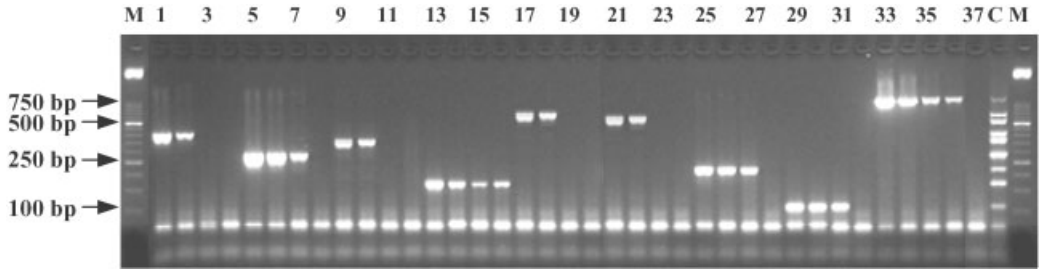

Fig. 1. Serial tenfold dilutions from 10^3 copies of the cloned amplified product of each virus and for the internal control, as follows: human parainfluenza type 1 (**lanes 1–4**), type 2 (**lanes 5–8**), type 3 (**lanes 9–12**), and type 4A (**lanes 13–16**) (C-35, Greer, C-243, and M-25, respectively); coronaviruses strain 229E (**lanes 17–20**) and OC43 (**lanes 21–24**); human echovirus 30 (**lanes 25–28**) and rhinovirus serotype 14 (**lanes 29–32**); internal control (**lanes 33–36**). **Lane 37**: water control; **lane C**, molecular size marker made-in-house (837 bp for internal control, 630 bp for coronavirus 229E, 587 bp for coronavirus OC43, 439 bp for parainfluenza type 1, 390 bp for parainfluenza type 3, 297 bp for parainfluenza type 2, 200–232 bp for enteroviruses, 174 bp for parainfluenza type 4, and 100–120 bp for rhinoviruses); **lanes M**, 50 bp ladder XIII Molecular Weight Marker (Roche Molecular Diagnostics).

serum, 200 UI/ml penicillin, and 0.1 mg/ml streptomycin. MDCK cells were also maintained in Eagle's minimal essential medium with penicillin–streptomycin but supplemented with 3 μg/ml of trypsin. NCI-H292 cells were maintained in Eagle's minimal essential medium supplemented with 1.5 μg/ml of trypsin.

Indirect Immunofluorescence Assay

Cells were collected and stained by standard methods [Kendal et al., 1982]. The monoclonal antibodies used for detection of fusion protein (F0/F1) of all strains of parainfluenza virus type 1, and haemagglutinin of all strains of parainfluenza viruses types 2 and 3, were obtained from Chemicon International Inc. (Temecula, CA). The monoclonal antibodies used for detection of enteroviruses were also obtained from Chemicon. The IFA was carried out with fluorescein isothiocyanate (FITC)-conjugate goat anti-mouse IgG (Sigma).

Statistical Analysis

Comparisons between the results obtained by the proposed multiplex RT-nested PCR assay and by isolation in cell culture, IFA, and alternative multiplex RT-PCR assay for human parainfluenza viruses, were performed by the computation of the percentage sensitivity and specificity [Griner et al., 1981]. Comparisons between these diagnostic assays were evaluated by McNemar's test.

RESULTS

Optimisation of Multiplex RT-PCR

The primers designed for the multiplex RT-nested PCR assay amplified the haemagglutinin genes of human parainfluenza viruses types 1, 2, 3, 4A, and 4B, the coronavirus spike protein genes, and partial 3′ end of 5′-NCR of *VP4* gene and partial 5′ end of *VP2* gene of enteroviruses and rhinoviruses. These primers were examined to ensure they all met the essential criteria for optimal PCR primers [Dieffenbach et al., 1993; Edwards and Gibbs, 1994; Henegariu et al., 1997] and to check they could be used together in a multiplex reaction under similar conditions for amplification. Primer annealing temperatures and concentrations were calculated empirically and standardised by experimentation. The G + C contents, melting temperatures, and lengths of the primers were chosen and analysed by using PrimerSelect v3.04a (DNAstar, Inc., Madin, WI). The primers were also tested for possible primer interactions and hairpins, and no significant theoretical mispriming was identified on any template. To test for possible palindrome sequences, all the primers used were aligned with the sequence databases of the National Centre for Biotechnology Information (NCBI) using the Basic Local Alignment Search Tool (BLAST).

All primers were designed to ensure that the final reaction products could be easily differentiated on the basis of their size. As shown in Table III, the primers selected were 23–30 bp and had G + C contents less than or equal to 68%, thus having an annealing temperature of 49–64°C. Thermocycling parameters (denaturation, annealing, and extension times) were systematically optimised. Annealing temperatures of 55°C in the first and second rounds of amplification, respectively, were selected to give maximum product yield and specificity (data not shown). The location of the oligonucleotide primers designed to amplify all these viruses allowed the typing of parainfluenza viruses into 1, 2, 3, and 4AB types, coronaviruses into serogroups 229E or OC43, and a generic detection of human enteroviruses and rhinoviruses.

As described above, the specific primers for simultaneous detection of enteroviruses and rhinoviruses have been designed on 5′-NCR and VP4/VP2 regions of the polyprotein gene. Descriptions of both sense primers for RT-PCR and nested PCR assays for specific amplification of a fragment of enteroviruses 5′-NCR and VP4/VP2 can be found [Arola et al., 1996; Huttunen et al., 1996]. However, to improve further differentiation by electrophoresis in those assays, we also included the corresponding antisense degenerated primers (see Table III), thus taking advantage of their different relative sizes. Likewise, primers to amplify the coronavirus spike protein were designed to produce fragments with 50 bp of difference between both serogroups 229E and OC43, in order to permit their identification by electrophoresis.

Specificity and Sensitivity of the Assay

The multiplex RT-nested PCR assay was tested for its specificity on all viral targets (Table I; Fig. 1). No mispriming was observed when all primer sets were mixed with either human parainfluenza viruses types 1, 2, 3, 4A, and 4B, coronaviruses types 229E and OC43, and enteroviruses and rhinoviruses template. A product of the expected size was obtained for each viral template in the presence of all the primers, the specific products being clearly separated and identified on a 3.5% Seakem agarose gel both for virus control material (cell culture-grown parainfluenza viruses, coronavirus 229E, enteroviruses and rhinoviruses, and tissue culture grown coronavirus OC43) and for clinical specimens containing wild type strains (Fig. 2).

The product specificities of the amplicons obtained were also confirmed by sequence analysis—especially for rhinoviruses and coronaviruses—due to the lack of easy and reliable methods of detection. As expected, the analysis of specimens containing other respiratory viruses, influenza, RSV, adenovirus, citomegalovirus, with the multiplex assay 2 showed no amplified product in any case (data not shown).

The sensitivity of detection of the human parainfluenza viruses, coronaviruses, enteroviruses, and rhinoviruses with the nested primer sets used individually was similar to that of the multiplex reaction. Sensitivity was evaluated by using serial tenfold dilution series of cloned PCR products obtained for each virus after first reaction. Detection levels of 1–10 molecules of cloned amplified product were achieved for parainfluenza virus

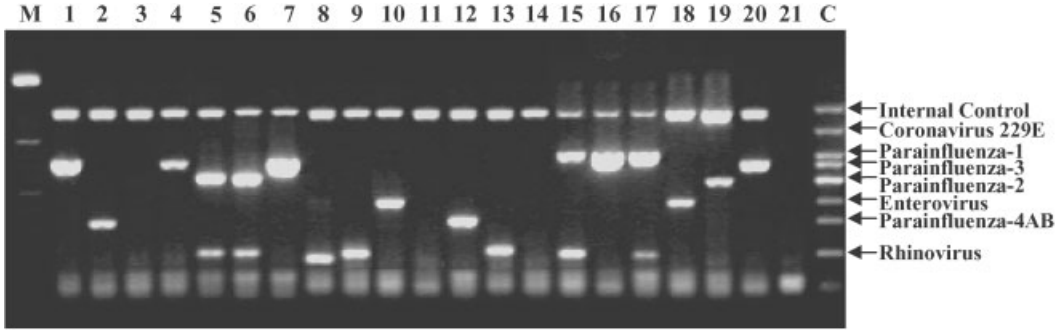

Fig. 2. Analysis of a panel of clinical specimens by multiplex RT-nested PCR assay. **Lanes 1–20**: Combined nose and throat swabs, and nasopharyngeal aspirates; lanes 3, 11, and 14, negative samples; lanes 1, 4, 7, and 20, parainfluenza type 3; lanes 2 and 12, parainfluenza type 4; lanes 5, 6, and 19, parainfluenza type 2, coinfected with rhinovirus in lanes 5 and 6; lanes 8, 9, and 13, rhinovirus; lanes 10 and 18, enteroviruses; lanes 15–17, parainfluenza type 1, and simultaneous coinfection with rhinovirus in lanes 15 and 17; **lane 21**, water control. **Lane M**: 50 bp ladder XIII Molecular Weight Marker (Roche Molecular Diagnostics); **lane C**, molecular size marker made-in-house (837 bp for internal control, 630 bp for coronavirus 229E, 587 bp for coronavirus OC43, 439 bp for parainfluenza type 1, 390 bp for parainfluenza type 3, 297 bp for parainfluenza type 2, 200–232 bp for enteroviruses, 174 bp for parainfluenza type 4, and 100–120 bp for rhinoviruses).

type 2 (Fig. 1, lanes 5–8), parainfluenza virus type 4A (lanes 13–16), echovirus 30 (lanes 25–28), and rhinovirus serotype 14 (lanes 29–32). For parainfluenza viruses types 1 and 3 (Fig. 1, lanes 1–4 and 9–12, respectively), and both human coronavirus strains (lanes 17–24), the limits of detection were 10–100 molecules of cloned amplified product. In addition, a comparison of the results obtained in the multiplex assay with those obtained in virus isolation in cell culture using tenfold dilutions of reference strains was made to evaluate the sensitivity of both assays. The sensitivities obtained were as follows: 0.001 50% tissue culture infective dose ($TCID_{50}$) for parainfluenza viruses types 1 and 3, and echovirus 30, and 0.0001 $TCID_{50}$ for parainfluenza viruses types 2 and 4AB, and rhinovirus serotype 14 (data not shown).

Evaluation of the Assay Using Respiratory Specimens

The multiplex RT-nested PCR assay was evaluated using a panel of 201 combined nose and throat swabs and nasopharyngeal aspirates obtained from the 1994–2003 seasons. The majority of the specimens were obtained from infants under two years of age, although all ages were represented in this study (Table IV). Results showed that most of the specimens were infected with parainfluenza virus type 3 and/or any rhinovirus type, and these samples came from children under 24 months. The nucleic acids were extracted for cDNA synthesis and an independent aliquot was taken for IFA and isolation in cell cultures, which were set up in the same day. The synthesis and amplification of cDNA were performed by the multiplex assay 1 for detection of influenza A, B, and C viruses, RSV types A and B, and a generic detection of adenoviruses, and also by the multiplex assay 2 described in this work. Most of specimens (185/201) were selected because they were negative by the multiplex assay 1, and the rest (16/201) were positive for influenza B virus, RSV types A and B, and adenovirus. These last samples were used to confirm the specificity of the multiplex assay and also to detect the possibility of coinfection.

Respiratory syndromes and laboratory results obtained by the different techniques used in this study are shown in Table IV. Detection of infective viruses in cell cultures and/or detection of antigens by IFA provided 40 positive results (19.9%, 40/201) distributed as follows: 24 (11.9%, 24/201) were positive for parainfluenza viruses, one (0.5%, 1/201) was positive for coronaviruses (detected by cell culture), two (1%, 2/201) were positive for enteroviruses, and two (1%, 2/201) were positive for rhinoviruses (detected by cell culture but not identified by conventional methods). Ten specimens (5%,10/201) were positive for other respiratory viruses (influenza B virus, RSV type B, and adenovirus). In one specimen (0.5%, 1/201) coinfection by parainfluenza virus and RSV was detected. No virus was detected in 161 (80.1%, 161/201) specimens.

All the specimens positive by IFA and isolation in cell culture were also confirmed by the multiplex assay 2. Additionally, this new approach was able to detect 63 positive specimens more (31.3%, 63/201): parainfluenza virus type 2 in 2 additional specimens (1%, 2/201), parainfluenza virus type 3 in 9 (4.4%, 9/201), and parainfluenza virus type 4 in 5 additional specimens (2.5%, 5/201). Additional 43 rhinoviruses (21.4%, 43/201) and 4 enteroviruses (2%, 4/201) were also detected only by the multiplex assay 2 and confirmed by sequencing of nested PCR fragment. In four of these specimens the presence of virus was detected by growing in cell culture, but not by IFA. The multiplex assay 2 confirmed that one specimen was infected with enterovirus and the other three with rhinoviruses. All the positive results for parainfluenza viruses (7.9%, 16/201) were confirmed by an alternative multiplex RT-nested PCR developed for detection of all parainfluenza viruses types [Aguilar et al., 2000]. In addition, one specimen infected with a virus that grew in cell culture but could not be identified, was also detected by multiplex assay

TABLE IV. Results Obtained in the Analysis of Respiratory Specimens of Patients Presenting Respiratory Illnesses

Age range	Number of specimens tested	Number of specimens positive for one virus with all methods (%)	Parainfluenza detected by					Coronaviruses detected by			Enteroviruses detected by		Rhinoviruses detected by		Other viruses detected by	
			IFA/CC and both RT-PCRs	Parainfluenza and multiplex RT-PCRs (%)				CC and multiplex RT-PCR	Multiplex RT-PCR (%)		IFA/CC and multiplex RT-PCR	Multiplex RT-PCR (%)	CC and multiplex RT-PCR	Multiplex RT-PCR (%)	IFA/CC and RT-PCR[a]	Multiplex RT-PCR[a] (%)
				1	2	3	4		229E	OC43						
0–6 m	65 (32.3)	33 (16.4)	11	0	1 (0.5)	14 (6.9)	2 (1.0)	1	1	0	1	4 (2.0)	1	8 (3.9)	4	4 (2.0)
7–12 m	47 (23.3)	28 (13.9)	8	4 (2.0)	1 (0.5)	3 (1.5)	3 (1.5)	0	0	0	0	0	1	16 (8.0)	1	1 (0.5)
13–24 m	39 (19.3)	24 (11.9)	5	0	2 (1.0)	6 (3.0)	0	0	0	0	0	0	0	13 (6.5)	2	2 (1.0)
>2–10 y	14 (6.9)	7 (3.5)	0	0	1 (0.5)	0	1 (0.5)	0	0	0	1	2 (1.0)	0	2 (1.0)	1	1 (0.5)
11–20 y	8 (3.9)	3 (1.5)	0	0	0	0	1 (0.5)	0	0	0	0	0	0	0	2	2 (1.0)
21–40 y	11 (5.4)	2 (1.0)	0	0	0	0	0	0	0	0	0	0	0	2 (1.0)	0	0
>40 y	14 (6.9)	3 (1.5)	0	0	0	1 (0.5)	0	0	0	0	0	0	0	2 (1.0)	0	0
Unknown	4 (2.0)	2 (1.0)	0	0	0	0	0	0	0	0	0	0	0	2 (1.0)	0	0
Total	201 (100)	102 (50.7)	24	4 (2.0)	5 (2.5)	24 (11.9)	7 (3.5)	1	1 (0.5)	0	2	6 (3.0)	2	45 (22.4)	10	10 (5.0)

[a]Multiplex RT-nested PCR assay previously described [Coiras et al., 2003] to simultaneous detection of influenza A, B, and C viruses, RSV types A and B, and a generic detection of adenovirus. Specimens infected only with one virus (parainfluenza, coronavirus, rhinovirus, enterovirus, or other respiratory virus) are grouped according to the type of assay used in the analyses. m, month; y, year; IFA/CC, immunofluorescence assay/cell culture; RT-PCR, reverse transcription-polymerase chain reaction.

2 and identified as a coronavirus. Sequencing of the fragment obtained in nested PCR confirmed its similarity to the 229E strain. In 18 specimens (8.9%, 18/201), coinfection by two different viruses was determined. In addition, no virus was detected in a total of 80 (39.8%, 80/201) specimens.

Of the 201 specimens assayed by multiplex assay 1, 3 (1.5%) were positive for RSV type A (see Table IV), 3 (1.5%) for RSV type B, 2 (1%) for adenovirus, 2 (1%) for influenza B, and no virus was detected in 185 (92%) specimens.

In total, 121 specimens (60.2%) from the panel of 201 were positive for some virus by combination of all methods, and 80 (39.8%) specimens were negative. In these samples, no detectable PCR products were seen, although interestingly a citomegalovirus from one specimen was grown in cell cultures.

The percentage of sensitivity and specificity of the developed multiplex RT-PCR was computed as previously described [Griner et al., 1981]. Comparison between the multiplex 2 assay results and the detection by IFA and cell culture showed the new multiplex RT-nested PCR assay to be far more sensitive than conventional methods ($P < 0.000$ [McNemar's test]) and that it absolutely agrees with the alternative multiplex RT-PCR method assayed.

Detection of Dual Infections

The ability of the multiplex reaction mixture to detect the presence of more than one virus template in the same starting material was assessed by the preparation of various combinations of viral templates (data not shown). Detection capabilities were further improved by combination of multiplex assays 1 and 2. Multiplex reaction 2 was able to detect all eight different viruses, as well as the internal control (Fig. 2, lane C). Multiple respiratory viruses were observed in 19 specimens (see Table V): RSV and enterovirus in one specimen, RSV and parainfluenza virus type 3 in one specimen, RSV and parainfluenza virus type 4 in two specimens (all parainfluenza viruses were detected by the multiplex RT-PCR assay, but only RSV could be isolated in cell culture and detected by IFA). Adenovirus and parainfluenza virus type 3 were detected in two specimens but only in one of them could the adenovirus be isolated; parainfluenza virus, however, could not be detected by cell culture or IFA. Rhinoviruses were detected with parainfluenza virus type 1 in two specimens (both parainfluenza viruses confirmed by viral culture), with parainfluenza virus type 2 in four specimens (only in two specimens the parainfluenza viruses were detected by viral culture), with parainfluenza virus type 3 in five specimens (only in three specimens the parainfluenza viruses were confirmed by viral culture), and with parainfluenza virus type 4 in one specimen (no parainfluenza virus could be confirmed by cell culture or IFA). All parainfluenza viruses were confirmed by the multiplex assay for human parainfluenza viruses [Aguilar et al., 2000]. Interestingly, coinfection with two different

TABLE V. Viruses Identified in Mixed Infections

Viruses identified in mixed infections	Number of occurrences in mixed infections	Number of occurrences with		
		RSV	Adenovirus	Rhinovirus
RSV	4	—	—	—
Adenovirus	2	—	—	—
Rhinoviruses	13	—	—	1
Enteroviruses	1	1	—	—
Parainfluenza 1	2	—	—	2
Parainfluenza 2	4	—	—	4
Parainfluenza 3	8	1	2	5
Parainfluenza 4	3	2	—	1

rhinoviruses was found in one specimen. This coinfection was detected because both rhinoviruses showed a different PCR product size by electrophoresis in agarose gel (see above, Materials and Methods). It was confirmed by sequencing of both fragments obtained in nested PCR. Their typing was not possible due to nonavailability of public sequences for most rhinoviruses.

DISCUSSION

The circulation of different respiratory viruses during the same period of the year makes very complex to elucidate their individual contributions to the global respiratory disease. Moreover, the pattern of the respiratory virus activity seems to change within the different age groups or among the patients enclosed on the high-risk groups. Therefore, rapid and accurate etiological diagnosis of viral infections is essential for choosing adequate antiviral therapy and preventing nosocomial spread or opportunistic secondary infections. These control measures would undoubtedly reduce hospital stay, treatment costs, and unnecessary use of antibiotics [Woo et al., 1997]. Furthermore, the addition of this assay to the routine diagnosis would be helpful to define the role of every respiratory virus in relation to different population groups and therefore to perform epidemiological studies.

A variety of diagnostic tests are available currently for detection of one or some of the most important viral respiratory pathogens [Gilbert et al., 1996; Freymuth et al., 1997; Valassina et al., 1997; Eugene-Ruellan et al., 1998; Osiowy, 1998; Gröndahl et al., 1999; Aguilar et al., 2000; etc]. A commercially available multiplex RT-PCR assay for detection of respiratory viruses was described by Fan et al. [1998]: prodesse hexaplex assay to detect simultaneously RSV types A and B, influenza A and B viruses, and parainfluenza viruses types 1, 2, and 3. However, it does not detect adenovirus, important respiratory tract pathogens [Kehl et al., 2001]; rhinoviruses or coronaviruses, which constitute both the most common upper respiratory tract pathogens and could cause serious complications [Andréoletti et al., 2000; Arbour et al., 2000]; or parainfluenza virus type 4, apparently more frequent and virulent than had been described [García García et al., 2002]. Besides, while an Hexaplex assay is relatively rapid and more sensitive than currently available direct techniques, it is laborious and costly. Moreover, viruses such as enteroviruses, rhinoviruses, or coronaviruses are repeatedly found in clinical specimens from patients with respiratory infections but its role on them is not well defined, because it is difficult to detect them with current methods.

In this paper, a new multiplex RT-nested PCR assay that is able to identify simultaneously eight different viruses of the respiratory tract is described and evaluated. Primers were designed in conserved regions of the haemagglutinin genes of the parainfluenza viruses types 1, 2, 3, 4A, and 4B, the spike protein genes of the coronaviruses 229E and OC43 genomes, and the partial 5′-NCR of *VP2* gene of rhinoviruses and enteroviruses genome. The sensitivity of detection of parainfluenza viruses, coronaviruses, rhinoviruses, and enteroviruses with the nested primer sets used in multiplex reaction was tested. Detection levels of 1–10 molecules of cloned-DNA of parainfluenza viruses type 2 and 4A, rhinovirus serotype 14, and echovirus 30, and 10–100 molecules of cloned-DNA of parainfluenza viruses 1 and 3, and coronavirus 229E, were achieved (Fig. 1). Additionally, 0.001 $TCID_{50}$ for parainfluenza viruses types 1 and 3, and echovirus 30, and 0.0001 $TCID_{50}$ for parainfluenza viruses types 2 and 4AB, and rhinovirus serotype 14, were detected (data not shown). Cross-reactions with other respiratory viruses such as influenza, RSV, adenovirus, or citomegalovirus were not found, demonstrating the specificity of the assay.

To detect as much respiratory viruses as possible, it was proposed to analyse the clinical specimens using the multiplex assay described above (multiplex assay 2) and the one described by Coiras et al. [2003] (multiplex assay 1). Using both multiplex RT-nested PCR assays, it is possible to detect simultaneously and identify fourteen respiratory viruses. The test requires approximately 10 hr of processing time and is capable of screening simultaneously for a high quantity of clinical specimens instead of performing different assays to detect and type each of them separately. Therefore, it decreases costs and increases the throughput of specimens. The simultaneous use of both multiplex assays is the first step to perform epidemiological studies to determine how frequently every pathogen could cause serious complications in children under 2 years old, elderly people, and

immunocompromised patients. Furthermore, its accurate diagnosis would effectively prevent unnecessary antibiotic treatment.

Two hundred and one specimens from 1994 to 2003 seasons were used to validate the new multiplex assay (see Fig. 2). Most of them had been frozen at −70°C for several years, and the majority were obtained from children under 2 years of age (see Table IV). All the specimens were initially tested in parallel with the multiplex assay 1, IFA, and/or isolation in cell culture. Fifty-six specimens were positive with one or all these methods: sixteen (8%, 16/201) were positive with the multiplex assay 1 and IFA or isolation in cell culture; and 40 (19.9%, 40/201) specimens were positive only with IFA or isolation in cell culture because they presented some different virus from those that can be detected with multiplex assay 1. Among them, one (0.5%, 1/201) was coinfected with parainfluenza viruses and RSV. Therefore, there were 145 (72.1%, 145/201) specimens that were negative with all these methods.

The multiplex assay 2 detected the presence of respiratory viruses in 63/201 (31.3%) additional specimens, which were negative or could not be evaluated by IFA and viral isolation (see Table IV). These differences could be due not only to the limited sensitivity of these conventional methods, but also because nose and throat swabs are not the optimal clinical samples for detection of viruses by IFA compared to nasopharyngeal aspirates [Ellis et al., 1997]. In case of isolation in cell cultures, the loss of infectivity could be related to different factors such as deficient transport and/or conservation of the samples or the concomitant microbial contamination of the clinical specimens. In total, 121/201 (60.2%) specimens were positive with all methods used, and among them 19/201 (9.5%) were coinfected with two different viruses (see Tables IV and V).

Therefore, although IFA has been used mostly for diagnosis of respiratory infection caused by parainfluenza viruses types 1, 2, and 3, and virus isolation has been considered the "gold standard" for parainfluenza viruses detection, they are sometimes unable to confirm the positive specimens for parainfluenza viruses obtained using the multiplex assay 2. In an attempt to determine if the viral culture- and IFA-negative, multiplex assay-positive specimens for parainfluenza viruses were proper positive, the multiplex assay described by Aguilar et al. [2000] was used to confirm all positive results. In case of enteroviruses, the "gold standard" to detect them is viral culture followed by IFA identification to confirm the cytopatic effect obtained. However, the diagnosis of rhinoviruses respiratory tract infections is limited because the poor sensitivity of cell cultures and the necessity of confirmation using an acid sensitivity assay. Serologic diagnosis is virtually impossible as well, due to the abundance of rhinoviruses serotypes [Hyypia et al., 1998]. As infections caused by rhinoviruses are among the most common infectious diseases worldwide it is important to have a reliable diagnostic tool capable of detecting their presence in clinical specimens. The relevance of the molecular assays—such as the RT-PCR used to detect both rhinoviruses and enteroviruses in nasopharyngeal aspirates from infants with respiratory tract infection—has already been demonstrated [Atmar and Georghiou, 1993; Johnson et al., 1993; Halonen et al., 1995]. The present study incorporates the detection of rhinoviruses and enteroviruses in a RT-PCR assay that also allows the identification of parainfluenza viruses and coronaviruses. In fact, it was found that 28.8% of respiratory infections within the 201 specimens used to validate the multiplex assay had been caused by rhinoviruses.

On the other hand, it has been described that coronaviruses antibodies rose against coronaviruses 229E strains (serogroup 1) could not be protective against coronaviruses OC43 strains (serogroup 2) and vice versa [Macnaughton, 1982]. For this reason, to perform epidemiological studies it is important to distinguish between both serogroups. Therefore, primers to detect the presence of coronaviruses in respiratory specimens were also included in the mixture reaction. Nevertheless, although it is described that coronaviruses produces 10% of upper respiratory tract infections, only one specimen of 201 tested (0.5%) was infected with coronavirus 229E-like. The underdiagnosis of the respiratory infections caused by coronaviruses in this study could be due to the fact that most of the specimens analysed belonged to children under two years old with severe lower respiratory tract infectious disease.

The number of dual infections found in the validation of the new multiplex assay was high: 19/201 (9.5%), and definitively most of them could not be detected by other assays. It is likely that dual infections play a greater role in disease than described previously [Kehl et al., 2001]. Most of the respiratory infections observed with both multiplex assays 1 and 2 were confirmed by other methods. RSV was found in one specimen with enterovirus and in three specimens with parainfluenza viruses types 3 and 4; adenoviruses were found in two specimens with parainfluenza virus type 3; and 13 coinfections of rhinoviruses with all types of parainfluenza viruses and even with other type of rhinoviruses have also been detected (see Table V). As the clinical aspects of distinct viral respiratory infections are indistinguishable [Yun et al., 1995; Garcia Garcia et al., 2001], a diagnostic tool able to detect dual infections in small quantities of clinical specimens is extremely useful to evaluate the clinical consequences of simultaneous presence of several pathogens in the respiratory tract. In that sense, the analysis of simulated clinical samples and original specimens reveals both multiplex RT-nested PCR assays to be clearly capable of simultaneously detecting the presence of fourteen viruses at both high and low copy numbers. Other detection methods, such as IFA or viral isolation are less adequate because although IFA can reveal the presence of influenza A and B viruses, RSV, parainfluenza viruses, and their antigens in epithelial cells from the clinical specimens, it is not widely used in the diagnosis of adenovirus, coronaviruses, enteroviruses, and rhinoviruses.

Neither the isolation in cell culture is appropriated to detect coinfections because the most virulent virus can destroy the cell monolayer before the slower growth of the other virus could be evident. It would make difficult to identify the coinfection of a cell culture by some respiratory viruses.

A comparison of the new multiplex RT-nested PCR assay with conventional methods such as viral isolation in cell culture and viral antigen detection by IFA, or the individual RT-PCR assay for parainfluenza viruses detection, was performed. The statistical analysis of the results proved that the new multiplex assay was more sensitive than conventional methods ($P < 0.000$), and presented the same sensitivity as the RT-PCR assay for parainfluenza viruses detection. Accordingly, detection of respiratory pathogens by molecular methods proved to be more sensitive than conventional viral culture or IFA methods, with the advantage that all viruses can be tested at the same time with a single technique. These findings are consistent with those of other studies reported previously [Tantivanich et al., 1995; Atmar et al., 1996; Gilbert et al., 1996; Freymuth et al., 1997; Liolios et al., 2001]. Additionally, the combination of RT-PCR assay with a nested PCR potentially increases the sensitivity of the whole approach. For this reason, both multiplex assays 1 and 2 detected all viral culture—and IFA-positive clinical specimens and additional positive samples, which would otherwise have been missed by conventional methods.

Nevertheless, it is necessary to consider the potential problems inherent to PCR, such as false negatives due to reaction failure or false positives caused by contamination, which appeared as unexpected bands in the negative controls. Complete PCR failure that presumes a false negative can be distinguished from a non-amplification result by adding a control template non-related with the target sequence [Ballabio et al., 1990; Edwards and Gibbs, 1994]. Thus, the addition of 100 copies of the internal control in every reaction tube ensured that the multiplex assay was accurately working.

In conclusion, the testing of respiratory specimens using the multiplex RT-nested PCR assay described previously by Coiras et al. [2003] with that described above permits the simultaneous detection and identification of fourteen respiratory viral pathogens in clinical specimens. Both multiplex assays constitute a more rapid, sensitive, specific, and less expensive alternative to conventional methods, avoiding the application of many different assays to analyse one specimen for fourteen different respiratory viruses. They can improve the rapid diagnosis of respiratory diseases, and accordingly reduce nosocomial transmission, limit unnecessary antibiotic use, and improve clinical assistance as a result of an appropriated therapy following accurate diagnosis of viral infections that produce similar clinical symptoms. They also could be applied for epidemiological studies and surveillance of respiratory viruses involves. Because of their ability to detect dual infections and identify the pathogens implied, the assays can be used to determine their importance to produce serious complications in some groups of patients. The results presented above also indicate that such multiplex approaches have great potential for the detection of groups of pathogens causing clusters of diseases with similar signs and symptoms.

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