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Development of three multiplex RT-PCR assays for the detection of 12 respiratory RNA viruses

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Abstract

Three multiplex hemi-nested RT-PCR assays were developed to detect simultaneously 12 RNA respiratory viruses: influenza viruses A, B and C, human respiratory syncytial virus (hRSV), human metapneumovirus (hMPV), parainfluenza virus types 1–4 (PIV-1, -2, -3 and -4), human coronavirus OC43 and 229E (HCoV) and rhinovirus (hRV). An internal amplification control was included in one of the RT-PCR assays. The RT-PCR multiplex 1 and the hemi-nested multiplex 1 detected 1 and 0.1 TCID50 of RSV A, respectively, and 0.01 and 0.001 TCID50 of influenza virus A/H3N2, respectively. Two hundred and three nasal aspirates from hospitalised children were retrospectively tested in comparison with two conventional methods: direct immunofluorescence assay and viral isolation technique. Almost all samples (89/91) that were positive by immunofluorescence assay and/or viral isolation technique were detected by the multiplex assay. This method also detected an additional 85 viruses and 33 co-infections. The overall sensitivity (98%), rapidity and enhanced efficiency of these multiplex hemi-nested RT-PCR assays suggest that they would be a significant improvement over conventional methods for the detection of a broad spectrum of respiratory viruses.

Keywords: Multiplex RT-PCR assay; Respiratory syncytial virus; Influenza virus; Parainfluenza virus; Human metapneumovirus; Rhinovirus; Human coronavirus

1. Introduction

Human respiratory tract infections are caused by numerous viruses, including influenza viruses A, B and C, parainfluenza viruses 1–4 (PIV-1, -2, -3 and -4), human respiratory syncytial virus (hRSV), human metapneumovirus (hMPV), human coronaviruses OC43 and 229E (HCoV), human rhinoviruses (hRV), adenoviruses and some human enteroviruses (hEV).

The direct diagnosis of such viral respiratory infections is based on the use of conventional methods such as isolation by cell culture and antigenic detection (Gardner and McQuillin, 1968). Even though these methods are effective and often complementary, they have some disadvantages. Cell culture, often considered to be the gold standard, is delicate and sometimes too slow for it to be useful for diagnosis. Antigenic detection is sometimes insufficiently sensitive or specific. Even when these methods are combined, some samples remain negative even though there is clinical or epidemiological evidence of viral respiratory infection (Freymuth et al., 1995).

The detection of respiratory viruses can be improved by using molecular biology techniques. Numerous studies have developed and evaluated PCR- or RT-PCR-based methods for the detection and typing of respiratory viruses (Donofrio et al., 1992; Eugene-Ruellan et al., 1998; Freymuth et al., 1997; Gilbert et al., 1996). Given the number of respiratory viruses and the fact that clinical and virological results are not always the same, multiplex RT-PCR methods have been developed

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with the aim of providing a tool capable of detecting an increasingly complete panel of viruses (Aguilar et al., 2000; Coiras et al., 2003, 2004; Echevarria et al., 1998; Fan et al., 1998; Grondahl et al., 1999; Osiowy, 1998; Templeton et al., 2004).

Here, we describe the development and evaluation of three multiplex RT-PCR methods for the detection of RNA viruses involved in respiratory diseases.

2. Materials and methods

2.1. Respiratory specimens and virus strains

This retrospective study tested 203 nasal aspirates from children hospitalised in paediatric units of the University Hospital of Caen and Flers Hospital between October 2002 and March 2003. Each nasal aspirate was collected in 4 ml of viral transport medium (Eugene-Ruellan et al., 1998) and a 2 ml aliquot was frozen at −80 °C. Two groups of specimens, positive and negative, were assessed.

The first group included 111 specimens positive for a respiratory virus, selected by chronological order. Ninety-one contained viruses detected by conventional methods: direct fluorescence assay and viral isolation technique. The number of samples has been limited, according to the representativeness of the most frequent viruses in respiratory diseases. This group comprised 30 samples positive for hRSV, 20 for influenza A virus, 10 for influenza B virus, 15 for hRV, 1 for PIV-1, 3 for PIV-2, 10 PIV-3 and 2 for HCoV OC43. Twenty samples positive for hMPV detected by a specific PCR (Freymuth et al., 2003a, b) were included in this group. No influenza C virus, HCoV 229E, or PIV-4 were identified during the period of study. The negative group included 92 randomly selected clinical specimens not found to contain any viruses according to conventional methods.

The following reference strains were used as positive controls to determine the sensitivity and specificity of our methods: hRV-31, ATCC VR-506; influenza C virus: C/Paris/145/91; influenza A virus: A/H3N2/Panama/2007/99; influenza B virus: B/Victoria/1987; hRSV A (ATCC VR-26); hMPV Canada/S29; HCoV OC43 (ATCC VR-759); HCoV 229E (ATCC VR-740); PIV-1 Sendaï 431.E72; PIV-2°: Lyon/26632/97; PIV-4: Lyon/154/01.

2.2. Isolation and identification of viruses by immunofluorescence assay and viral isolation technique

Immonofluorescence assay was used to detect viruses as previously described (Freymuth et al., 1987), using fluorescein-conjugated monoclonal antibodies directed against influenza viruses A and B, hRSV, PIV-1, -2, -3 and adenovirus (Ad) (IMAGEN©; Dako Diagnostics). Slides were examined under a microscope using a hemi-quantitative method.

For viral isolation, embryonic lung fibroblasts (MRC5) were cultured in 25-cm^2 flasks. They were then inoculated with 0.25 ml of each sample, incubated at 35–36 °C and observed for cytopathology during 4 weeks. Ad, hRSV and some hRV strains are likely to replicate in this cellular type. When samples were negative in immunofluorescence assay, we also attempted to isolate them using HuH7 cells (Nakabayashi et al., 1977) that had been grown in 48-well tissue culture plates, as described previously (Vabret et al., 2001). After 4 days of incubation, cultures were examined for cytopathogenic effects and the cells were scraped and tested by immunofluorescence assay. When immunofluorescence assay was negative but the culture was positive, PCR specific for hRV (Savolainen et al., 2002), HcoV 229E and HcoV OC43 (Vabret et al., 2001) were carried out using culture supernatants.

2.3. RNA preparation

RNA was extracted from 140 μl of each sample, using a commercial reagent (QIAamp viral RNA mini kit®, Qiagen). Whenever possible, the extracts were tested immediately after extraction. If this was not possible, they were divided into aliquots and kept frozen at −80 °C. Each aliquot was used only once to avoid the loss of viral genomic material during repetitive freezing and thawing.

2.4. Multiplex RT-PCR

Three multiplex RT-PCR methods, targeting 12 respiratory viruses, were developed (Fig. 1). Each multiplex method detected four viruses: influenza viruses A, B, hMPV (A and B) and hRSV (A and B) for multiplex 1; PIV-1, -2, -3 and -4 (A and B) for multiplex 2; hRV, influenza C virus, HCoV OC43 and 229E for multiplex 3. An internal control was included in multiplex 2 to check the extraction step and the presence of inhibitors of the RT-PCR assay. This control consisted of glyceraldehyde-3-phosphate dehydrogenase (GAPDH) gene, which is normally transcribed in nasal mucosis cells. This gene was amplified with specific primers (Table 1).

Primers targeted specifically the haemagglutinin neuraminidase genes of PIV-1, -2 (Echevarria et al., 1998) and -3 (Karron et al., 1994), the phosphoprotein gene of VIP-4A and -4B (Aguilar et al., 2000), the nucleocapsid gene of hRSV sub-groups A and B (Cane and Pringle, 1991; Freymuth et al., 1995), the matrix protein genes of influenza viruses A and B (Donofrio et al., 1992), the matrix protein gene of hMPV (this study), the haemagglutinin-esterase gene of influenza C virus (Zhang and Evans, 1991), the M gene of OC43 and 229E (Vabret et al., 2001) and the VP4/VP2 and hypervariable region in the 5′-non-coding region of hRV (Savolainen et al., 2002). The sequences of the primers, as well as their annealing temperatures and amplicon sizes are given in Table 1.

Positive controls were included in each multiplex RT-PCR. These consisted of four RNAs extracted from virus-

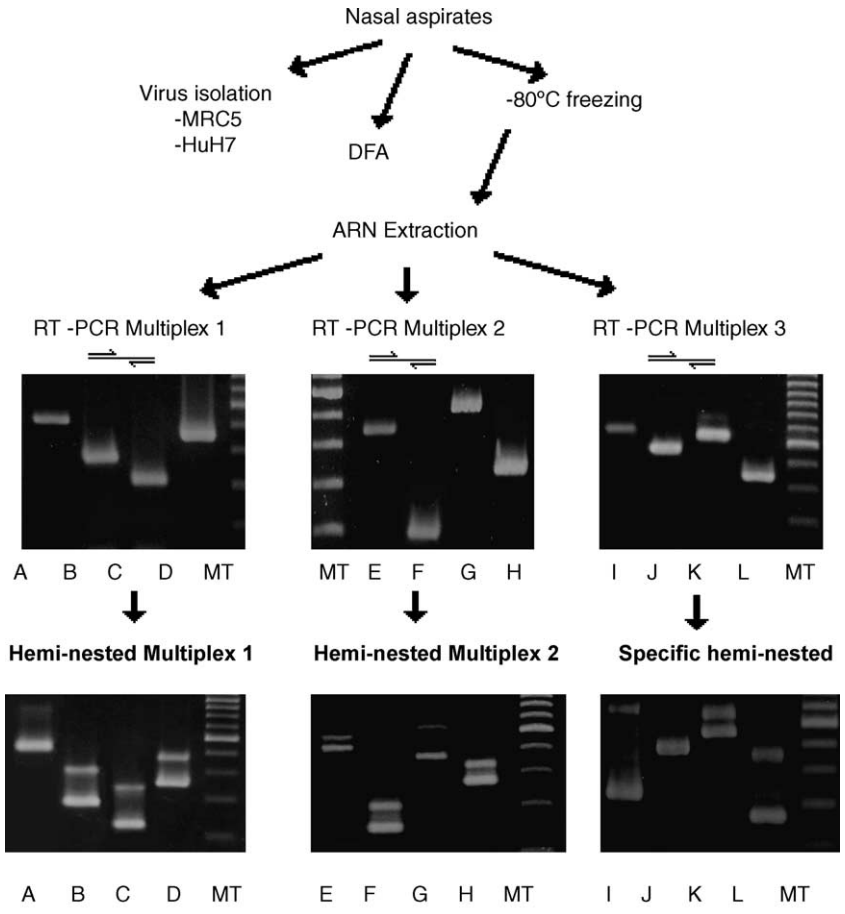

Fig. 1. Procedure and results of the multiplex RT-PCR and hemi-nested PCR.

infected cells and mixed together. For example, the multiplex 1 positive control was a mixture of influenza A and B RNA, hRSV RNA and hMPV RNA. As a negative control, H_2O was used instead of nucleic acid.

Each multiplex RT-PCR was a single-step combined RT-PCR amplification, performed using the one-step RT-PCR kit from QIAGEN. The reaction mixture contained 5 μl of 5× RT-PCR buffer (2.5 mM $MgCl_2$), 0.4 mM dATP, dGTP, dCTP and dTTP, 0.5 μM of each of the 8 primers (10 primers in the multiplex 2 for the negative group) and 1 μl of enzyme mix. A 2.5 μl aliquot of RNA extract was added to give a final volume of 25 μl. The cycling conditions for the three RT-PCRs were: an initial cycle at 50 °C for 30 min and 94 °C for 15 min; followed by 40 cycles at 94 °C for 30 s, 55 °C (58 °C for multiplex 3) for 30 s and 72 °C for 1 min; and a final incubation at 72 °C for 10 min.

The Q-solution provided in the kit was used for multiplexes 1 and 3 (3 μl/reaction). Our preliminary assays showed that Q-solution was not necessary in multiplex 2. Multiplex RT-PCR products were visualised after electrophoresis on an ethidium bromide-stained 2% agarose gel.

2.5. Hemi-nested multiplex PCR

The products of multiplex RT-PCRs 1 and 2 were subjected to hemi-nested multiplex PCR. The principle is to amplify part of one or several DNA fragments resulting from RT-PCR. For each virus, an internal primer was designed and used together with the corresponding anti-sense primer used for RT-PCR (Table 1).

For hemi-nested multiplex PCR 1, the reaction mixture contained: 10% buffer (Applied Biosystems, Roche®), 0.2 mM dNTPs, 0.4 μM each "hemi-nested" primer (MIA3, MIB3, hMPV3 and VRSi), 0.2 μM each of the following primers: P1 Cane, MIA2, MIB1 and hMPV2, 0.625U of Amplitaq® DNA polymerase (Applied Biosystems, Roche®) and 25 μl water q.s.p. We added 0.5 μl of each multiplex RT-PCR 1 product to this mixture. For hemi-nested multiplex PCR 2, the reaction mixture contained: 10% buffer (Applied Biosystems, Roche®), 0.2 mM dNTPs, 1 μM each hemi-nested primer (PiS1i, Para2i, Para3i, Pi4i), 0.2 μM each following primers: PIS1−, PIP2−, Para3–1, PIP4+, 0.625U of Amplitaq® DNA polymerase (Applied Biosystems, Roche®)

Table 1
Primers used for multiplex RT-PCR and hemi-nested PCR

	Virus	Primers	Sequence (5′ → 3′)	Gene	Amplicon size (bp)	Melting temp (°C)	Authors
Primers multiplex 2							
RT-PCR	hRSV	vrs P1	GGA ACA AGT TGT TGA GGT TTA TGA ATA TGC	Nucleocapsid	279 pb	60	Cane and Pringle (1991)
		vrs P2	TTC TGC TGT CAA GTC TAG TAC ACT GTA GT			55	
	Influenza A virus	mia 1	CAG AGA CTT GAA GAT GTC TTT GCT GG	Matrix protein	212	68	Donofrio et al. (1992)
		mia 2	GCT CTG TCC ATG TTA TTT G			68	
	Influenza B virus	mib 1	AAA ATT ACA CTG TTG GTT CGG TG	Matrix protein	362	70	Donofrio et al. (1992)
		mib 2	AGC GTT CCT AGT TTT ACT TG			72	
	hMPV	hmpv 1	CCC TTT GTT TCA GGC CAA	Matrix protein	416	54	This study
		hmpv 2	GCA GCT TCA ACA GTA GCT G			58	
Hemi-nested	hRSV	vrs i	GGT GTA CCT CTG TAC TCT C	Nucleocapsid	180	58	This study
	Influenza A virus	mia 3	CTC TGA CTA AGG GGA TTT TG	Matrix protein	130	58	This study
	Influenza B virus	mib 3	CAT GAA ARC TCA CAC ATC T	Matrix protein	260	53	This study
	hMPV	hmpv 3	AGG CCA ACA CAC CAC CAG	Matrix protein	410	58	This study
Primers multiplex 2							
RT-PCR	Parainfluenza virus 1	PIS1+	CCG GTA ATT TCT CAT ACC TAT G	Hemagglutinin-	317 pb	48	Echevarria et al. (1998)
		PIS1−	CCT TGG AGC GGA GTT GTT AAG	Neuraminidase		51	
	Parainfluenza virus 2	PIP2+	AAC AAT CTG CTG CAGCAT TT	Hemagglutinin-	507	56	Echevarria et al. (1998)
		PIP2−	ATG TCA GAC AAT GGG CAA AT	Neuraminidase		56	
	Parainfluenza virus 3	Para3.1	CTC GAG GTT GTC AGG ATA TAG	Hemagglutinin-	189	46	Karron et al. (1994)
		Para3.2	CTT TGG GAG TTG AAC ACA GTT	Neuraminidase		48	
	Parainfluenza virus 4	PIP4+	CTG AAC GGT TGC ATT CAG GT	Phosphoprotein	451	60	Aguilar et al. (2000)
		PIP4−	TTG CAT CAA GAA TGA GTC CT			56	
	Internal control	GAPDH1	TCA TCC ATG ACA ACT TTG GTA TCG TG	GAPDH	564	59	Gueudin et al. (2003)
		GAPDH2	CTC TTC CTC TTG TGC TCT TG			60	
Hemi-nested	Parainfluenza virus 1	PiS1i	AGC TGC AGG AAC AAG GGG	Hemagglutinin-N	261	58	This study
	Parainfluenza virus 2	Para2i	CTA GCT GAA CTG AGA CTT G	Hemagglutinin-N	340	56	This study
	Parainfluenza virus 3	Para3i	GCT AGA GAA CAT GAC TTC C	Hemagglutinin-N	145	56	This study
	Parainfluenza virus 4	Pi4i	GTC TGA TCC CAT AAG CAG C	Phosphoprotein	390	58	This study
Primers multiplex 3							
RT-PCR	hRV	SRHI1	GCA TCI GGY ARY TTC CAC CAC CAN CC	VP4/VP2/5'NC	549	62	Savolainen et al. (2002)
		SRHI2	GGG ACC AAC TAC TTT GGG TGT CCG TGT			65	
	HCoV 229E	MD1	TGG CCC CAT TAA AAA TGT GT	Gene M	573	60	Vabret et al. (2001)

Table 1 (*Continued*)

	Virus	Primers	Sequence (5′ → 3′)	Gene	Amplicon size (bp)	Melting temp (°C)	Authors
		MD3	CCT GAA CAC CTG AAG CCA AT			60	
	HCoV OC43	MF1	GGC TTA TGT GGC CCC TTA CT	Gene M	335	58	Vabret et al. (2001)
		MF3	GGC AAA TCT GCC CAA GAA TA			58	
	Influenza C virus	CHAA	ACA CTT CCA ACC CAA TTT GG	Hemagglutinin-esterase	485	58	Zhang and Evans (1991)
		CHAD	CCT GAC AGC AAC TCC CTC AT			62	
Hemi-nested	hRV	Nestrhi1	ATG GGN GCW CAN GTN TCH ANH CA	VP4/VP2/5′NC	450	53	This study
	HCoV 229E	MD2i	CCG TAT CAA CAC TCG TTA TGT GG	Gene M	230	54	This study
	HCoV OC43	MF2i	CTC CAA AAA CTT CCA GTT C	Gene M	170	54	This study
	Influenza C virus	MICi	GAG GAT GTG GCA ACT ACT	Haemagglutinin-E	391	54	This study

and 25 μl water q.s.p. We added 0.5 μl of each multiplex RT-PCR 2 product to this mixture. The cycling conditions for hemi-nested PCRs 1 and 2 were: 94 °C for 5 min; followed by 40 cycles of 94 °C for 10 s, 55 °C for 10 s, 72 °C for 30 s; and a final incubation at 72 °C for 10 min. Hemi-nested PCR products were visualised after electrophoresis on an ethidium bromide-stained 2% agarose gel.

2.6. Hemi-nested mono-specific PCRs

When a multiplex RT-PCR 3 product was thought to be a virus (due to its size), a specific hemi-nested PCR was carried out to confirm the virus identity. The hemi-nested PCR mixtures were similar for all four viruses (HCoV OC43 and 229E, hRV and influenza C virus): 10% buffer (Applied Biosystems, Roche®), 0.2 mM dNTPs, 1 μM of each primer (Table 1), 0.625U of Amplitaq® DNA polymerase (Applied Biosystems, Roche®) and 25 μl water q.s.p. We added 0.5 μl of each multiplex RT-PCR 3 product to this mixture. Cycling conditions were: 94 °C for 5 min; followed by 40 cycles of 94 °C for 10 s, 58 °C for 10 s, 72 °C for 30 s; and a final incubation at 72 °C for 10 min. Hemi-nested PCR products were visualized after electrophoresis on an ethidium–bromide stained 2% agarose gel.

3. Results

3.1. Development and optimisation of the multiplex RT-PCRs

The primers used for RT-PCR have been described and individually evaluated in the original publications (Table 1). The primers used for hemi-nested PCR were designed using BLAST (http://www.ncbi.nlm.nih.gov/) and the PROLIGO site. These primers were designed to optimise amplification (G + C content, melting temperature and length) and to be usable in identical amplification conditions, to amplify fragments of sizes sufficiently different to allow them to be distinguished on a gel and to avoid the formation of primer–dimers whenever possible (Elnifro et al., 2000). A second fragment is frequently visible in the hemi-nested PCR assays. It corresponds to an additive amplification of the RT-PCR fragment, because of the persistence of RT-PCR primers in the RT-PCR products.

The presence of several pairs of primers in a PCR increases the probabilities of mispairing and obtaining non-specific amplification products, in particular the formation of primer–dimers. The Oligo6 software can theoretically detect such interactions. In practice, this is one of the major difficulties encountered when designing multiplex PCRs. The use of Q-solution, supplied in the one-step RT-PCR QIAGEN©kit, reduced this phenomenon. Q-solution was included in multiplex RT-PCR strategies 1 and 3; preliminary studies showed that it was not necessary in multiplex RT-PCR 2. This solution reduces the number of non-specific reactions, but in some cases it can affect the hybridisation of primers and thus reduce amplification efficacy. A range of Q-solution concentrations were used to determine the concentration that reduced non-specific reactions maximally without affecting sensitivity: a concentration of 3 μl/reaction mix was found to be optimal.

The analytical sensitivity of the method was assessed first by testing successive dilutions of various viral strains (influenza virus A, hRSV A, PIV-3, hRV and HCoV OC43) with multiplex RT-PCRs 1, 2 and 3 and classical RT-PCR specific for each virus tested (as described in the original publications). For influenza A virus and hRSV, the signal was lost at the same dilution in multiplex RT-PCR 1 and mono-specific RT-PCR, showing that the sensitivity of these two methods was identical. Similar results were obtained with

Table 2
Analytical sensitivity of the multiplex RT-PCR versus mono-specific RT-PCR

	Detection threshold	
	Specific RT-PCR	Multiplex
Influenza A virus	10^{-4}	10^{-4}
hRSV A	10^{-4}	10^{-4}
Parainfluenzavirus 3	10^{-2}	10^{-3}
Rhinovirus	10^{-3}	10^{-3}
HCoV OC43	10^{-3}	10^{-4}

hRV. However, the multiplex method was found to be more sensitive than the mono-specific method for the detection of PIV-3 and HCoV OC43 (Table 2). The analytical sensitivity of the method was also assessed by quantifying two prototype strains (influenza virus A/H3N2 and hRSV A) by TCID50 and RT-PCR. The RT-PCR multiplex 1 and the hemi-nested multiplex 1 detected 1 and 0.1 TCID50 of RSV A, respectively, and 0.01 and 0.001 TCID50 of influenza virus A/H3N2, respectively.

The ability of the multiplex methods to detect several viruses in the same extract was assessed by testing combinations of four viral strains prepared from culture supernatants. Each multiplex assay simultaneously detected all four viruses: four distinct bands of the expected sizes (Table 1) were visible on the electrophoresis gels (Fig. 1). However, we noted that HCoV 229E and hRV amplicons were too similar in size to be distinguished accurately (respectively, 573 and 549 bp). A hemi-nested PCR is therefore indispensable to distinguish between these two viruses.

The analytical specificity of the method was checked by including the following in each multiplex RT-PCR: an appropriate positive control, a control associating a strain of *Chlamydia pneumoniae*, a strain of *Mycoplasma pneumoniae* and a strain of adenovirus (Ad2). No non-specific amplification products were observed (data not shown).

3.2. Evaluation of the multiplex RT-PCRs using clinical samples

The multiplex methods were assessed on 203 samples (111 positive and 92 negative nasal aspirates) collected from children hospitalised in the Caen University Hospital or Flers Hospital between October 2002 and March 2003.

The 111 positive samples included 91 viruses detected by the classical immunofluorescence assay and cell culture methods, and 20 hMPV strains detected by an RT-PCR. Most (89/91) of the viruses detected by conventional methods were also detected by the multiplex method (Table 3).

All the samples positive for hRSV (30), influenza A virus (20), influenza B virus (10), PIV-1 (1), PIV-2 (3), PIV-3 (10) and HCoV OC43 (2) were detected by the multiplex method. In comparison with the conventional methods, the negative predictive value and sensitivity of the multiplex method for the detection of these viruses were 100% (with a confidence interval of 0.83 to 1 with a 5% risk for influenza A virus, 0.69 to 1 for influenza B virus; 0.88 to 1 for hRSV, 0.2 to 1 for PIV-1, 0.4 to 1 for PIV-2, 0.70 to 1 for PIV-3 and 0.33 to 1 for HCoV OC43). The multiplex method detected hRV in 13 of the 15 samples positive for this virus. The two others, in which hRV was detected by culture in MRC5 cells, were not confirmed; moreover, one hMPV was detected in one of these two samples and one PIV-4 in the other. All the samples positive for hMPV was confirmed by the multiplex method, giving a sensitivity of 100% compared to the specific RT-PCR that detected the virus in the previous study.

Nearly all of the viruses (100/109) were detected during the first stage of the multiplex reaction, i.e., before the hemi-nested step. In the other nine cases, the virus was only detected at the hemi-nested step: 7 hRSV and 2 PIV-2. Six of the seven negative results for hRSV and the two PIV-2 corresponded to samples that were positive according to the culture method but negative according to immunofluorescence assay (Table 3), showing that the viral load in the samples was probably low. The viruses that were only detected during the second step of the multiplex PCR were all confirmed by mono-specific hemi-nested PCR.

In addition to the 89 viruses detected by the conventional methods and the 20 hMPV detected by RT-PCR, the multiplex method detected 27 further viruses, consisting of 26 co-infections (26/111 or 23.4% of aspirates): 25 co-infections associating two viruses and one co-infection associating three viruses (hMPV, hRSV and hRV) (Table 4). Among these 27 viruses, 17 could potentially be detected by the usual cell culture and/or immunofluorescence assay: 15 hRV, 1 hRSV and 1 HCoV OC43. Fifteen of the 26 (57.6%) co-infections involved an hRV.

In the negative group, the multiplex assays identified 58 viruses in 49 of the 92 samples, i.e., in 53% of them. Forty-two of these 58 viruses could theoretically be detected by the conventional methods: 26 hRV, 7 hRSV, 3 PIV-3, 2 PIV-1, 2 influenza A virus, 2 influenza B virus. The 16 others were: 14 hMPV and 2 PIV-4 (Table 5). Furthermore, seven co-infections were detected by the multiplex method, which is equivalent to 8% of the extracts tested (7/92) and 12% of the viruses detected (7/58). All these co-infections involved an hRV. Two of them involved three viruses: PIV-1, hRV and hMPV in one case, and hRSV, hRV and hMPV in the other (Table 5).

4. Discussion

A wide range of viruses can cause respiratory infections and currently 20–30% of these aetiologies remain unidentified in hospitalised children (Freymuth et al., 1987). This may be due to the lack of sensitivity of some of the detection methods and to the fact that some respiratory viruses are not systematically sought (e.g., PIV-4, influenza C virus and HCoV). The aim of this study was to develop rapid, sensitive and specific molecular methods for the detection of a large panel of respiratory RNA viruses that are more powerful than

Table 3
Results obtained for the positive group with conventional and molecular methods

Viruses	No. of specimens tested	DFA	No. of positive on MRC5	No of positive on HuH7	hMPV RT-PCR positive	No. of specimens positive with RT-PCR multiplex 1	No. of specimens positive with hemi-nested PCR multiplex 1	No. of specimens positive with RT-PCR multiplex 2	No. of specimens positive with hemi-nested PCR multiplex 2	No. of specimens positive with RT-PCR multiplex 3	No. of specimens positive with specific hemi-nested PCR
Influenza A virus	4	+	–			4	4	–	–	–	
	5	++	–			5	5	–	–	–	
	3	+++	–			3	3	–	–	–	
	8	–	–	8		8	8	–	–	–	
Influenza B virus	5	+	–			5	5	–	–	1hRV	1hRV
	2	++	–			2	2	–	–	–	
	3	–	–	3		3	3	–	–	–	
hMPV	20	–	–	–	20	20	20	–		2hRV	2hRV
hRSV	5	+	5			5 + 1hMPV	5 + 1hMPV	–	–	–	
	7	++	7			6 + 2hMPV	7 + 2hMPV	–	–	1hRV	1hRV
	2	+++	2			2	2	–	–	–	
	3	+	–			3	3	–	–	–	
	2	++	–			2	2	–	–	–	
	4	+++	–			4 + 1hMPV	4 + 1hMPV	–	–	1hRV	1hRV
	7	–	7			1	7	–	–	5hRV	5hRV
PIV-1	1	–	–	1		–	–	1	1	–	
PIV-2	3	–	–	3		1hRSV	1hRSV	1	3	1hRV	1hRV
PIV−3	5	+	–			1hMPV	1hMPV	5	5	2hRV + 1OC43	2hRV + 1OC43
	2	++	–			–	–	2	2	1hRV	1hRV
	2	+++	–			–	–	2	2	–	
	1	–	–	1		–	–	1	1	1hRV	1hRV
	8	–	8			2hMPV	2hMPV	–	–	8	8
hRV	5	–	–	5		1hMPV	1hMPV	–	–	5	5
	1	–	1			1hMPV	1hMPV	–	–	–	–
	1	–	1			–	–	1 PIV-4	1 PIV-4	–	–
HCoV OC43	2	–	–	2		–	–	–	–	2	2

DFA+: low positive intensity; DFA++:medium positive intensity; DFA+++: strong positive intensity; (+): positive result; (−): negative result. Bold: detected only with multiplex method.

Table 4
Co-infections in the positive group

Viruses detected with conventional methods (except for hMPV) and multiplex RT-PCR		Viruses detected with multiplex RT-PCR only	No. of co-infected specimens
hRSV	+	hRV	6
hRV	+	hMPV	4
PIV-3	+	hRV	4
hRSV	+	hMPV	3
hMPV	+	hRV	2
Influenza B virus	+	hRV	1
hRSV	+	hMPV + hRV	**1**
PIV-3	+	hMPV	1
PIV-3	+	OC43	1
PIV-2	+	hRV	1
PIV-2	+	hRSV	1
hRV	+	PIV4	1
Total			26

Table 5
Viruses detected by multiplex RT-PCR in the negative group

Mono-infections	
Influenza A virus	1
Influenza B virus	2
hMPV	12
hRSV	4
VIP-3	2
VIP-4	2
hRV	19
Co-infections	
hRV + PIV-3	1
hRV + PIV-1	1
hRV + PIV-1 + hMPV	1
hRV + hRSV + hMPV	1
hRV + influenza A virus	1
hRV + hRSV	2
Total no. of positive samples	49

the classical immunofluorescence assay and culture methods. During preliminary trials, we attempted to adapt the multiplex method to the detection of adenovirus, which cause frequent respiratory tract infections (Freymuth, 2001). But this affected the detection of other viruses, and we considered that it was preferable to search for adenovirus in a multiplex PCR assay including other DNA respiratory pathogens.

In this study, three multiplex methods for the detection of 12 respiratory viruses were developed and tested on 203 nasal aspirates from hospitalised children. All the viruses initially detected by the conventional methods were confirmed by the multiplex method, with the exception of two hRV identified after culture in MRC5 cells. It was not possible to check this result by repeating the cell culture (insufficient sample volume). The absence of inhibitors in the RT-PCR step was confirmed by the internal control. Given the large genotypic diversity of hRV, it is probable that the primers used here were not adapted to some genotypes, even though they have been shown to amplify over 60 serotypes (Savolainen et al., 2002).

The overall sensitivity of the multiplex method (RT-PCR and hemi-nested PCR) was 98% compared to conventional methods, but the non-nested multiplex RT-PCR had a sensitivity of only 88%. In fact, seven hRSV and two PIV-2 were not detected by the first step of the multiplex method; this was undoubtedly due to a low viral load. It is also very likely that the freezing and thawing steps altered the samples. The hemi-nested multiplex PCR gave a sensitivity of 100% for these two viruses. In an evaluation of the commercially available multiplex RT-PCR (Hexaplex©, Prodesse), Hindiyeh et al. found that hRSV were more difficult to detect (sensitivity of 91%) than influenza A virus (98.6%), influenza B virus (100%) and PIV1-3 (100%) (Hindiyeh et al., 2001). For the other viruses (influenza A, B and C viruses, PIV-1, -3, hRV and OC43) the first step of our method (RT-PCR) alone gave a sensitivity equivalent to that obtained with the conventional tools. A hemi-nested multiplex PCR 3 was not developed due because of the persistence of non-specific amplification products.

Several multiplex methods for the simultaneous detection of several respiratory viruses have been published. Grondahl et al. described a multiplex RT-PCR-hybridisation method targeting nine microorganisms (hEV, influenza viruses A and B, hRSV, PIV-1, -3, Ad, *M. pneumoniae* and *C. pneumoniae)*. They found that its sensitivity was low, particularly for hRSV: 24 of the 140 hRSV detected by EIA were negative (Grondahl et al., 1999). Puppe et al. assessed this method in 2004 and confirmed that the sensitivity never exceeded 90% and was particularly low for PIV-3 (23%) (Puppe et al., 2004). The multiplex RT-PCR-hybridisation technique, Hexaplex was described in 1998 by Fan et al. for the detection of seven respiratory viruses: hRSV A and B, influenza viruses A and B, PIV-1, -2 and -3 (Fan et al., 1998). Four studies have found that the Hexaplex©method had a good sensitivity (91–100% depending on the study and the virus) and was more efficient than conventional methods (Fan et al., 1998; Hindiyeh et al., 2001; Kehl et al., 2001; Liolios et al., 2001). Two multiplex nested RT-PCR methods were

developed by Coiras et al. for the detection of 14 respiratory viruses. The first one was able to detect six viruses (influenza viruses A, B and C, hRSV-A, -B and adenovirus) more efficiently than conventional methods, and the method detected 46 additional viruses, 34 of which were also detected by individual RT-PCRs. The sensitivity and specificity of the methods were, respectively, 100 and 87% (Coiras et al., 2003). In 2004, the authors described a second multiplex RT-PCR, which detected eight other respiratory viruses: PIV-1, -2, -3 and -4, HCoV OC43 and 229E, hRV and hEV. All samples found to be positive by immunofluorescence assay and/or cell culture (40/201) were confirmed by multiplex 2, which also detected 63 additional viruses (Coiras et al., 2004). Finally, in 2004, Templeton et al. described two real-time multiplex RT-PCR methods for the detection of seven respiratory viruses: influenza viruses A and B, hRSV, PIV-1, -2, -3 and -4 (Templeton et al., 2004), and Syrmis et al. an RT-PCR-hybridisation method for the detection of seven viruses: influenza viruses A and B, Ad, PIV-1, -2, -3 and hRSV (Syrmis et al., 2004).As well as being highly sensitive, our multiplex methods can identify a large number of viruses that are not detected by the conventional methods: 27 viruses among the 26 aspirates in the positive group and 58 viruses among the 49 aspirates in the negative group, which is equivalent to 23.4 and 53.3% of samples, respectively. According to statistical rules, the selection of test samples does not allow the calculation of the positive predictive value or of the specificity of the multiplex method. Furthermore, as believed by other authors (Vabret et al., 2000), the specificity of the method is difficult to interpret given that the reference method itself has limits. There are several possible explanations why these samples were negative according to conventional methods (a). The multiplex method can have given false positive result. Substantial precautions have been taken to prevent contaminations of reaction tubes with previously amplified products or target RNA or DNA from other specimens, and the existence of false positives due to contaminants was ruled out by the absence of unexpected bands in the negative controls (b). The immunofluorescence assay to detect antigens is associated with sensitivity problems when the viral load is low (Casiano-Colon et al., 2003), whereas molecular methods are theoretically more sensitive (c). Using the cell culture method it is difficult to detect several viruses in a given sample, as the development of one virus can mask or inhibit that of another (d). Conventional methods only detect replicative viruses, which gives them a good diagnostic value. The hypothesis that the viruses detected uniquely by the multiplex method are non-replicative is probably true in some cases (e). The most consistent argument is that cell culture methods are not adapted to all viruses, particularly to hRV, hMPV, PIV, hCoV and influenza virus C.

In this study, hRV that were detected uniquely by the multiplex method (26 in the negative group and 15 in the positive group) are likely to be serotypes that are difficult or impossible to culture. However, only MRC5 and HuH7 cells were used for viral isolation technique, and the traditional cell lines, such as MDCK, HeLa R or NCI-H292 cells, were not used. This could explain the greater quantity of viruses that were found using the multiplex RT-PCR assay in relation to the cell cultures. The detection of hRV is considerably improved by the use of molecular biology techniques (Savolainen et al., 2003; Coiras et al., 2004; Gilbert et al., 1996). A retrospective study carried out in Caen between 1998 and 2000 revealed 211 hRV infections in hospitalised children, 53% of which were identified by cell culture and 47% of which were identified only by RT-PCR (Guittet et al., 2003). Nevertheless, it is important to point out that the detection of hRV in nasal aspirates may be associated with interpretation problems, given that this virus can persist for 2 weeks or longer after the acute phase (Jartti et al., 2004). The multiplex RT-PCR1 was able to detect all the hMPV previously identified. Furthermore, a high proportion of hMPV was found in the negative group: 14/92, i.e., 15% of samples and nine others in the positive group. hMPV is responsible for 5–7% of viral respiratory tract infections in hospitalised children worldwide (Van Den Hoogen et al., 2004; Boivin et al., 2003; Freymuth et al., 2003a, b). Until now hMPV was not targeted by any multiplex protocol. Its clinical impact and its prevalence fully justify its detection in routine diagnosis, alongside hRSV. Three type 4 PIV were detected by this method: one in the positive group and two in the negative group. It is very difficult to culture this serotype in vitro and immunofluorescence assay has only been possible for a short while, since the production of a first monoclonal anti-PIV-4 antibody. For these reasons PIV-4 is practically never sought in virology laboratories (Echevarria et al., 1998). But it has been shown that it can cause bronchiolitis or pneumonia in young children and immunodepressed subjects and its prevalence appears to be higher than originally thought (Aguilar et al., 2000; Lindquist et al., 1997). Three HCoV OC43 were detected by the multiplex PCR, two of which were positive with the cell culture method. The HCoV OC43, which are generally considered to cause colds, have been described to cause lower respiratory tract infections (pneumonia and bronchiolitis) in infants and the elderly (Vabret et al., 2003). Given that few virology laboratories seek HCoV, it is probable that their pathogenic role is underestimated (Vabret et al., 1998). The influenza C virus is generally considered to cause non-severe influenza. However, its pathogenic potential is not well known and its frequency is undoubtedly highly underestimated, as shown by the presence of anti-influenza C antibodies in a large proportion of the French population (Manuguerra et al., 1992).

GAPDH, transcribed by nasal mucous cells and used as an internal control in the multiplex RT-PCR, has the advantage of being always present in the sample. This internal control was used in multiplex 2 only, as it caused disturbances in amplifications results of the other RT-PCR multiplex assays and in the hemi-nested PCR multiplex 2. Theoretically, since those RT-PCR multiplex assays used the same amplification kit and the same extraction protocol, the amplification of GAPDH would be similar in the three RT-PCR assays.

A GAPDH amplification product was observed for all nasal aspirates tested, indicating that no enzyme inhibitors were present. However, it has never been validated for use as an internal control in respiratory samples. The techniques described by Coiras et al. include an internal control supplied with the Promega©kit; once again no amplification inhibitors were detected during clinical evaluations (Coiras et al., 2003, 2004). Syrmis et al. used an endogenous human retrovirus (ERV-3) as an internal control and found that only 5 out of 396 samples tested were amplification negative (Syrmis et al., 2004). Dingle et al. created a stable internal control based on a modified RNA fragment of hepatitis Delta (Dingle et al., 2004). Among the 324 respiratory samples tested, only two cases of inhibition were detected. It is possible that the dilution of samples in the transport medium overcomes the effect of inhibitors (Syrmis et al., 2004).

The multiplex methods described in this study detected numerous co-infections in the positive (23%) and negative (8%) groups. The RT-PCR 1 and 2 described by Coiras et al., respectively, detected 4.5 and 21% of co-infections among the positive samples (Coiras et al., 2003, 2004). The method described by Templeton et al. detected 2.3% of co-infections (Templeton et al., 2004). Of the four studies that have evaluated the Hexaplex technique, only that by Kehl et al. (2001) reported cases of co-infection: 10% among the positive samples, none of which were confirmed by cell culture. The studies by Osiowy (1998), Puppe et al. (2004) and Syrmis et al. (2004) did not describe any cases of co-infection. In our 77% of co-infections in the positive group and 100% of co-infections in the negative group involved hRV. The rate of viral co-infection among hRV infections appears to be very variable: from 5 to 40% (Guittet et al., 2003). Three cases of triple infection were observed: one in the positive group and two in the negative group (cf. Tables 4 and 5). Two cases associating hRSV, VIP3 and hRV were previously described by Gilbert et al. (1996) who used a protocol involving three distinct RT-PCRs, specific for each virus.

The studies that have revealed co-infections have tended to find that they are not a factor associated with severity. Brouard et al. (2000) studied samples from 202 newborns hospitalised for bronchiolitis and detected viral co-infection in 27% of them, with a predominance of hRSV (39%). The analysis of various clinical and radiological parameters in these children showed that viral co-infections are not associated with more severe forms (Brouard et al., 2000). Other authors found no difference in the duration of hospitalisation or the intensity of clinical signs between co-infected children and those infected with only one virus (Maletzky et al., 1971; Portnoy et al., 1965). In children, co-infections associating hRSV and influenza A virus, hRV or PIV are not associated with more severe signs than mono-infections, whereas hRSV-Adenovirus co-infections may be associated with a worse prognosis (Glezen et al., 1980; Meissner et al., 1984; Tristram et al., 1988; Valenti et al., 1982). To date, the study of viral co-infections has not revealed any particular clinical impact, however most of the methods used have undoubtedly underestimated the proportion of co-infections, either due to lack of sensitivity (i.e., conventional methods) or because the panel of viruses sought was too restricted. It is probable that the use of a multiplex RT-PCR approach will give results that are closer to reality and provide interesting information on the existence of associated or successive infections, their role and their clinical, prognostic and epidemiological effects.

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