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Simultaneous detection of respiratory viruses in children with acute respiratory infection using two different multiplex reverse transcription-PCR assays

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A 4-tube multiplex RT-PCR (mRT-PCR), which showed higher sensitivity over conventional methods, was previously developed for the diagnosis of 14 viral pathogens of the respiratory tract. Herein the mRT-PCR was compared to the commercial Luminex mPCR-microsphere flow cytometry assay (Resplex II) which allows the detection of 12 different viruses. Eleven different viruses were identified in 91 nasopharyngeal swabs of children with acute respiratory infection, influenza A (IAV) and B, respiratory syncytial virus (RSV), human rhinovirus (hRhV), human echovirus, parainfluenza viruses (PIV) 1, 2, 3 and 4, human metapneumovirus (hMPV), and human coronavirus NL63. The results of the two techniques showed 53 and 40 positive patients by the Resplex II assay and mRT-PCR, respectively, with a concordance in 35 positive and 33 negative patients (74.7%). Individual RT-PCR tests were performed to control viruses not simultaneously detected by the two multiplex assays. The major virus misdiagnosed by mRT-PCR was IAV whereas the major viruses misdiagnosed by Resplex II were PIV1, 3 and 4. The mRT-PCR remains a simple, rapid, and specific assay for the specific detection of respiratory viruses, and can be easily implemented with standards in clinical laboratories at a low cost.

1. Introduction

Acute respiratory infections in children are caused by a large panel of viruses and bacteria located in the upper and lower respiratory tracts. A viral etiology in children is most often suspected based on clinical signs and symptoms because laboratory investigation requires a biocontainment for the virus culture, qualified personnel, and expensive and time-consuming techniques, which provide late results with little impact on patient care (Brunstein and Thomas, 2006). Therefore, research for viral causes of acute respiratory infections is used rarely by clinicians. The recent development of molecular techniques and their advantage over cell culture, and the recent discovery of new viruses responsible of acute respiratory infections have increased the interest of clinical and molecular virology research laboratories for the study of viruses in clinical specimens (Bellau-Pujol et al., 2005; Briese et al., 2005; Fox, 2007; Freymuth et al., 2006; Liolios et al., 2001; Vabret et al., 2000; Weinberg et al., 2004). The most common viruses responsible for acute respiratory infections are respiratory syncytial virus (RSV), human rhinovirus (hRhV), human enterovirus (hEnV), influenza viruses A, B, and C (IAV, IBV, and ICV, respectively), human metapneumovirus (hMPV), human coronaviruses (hCoV) 229E, OC43, NL63, and HKU1, parainfluenza virus (PIV), adenovirus (AdV), and human bocavirus (hBoV). Several multiplex techniques developed so far have the capacity to identify the majority of these viruses in clinical samples (Bellau-Pujol et al., 2005; Brunstein and Thomas, 2006; Coiras et al., 2004; Freymuth et al., 2006; Lam et al., 2007; Lee et al., 2007; Li et al., 2007; Mahony et al., 2007; Nolte et al., 2007; Pabbaraju et al., 2008; Syrmis et al., 2004; Templeton et al., 2004) to identify co-infections (Brunstein et al., 2008) and to discover incidentally uncharacterized genotypes (Lamson et al., 2006). The choice of one technique over the other should consider the rapidity, specificity, and sensitivity of the method, but also whether it requires equipment and commercial kits too costly for routine diagnostic laboratories. In order to address these questions, a 3-tube RT-PCR-based multiplex (mRT-PCR) initially described for 11 viruses (Bellau-Pujol et al., 2005), and expanded with an additional tube of mRT-PCR targeting four other hCoVs (Vabret et al., 2005,

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2008) was used for diagnostic purposes. The sensitivity of the technique was tested using quantified RNA transcript fragments from 14 targeted viruses, excluding SARS-CoV. A total of 91 clinical specimens from children with acute respiratory infections were tested and the results obtained in the 4-tube mRT-PCR were compared to those obtained with a 1-tube tagged-enriched Resplex II assay using Luminex technology (Brunstein and Thomas, 2006; Brunstein et al., 2008; Li et al., 2007) for 12 common respiratory viruses and SARS-CoV detection. The results show that mRT-PCR offers the advantage of broader application at a lower cost over the ResPlex II assay for routine diagnosis of acute respiratory infections, albeit the former is less performant for IVA detection and the latter less performant for detection of PIV1, 3, and 4.

2. Materials and patients

2.1. Specimens and viruses

Clinical specimens from nasopharyngeal swabs were collected from children under 6 years of age experiencing acute respiratory infections and consulting the Paediatric Department of Shanghai Nanxiang Hospital. Ninety-one samples stored at −80 °C were randomly selected from the sample panel collected during the period between October 2006 and August 2007. This study was approved by the medical committee of Institut Pasteur in Paris and by the ethical committee of Shanghai Nanxiang hospital and received written informed consent from the parents of the children. Strict case definition according to WHO criteria was followed by the same paediatrician who assessed all patients.

2.2. Multiplex RT-PCR assay

Total RNA was extracted from nasopharyngeal swab specimens using the QIAamp viral RNA Mini Kit (Qiagen, Shanghai, China), and stored at −80 °C. A 4-tube mRT-PCR 1-4 was used, as described previously (Bellau-Pujol et al., 2005; Vabret et al., 2001, 2005, 2006, 2008). Tube 1 targeted IAV, IBV, hRSV, and hMPV. Tube 2 targeted PIV1–4. Tube 3 targeted hRhV and ICV. Tube 4 targeted 229E-hCoV, OC43-hCoV, NL63-hCoV, and HKU1-hCoV. The mRT-PCR 1-4 was performed, as described previously (Bellau-Pujol et al., 2005; Vabret et al., 2005), using the Qiagen on step RT-PCR kit. In brief, 2.5 μl of extracted RNA was mixed with a 5× buffer and 0.2 mM dNTP, 0.5 μM of each of the primers (Bellau-Pujol et al., 2005; Vabret et al., 2001, 2005, 2006), 1 μl of enzyme mix, and DEPC-treated ultrapure water to a final volume of 25 μl. After incubation at 50 °C for 30 min and at 94 °C for 15 min, the reactions were subjected to 40 cycles of 30 s denaturation at 94 °C, 30 s annealing at 55 °C, and 1 min extension at 72 °C, followed by a final extension at 72 °C for 10 min. The amplified products were analyzed in a 0.5 μg/ml ethidium bromide–2% agarose gel.

In order to assess the validity of viral RNA amplified by mRT-PCR, positive samples by mRT-PCR or Resplex II (*vide infra*) were controlled by a single RT-PCR (sRT-PCR) using the corresponding set of primers. The identity of the amplified DNA products in sRT-PCR negative by Resplex II was verified by cloning in a pMD18T vector (Takara, Shanghai, China), *E. coli* transformation, and sequencing of amplified plasmids from 3 colonies, according to standard molecular biology protocols.

The standard WHO IAV M RT-PCR protocol developed by the laboratory at the National Institute of Infectious Diseases (NIID), Tokyo, Japan, WHO Collaborating Centre for Reference and Research on Influenza was used as reference technique to compare the sensitivity of M gene sRT-PCR detection assay using the Qiagen on step RT-PCR kit. The reaction sample contained 10 μl of 5× Qiagen RT-PCR buffer, 2 μl dNTP mix, 6 μl (5 μM) of forward primer (5′ TTC TAA CCG AGG TCG AAA CG), 6 μl (5 μM) reverse primer (5′ ACA AAG CGT CTA CGC TGC AG), 2 μl enzyme mix, 0.5 μl RNase inhibitor (20 U/μl), 19 μl RNase-free water, and 5 μl viral RNA. The mixture was subjected to reverse transcription 30 min at 50 °C and, after an initial PCR activation 15 min at 95 °C, 40 cycles were operated with 3-step cycling of denaturation 30 s at 94 °C, annealing 30 s at 50 °C, and extension 30 s at 72 °C. A final extension was programmed for 2 min at 72 °C. The amplicon of about 232 bp in size was detected after agarose gel electrophoresis.

2.3. Resplex II assay

The Resplex II (Qiagen) assay simultaneously targets 12 viruses (IAV, IBV, RSVA and RSVB, hMPV, PIV1–4, hRhV, hEnV, and SARS-CoV. Specimen-extracted RNAs were tested in a single reaction using the ResPlex II assay following the protocol of the manufacturer (http://www1.qiagen.com/Products/ResPlexIIPanel.aspx), as described previously (Brunstein and Thomas, 2006; Li et al., 2007). Briefly, 5 μl of extracted RNA was mixed with 6 μl of ResPlex II SuperPrimers and 2 μl of QIAGEN OneStep RT-PCR Enzyme Mix (Qiagen) in a 50 μl final volume adjusted with 25 μl of water. Thermocycling started with 35 min of reverse transcription at 50 °C, followed by 15 cycles with sequences of 94 °C, 52 °C, and 72 °C, then another six 2-step cycle with 15 s denaturation at 94 °C, 1.5 min extension at 70 °C, finished by 30 cycles of 15 s denaturation at 94 °C, 15 s annealing at 52 °C, and 15 s extension at 72 °C, followed by a final extension at 72 °C for 3 min. Then, 5 μl of reaction product was mixed with a ResPlex II beads set and hybridized at 52 °C for 10 min. Ten microlitre of Streptavidin-PE was added to the reaction mixture and incubated for 5 min at 52 °C, and finally 120 μl of stop buffer was added and the tube was kept in the dark until tested in a Luminex L100 instrument (Austin, TX, USA), as described previously (Brunstein et al., 2008). The cut-off value suggested by the manufacturer was 250 mean fluorescence intensity (FI).

2.4. RNA extraction and preparation of RNA transcripts

DNA clones containing the sequences of each virus identified in the mRT-PCR were prepared from RNA extracted from virus culture supernatants or directly from the clinical specimens, using the primer sets published previously for mRT-PCR (*vide supra*). The origin of the viruses is indicated in Table 1. The amplified products were cloned in pGEM-T easy Vector (Promega, Madison, WI, USA) and *in vitro*-transcribed with T7 polymerase, as described previously (Wang et al., 2009). The RNA transcripts were quantified in a UV spectrophotometer (Biorad, Shanghai, China). The standard plasmids containing the amplified gene fragments of the 14 viruses tested are available upon request to the authors.

Table 1
Origin of plasmids used in this study.

Plasmid name	Virus strain	Origin (year)
pT7-MIA-6	IAV H3N2 Beijing	Caen, France (2005)
pT7-MIB-3	IBV Surn	Caen, France (2004)
pT7-FluC	ICV	Cambodia (2007)
pT7-VRS	RSV	Caen, France (ND[a])
pT7-Rho-2	hRhV31	Caen-France (2002)
pT7-HMPV	hPMV	Shanghai (2007)
pT7-PIV1–1	PIV1	Caen-France (2004)
pT7-PIV2–6	PIV2	Caen-France (2004)
pT7-PIV3–1	PIV3	Caen-France (2005)
pT7-PIV4	PIV4	Cambodia (2007)
pT7-OC43-4	OC43-hCoV	Caen-France (2005)
pT7-229E-3	229E-hCoV	Caen-France (2005)
pT7-NL63	NL63-hCoV	Cambodia (2007)
pT7-HKU1	HKU1-hCoV	Cambodia (2007)

[a] ND: not determined.

Table 2
Limit of detection of RNA transcript molecules by simplex (s) or multiplex (m) RT-PCR.

Test	Tube 1				Tube 2				Tube 3		Tube 4			
	Virus													
	IAV	IBV	RSV	hMPV	PIV1	PIV2	PIV3	PIV4	ICV	hRhV	229E	OC43	NL63	HKU1
Amplicon size (nt)	212	365	278	537	317	507	189	451	485	550	574	334	225	443
mRT-PCR	10^4[a]	10^2	10^4	10^4	10^3	10^3	10^2	10^3	10^2	10^3	10	10	10^3	10
sRT-PCR	10	10	10	10	10	10	10	10	10	10^3	10	10	10^3	10

[a] Number of RNA copies/reaction.

3. Results

3.1. Sensitivity of single and multiplex RT-PCR

The evaluation of the primers used for Tubes 1–4 of the mRT-PCR, and the specificities of the assays were described previously (Bellau-Pujol et al., 2005; Vabret et al., 2001, 2005, 2006). In order to assess the sensitivity of each reaction in mRT-PCR or sRT-PCR settings, RNAs were transcribed from cloned DNA-amplified fragments from each virus strain (Table 1), quantified, and serially diluted to provide 10^5–1 RNA copy per reaction. The limit of detection for each virus RNA molecule in the multiplex reaction is indicated in Table 2 and ranges from 10 to 10^4 copies per reaction, depending on the virus. However, sRT-PCR was generally more sensitive than mRT-PCR, in particular for viruses detected in Tube 1. The most sensitive mRT-PCR was observed for three hCoV, 229E, HKU1, and OC43. Detection of IAV, hMPV, and RSV RNA transcripts was less sensitive, with a limit of 10^4 RNA molecules detected by the mRT-PCR (Table 2). However, other than hRhV and NL63-hCoV, sRT-PCR detected 10 copies of RNA transcripts per reaction.

3.2. Evaluation of the multiplex RT-PCR using clinical specimens from children

The mRT-PCR was assessed on 91 nasopharyngeal swabs collected from children with acute respiratory infections. Each sample was tested first in the 4-tubes of the mRT-PCR (Table 3). The multiplex assay was negative for 51 samples, but 40 positive samples (43.9%) were detected. One single virus was detected in 33 specimens, 2 viruses in 5 samples, and 3 different viruses in 2 samples, for a total identification of 49 viruses (Table 3). Positive samples contained predominantly hMPV and RSV. Only two NL63-hCoVs were identified in the samples among the four hCoVs tested.

Table 3
Summary of results obtained on 91 nasopharyngeal swabs of children with ARI by two different detection assays.

Virus	mRT-PCR	Resplex II
hMPV	14	14
IAV	6	12
IBV	4	5
RSVA/RSVB	9	8
PIV1	5	4
PIV2	0	1
PIV3	4	3
PIV4	1	0
hRhV	3	3
hEcV	N/A[a]	7
ICV	1	N/A
SARS-hCoV	N/A	0
NL63-hCoV	2	N/A
229E-hCoV	0	N/A
OC43-hCoV	0	N/A
HKU1-hCoV	0	N/A
Positive viruses	49	57
Positive samples	40	53
Negative samples	51	38

[a] Not applicable.

3.3. Resplex II assay on clinical samples

The specimens tested in the mRT-PCR were also tested using the Resplex II assay (Table 3). This technique targets IAV, IBV, hMPV, RSVA, RSVB, hRhV, and PIV1–4, which are also detected in the mRT-PCR. In addition, Resplex II differentiates Coxsackie-A and -B, and echovirus (hEcV), which are grouped with hEnV. The mean FI value of the negative controls was about 7 times less than the threshold suggested by the manufacturer (250 FI) and the FI values for positive samples ranged from 250 to 5935 (data not shown). Fifty-three samples covering nine different viruses were identified by Resplex II (57.1%) and 38 were negative (Table 3). Fifty samples were positive for 1 virus, 2 samples had positive results for hRhV and hEcV, and 1 sample was co-infected with IAV, PIV1, and PIV3, for a total detection of 57 viruses (Table 3). Seven samples contained hEcV, including the two samples positive for both hRhV and hEcV.

3.4. Comparative evaluation of Resplex II and multiplex RT-PCR assays

Thirty-three of the nasopharyngeal specimens were negative by mRT-PCR and Resplex II (36.2%), whereas both methods detected similar virus(es) in 35 of the samples (38.4%), showing a concordance of 74.7% (data not shown). Resplex II did not contain primers for four hCoVs and ICV, and these viruses were not considered in the comparative study. Seven of the hEcVs detected by Resplex II, but not by the mRT-PCR, which is known to miss identification of the majority of non-hRhV hEnVs (Bellau-Pujol et al., 2005), were not included. Fifteen samples were positive by Resplex II for one among the nine viruses included in the comparative study and negative by the mRT-PCR (Table 4). However, nine of these samples were confirmed positive by sRT-PCR and six remained negative (Table 4). Among the false negatives by the mRT-PCR which turned positive in the sRT-PCR, there were five IVAs, two hMPVs, one IBV, and one hRhV. The six samples negative by the mRT-PCR and the sRT-PCR, but positive by the Resplex II contained low levels of RNA molecules, indicated by values below 1031 FI recorded by the Luminex assay (Table 4) and may correspond to false positive results. Three among these six viruses corresponded to PIV1, 2, and 3. Conversely, 11 viruses were identified by mRT-PCR, but not by Resplex II, and 7 corresponding to hRhV (2 samples), hMPV, PIV1 (2 samples), PIV3, and PIV4 were confirmed positive by the sRT-PCR and sequencing (Table 4). The remaining four positive viruses by the mRT-PCR were probably false positive. Overall, the majority of viruses showing false negative or false positive results by the mRT-PCR were IAV and RSV, respectively, whereas hRhV, and PIV 1, 2, and 3 were the most misdiagnosed viruses by Resplex II. Co-infection was detected by the two assays in one patient, and co-infection was detected in two other patients only by the mRT-PCR (Table 4).

The discrepancy between mRT-PCR and Resplex II diagnosis in the detection of IAV was assessed in more detail. The capacity of sRT-PCR, mRT-PCR and Resplex II to detect IAV M gene was compared

Table 4
Differences in comparative results between two RT-PCR assays for identification of respiratory viruses in nasopharyngeal swabs of children with acute respiratory infection.

Sample ID	mRT-PCR	Resplex II (FI value)	sRT-PCR
3070730005	PIV4[a]	–	PIV4
3061102007	PIV3	–	PIV3
3061120003	hRhV	–	hRhV
3061218001	hMPV	–	hMPV
3070521004	*PIV3*[b]	–	NL63-CoV
3070521005	*RSV*/hRhV	hEcV (2565)	hRhV
3061102002	–	hMPV (1041)	hMPV
3070205008	–	hMPV (1852)	hMPV
3070108003	*RSV*/PIV1/hMPV	hMPV (5247)	hMPV/PIV1
3070205004	–	IAV (1232)	IAV
3070122003	–	IAV (1417)	IAV
3070111006	–	IAV (1892)	IAV
3070122001	–	IAV (764)	IAV
3070802003	–	*IAV (784)*	–
3070122002	–	IAV (811)	IAV
3070326001	–	IBV (512)	IBV
3070823006	PIV3/IAV	*PIV1 (467)*/PIV3 (1842)/IAV (577)	PIV3/IAV
3061030010	–	*PIV2 (1031)*	–
3070129001	–	*PIV3 (357)*	–
3070205002	–	hRhV (593)	hRhV
3061026005	–	*hRhV (641)*	–
3070108001	RSV/PIV1	RSVA (291)	RSV/PIV1
3070118003	RSV/*hMPV*	RSVA (2941)	RSV
3061102005	–	*RSVA (304)*	–

[a] Underlined viruses correspond to negative results obtained in mRT-PCR or in Resplex II but confirmed positive in sRT-PCR, and are considered as positive results.
[b] Viruses in italic correspond to positive results obtained in mRT-PCR or in Resplex II but confirmed negative in sRT-PCR, and are considered as negative results.

to that of the standard WHO RT-PCR technique. Serial dilutions of H1N1 virus RNA extracted from infected cell culture supernatant were tested by the four techniques. Results are similar for sRT-PCR and WHO RT-PCR, show a lower sensitivity of mRT-PCR compared to sRT-PCR, and confirm a higher performance of Resplex II over mRT-PCR (Table 5). Performances of Resplex II and mRT-PCR were also compared using a series of limited dilutions of viruses (IBV, hEnV, and RSV) or of clinical samples (PIV1, PIV3, hRhV, and hMPV). Resplex II was more performant than mRT-PCR to detect hEnV and hMPV, and mRT-PCR was more performant than Resplex II to detect PIV1 and hRhV (Table 5). However, sRT-PCR was more sensitive for all viruses but IBV when compared to the multiplex assays.

Thirty-three of the 91 patients showed more severe disease and pneumonia, 19 were found positive by the multiplex study, but no virus was detected in 12 of the samples (data not shown). The most common virus identified in patients with pneumonia was hMPVs (8 cases), IAVs (3 cases), and RSVs (3 cases), but none of the children were hospitalized.

4. Discussion

Although detection of viruses by direct fluorescent assay and virus culture has been often used as standard for viral diagnosis and virus characterization, their inferiority over molecular methods for the diagnosis of acute respiratory infections is well-established (Bellau-Pujol et al., 2005; Brunstein and Thomas, 2006; Dominguez et al., 2008; Freymuth et al., 2006; Lam et al., 2007; Lee et al., 2007; Mahony et al., 2007; Nolte et al., 2007; Templeton et al., 2004). In this study, two techniques of multiplex were compared to identify different viruses prevalent in children suffering from acute respiratory infections. The sensitivity of the technique is usually compared to virus titres in cell culture, but rarely on quantified viral RNA molecules (Brunstein and Thomas, 2006; Freymuth et al., 2006; Li et al., 2007). In contrast to commercial kits containing all reagents and controls validated by the manufacturers, the reproducibility and sensitivity of in-house techniques requires controls that can be used for periodic standardization and validation in external laboratories. The number of RNA molecules detected by the mRT-PCR for different viruses differs notably, suggesting that the hybridiza-

Table 5
Limit of detection of viral RNA from different viruses by RT-PCR and Resplex II.

Virus origin (year)	Titration (TCID50/reaction)	Resplex II	mRT-PCR	sRT-PCR	WHO RT-PCR[a]
A					
IAV (H1N1)	3.0E−01	+	+	+	+
(A/WSN/33)	3.0E−02	+	–	+	+
	3.0E−03	–	–	+	+
	3.0E−04	–	–	+	+
	3.0E−05	–	–	–	–
hEnV (EV71)	7.0E+02	+	–	+	
Guangxi, PRC (2008)	7.0E+01	+	–	+	
	7.0E+00	+	–	–	
INFB	1.0E−02	+	+	+	
Shanghai, PRC (2007)	1.0E−03	+	+	+	
	1.0E−04	–	–	–	
RSV (RSVB)	1.40E−01	+	+	+	
Shanghai, PRC (2008)	1.40E−02	–	+	+	
	1.40E−03	–	–	+	

Specimen	Dilution in 10	Resplex II	mRT-PCR	sRT-PCR
B				
PIV1	−1	–	+	+
Shanghai, PRC (2008)	−2	–	–	+
	−3	–	–	+
PIV3	0	+	+	+
Shanghai, PRC (2008)	−1	+	+	+
	−2	–	–	+
hRhV	0	–	+	+
Shanghai, PRC (2007)	−1	–	–	+
	−2	–	–	–
hMPV	0	+	–	+
Shanghai, PRC (2007)	−1	–	–	+
	−2	–	–	+

[a] WHO reference test for IAV M gene (see Section 2).

tion temperature, amplification length, internal structures of the RNA transcript, and/or primer dimerization could specifically effect viral RNA amplification. The sRT-PCR usually showed higher sensitivity than mRT-PCR (Tables 2 and 5), supporting the last hypothesis. To increase the sensitivity of the mRT-PCR, a hemi-nested PCR was developed previously (Bellau-Pujol et al., 2005), but this second step of PCR was not applied in this study because of possible cross-contamination. Despite this, sRT-PCR was performed for confirmation of positive mRT-PCR results and when different results were obtained from Resplex II assay. Sequencing of DNA products confirmed in the sRT-PCR and negative by the Resplex II assessed the specificity of the test and the absence of contamination since all amplified products showed at least one mutation present in three different clones, considered as hallmark of unique species.

The Resplex II assay provides a rapid and easy test for semi-quantitation of viral RNA material present in biological samples (http://www1.qiagen.com/Products/ResPlexIIPanel.aspx). Only 68 samples of 91 (74.7%) showed concordant results with the mRT-PCR. The sensitivity reported by the supplier of Resplex II is about 500 viral genomes per reaction, a value in the order of that obtained by the mRT-PCR. The lack of detection of IVA by mRT-PCR in some clinical samples could be explained by the low amount of viral RNA in these samples and a higher performance of the Resplex II. This was confirmed by comparative analysis on serial dilution of H1N1 virus (Table 5). Our results suggest that the sRT-PCR for AIV should be added to the routine mRT-PCR. A real-time RT-PCR for IAV (Wang et al., 2009) was introduced recently in the panel of diagnosis of respiratory viruses. This technique confirmed the diagnosis of the 12 AIV-positive nasopharyngeal specimens detected in the present study by sRT-PCR and by Resplex II (data not shown). This test is now used in addition to the mRT-PCR diagnosis for seasonal H1N1 and H3N2 viruses and for avian H5 virus. Discrepant results between mRT-PCR and Resplex II on hMPV, hRhV and PIV were also confirmed by comparative analysis of serial dilutions of viruses or nasopharyngeal specimens. However, whether the discrepancies observed between the two techniques result from lower sensitivity or from lower specificity of primers and/or probes designed for each test is difficult to address since their specificity may show variability for different infecting virus genotypes.

Six strains of viruses positive by Resplex II were not detected by the mRT-PCR or sRT-PCR. One drawback of the Resplex II assay is its high sensitivity which may produce potentially false positive data (Lee et al., 2007). Conversely, seven samples were negative by Resplex II, but were positive by mRT-PCR and sRT-PCR. Our test performed on archive specimens did not allow for control of the presence of these viruses in nasopharyngeal samples of children by cell culture. A previous study using Resplex II showed co-infections in numerous samples (Brunstein and Thomas, 2006). Both tests performed in nasopharyngeal samples detected only one co-infection by Resplex II and three by mRT-PCR. The discrepancy between the two molecular assays reflects the difficulty to compromise multiplexing and sensitivity of RT-PCR for the diagnosis of a multitude of viruses causing respiratory infection. Recent technologies using mass-tag-PCR or DNA microarrays are able to detect viruses in samples remaining negative by conventional methods (Dominguez et al., 2008; Lamson et al., 2006; Palacios et al., 2007; Quan et al., 2007), but like Resplex II, these techniques require expensive equipment not available in the majority of clinical laboratories. A comparative study between an individual in-house real-time PCR and the Luminex xTAG respiratory viral panel showed a correlation of 72–100%, depending on the virus tested (Pabbaraju et al., 2008). The advantage of this new commercial kit based on Luminex technology over Resplex II is the number of viruses, including hCoV and AdV.

Only a single virus pathogen was detected in patients infected with IAV and IBV. Conversely, RSV, hMPV, or hRhV were detected associated with one or two other viruses (Table 3). However, detection of several common respiratory viruses, like hBoV and AdV, known to be associated frequently with co-infections, was not included in the comparative assay (Hindiyeh et al., 2008). Detection of these viruses has been introduced recently in the panel of the mRT-PCR diagnosis of respiratory viruses (Allander et al., 2007; Casas et al., 2005; Vabret et al., 2006). The present comparative study between two molecular methods confirms the difficulty in accurate diagnosis of pathogens responsible for acute respiratory infections. However, despite IAV detection, both of the nucleic acid-based assays carried out in this study offered similar performances. Therefore, mRT-PCR provides a solution to laboratories lacking resources and costly equipment or conducting monitoring of viral etiology and characterization in patients suffering respiratory infections.

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