Comparison of Multiplex PCR Assays and Conventional Techniques for the Diagnostic of Respiratory Virus Infections in Children Admitted to Hospital With an Acute Respiratory Illness

François Freymuth,[1]* Astrid Vabret,[1] Delphine Cuvillon-Nimal,[2] Sandrine Simon,[1] Julia Dina,[1] Loïc Legrand,[1] Stéphanie Gouarin,[1] Joëlle Petitjean,[1] Philippe Eckart,[2] and Jacques Brouard[2]

[1]*Laboratory of Human and Molecular Virology, University Hospital, Caen, France*
[2]*Department of Pediatrics, University Hospital, Caen, France*

The performances of four multiplex PCR (m-PCR) were compared to direct immunofluorescence assay (DFA) and HuH7 cell culture for the detection of viruses in 263 children admitted to hospital with an acute respiratory illness. One hundred fifty (57.6%) nasal aspirates were found DFA-positive; 188 (72.3%) were found positive by both DFA and HuH7 cell culture, and 242 (92%) were PCR-positive. The m-PCR detected 124 viruses which were not found by conventional methods: 68 rhinovirus, 17 human metapneumovirus, 15 respiratory syncytial virus (RSV), 8 parainfluenza virus (PIV), 5 coronavirus 229E, 3 OC43 and 3 NL63, 4 enterovirus, 2 influenza virus B and C virus. The m-PCR were more sensitive, had the advantages of a shorter delay in specific diagnosis, and a lower cost than DFA and culture. Using these m-PCR, the prevalence of each virus was compared between in-patient and out-patient groups of children attending the emergency unit of the hospital. Nasal aspirates from 411 (91.5%) children were found positive by the PCRs. RSV, rhinovirus, and influenza virus were the most frequent viruses detected in this population, representing 43.6%, 31.8%, and 8.8% of the virus found, respectively, followed by human metapneumovirus (4.4%), coronavirus (3.4%), parainfluenza virus (3.2%), adenovirus (2.3%), and enterovirus (2.1%). RSVs were detected more significantly in the in-patient group than in the out-patient group, and influenza viruses were detected more frequently in the out-patient group than in the in-patient group. Moreover, the use of m-PCR pointed out the frequency of rhinovirus and mixed viral detections in these patients. In conclusion, according to the requirements of speed and low cost of the methods, and to achieve the highest rate of detection of respiratory viruses, the combined use of DFA and m-PCR is today likely to be the best way to improve diagnosis of respiratory illnesses in children. *J. Med. Virol. 78:1498–1504, 2006.*
© 2006 Wiley-Liss, Inc.

INTRODUCTION

To date, antigen detection tests (immunofluorescence or immunoenzyme assays) have been used most frequently for the detection of respiratory viruses. These techniques are rapid and cheap, but they have limited performances in terms of the number of viruses detected and sensitivity [Freymuth et al., 1987]. Virus isolation techniques are also often associated with antigen detection tests for the optimal detection of respiratory viruses. However, the choice of the cell system is critical, as cell lines defined to grow one particular virus are not necessarily adapted to replicate other viruses. Primary kidney cells have long been the most efficient system to isolate respiratory viruses. Since these are no longer available, continuous cell lines such as LLC-MK2 [Frank et al., 1979; Shih et al., 1999], NCI-H292 [Hierholzer et al., 1993], A549 [Woods and Young, 1988], HuH7 [Freymuth et al., 2005] or hybrid cell cultures [Huang and Turchek,

2000] have been suggested as substitutes. The detection of respiratory viruses can also 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; Gilbert et al., 1996; Freymuth et al., 1997; Eugene-Ruellan et al., 1998; Fan et al., 1998; Kehl et al., 2001; Mullins et al., 2004; Rovida et al., 2005]. Given the number of respiratory viruses, multiplex RT-PCR methods (m-PCR) have been developed with the aim of providing a method capable of detecting an increasing number of viruses [Echevarria et al., 1998; Fan et al., 1998; Osiowy, 1998; Grondahl et al., 1999; Aguilar et al., 2000; Coiras et al., 2003, 2004; Templeton et al., 2004; Bellau-Pujol et al., 2005; Kuypers et al., 2006].

However, whether serological, culture, and molecular approach for diagnosing of respiratory tract infections are to be considered as alternative or complementary, and which of these three techniques is more efficient, rapid, and cost-effective, remains to be defined. In the present report, the performance and costs of four m-PCR were investigated along with conventional methods, immunofluorescence assay, and tissue culture in children suffering from an acute lower respiratory tract infection attending to hospital. The prevalence of the different virus was also investigated according to the hospitalization, or not, of the children.

MATERIALS AND METHODS

Patients and Samples

The patients were 449 children admitted to the emergency unit of the University Hospital of Caen between November 1st, 2003 and March 30th, 2004 for an acute lower respiratory tract infection with fever and cough. After clinical examination, and without considering the results of virological tests, the children were separated in two groups: 263 children were admitted to hospital (in-patients) and 186 were allowed to return home (out-patients). All patients had a nasal aspirate which was processed immediately by conventional methods for the in-patients group. The remaining sample was frozen at $-70°C$ and used to perform the m-PCRs in the following days.

Conventional Methods

The conventional methods included firstly a direct immunofluorescence assay (DFA). This immunofluorescence assay used commercially available monoclonal antibodies (Imagen®, Dako, UK) and permitted the detection of influenza virus types A and B, respiratory syncytial virus (RSV), parainfluenza virus (PIV) types 1, 2, and 3, and adenovirus. When the immunofluorescence assay was negative, the samples were inoculated onto HuH7 cell line grown in 48-well microplates. The procedures used for viral isolation in HuH7 cells have been described previously [Freymuth et al., 2005]. Briefly, after 4 days of incubation, HuH7 cells were harvested and used for an immunofluorescence

assay with the same antiviral antibodies. When a cytopathic effect was observed in the HuH7 cells, supernatants of the relevant wells were harvested and used in the multiplex PCR3 described above for the detection of rhinovirus, enterovirus, and human coronavirus.

Molecular Methods

The molecular methods included four multiplex RT-PCR and an adenovirus PCR. They were carried out directly on the respiratory samples. For the multiplex RT-PCR, RNA was extracted from 140 µl of each sample using the QIAamp viral RNA Mini Kit®, (Qiagen, Courtaboeuf, France). The first three m-PCR, targeting 13 respiratory viruses were described previously [Bellau-Pujol et al., 2005]. m-PCR1 detected influenza viruses A and B, human metapneumovirus (hMPV) and RSV; m-PCR2 detected PIV types 1, 2, 3, 4; m-PCR3 rhinovirus, enterovirus, influenza C virus, human coronavirus OC43 and 229E. An internal control consisting of glyceraldehyde-3-phosphate dehydrogenase (GAPDH) gene was included in the multiplex 2. The sequences of the primers, as well as their annealing temperatures, the amplicon sizes, and the details of the laboratory procedures were described by Bellau-Pujol et al. [2005]. Briefly, each m-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 primers, 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 m-PCRs were as follows: 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 m-PCR3) for 30 s and $72°C$ for 1 min; and a final incubation at $72°C$ for 10 min. The products of m-PCR 1 and 2 were subjected to heminested m-PCR. An internal primer was designed for each virus and used together with the corresponding anti-sense primer used for RT-PCR. Heminested PCR products were visualized after electrophoresis on an ethidium-bromide stained 2% agarose. The fourth multiplex (m-PCR4) was developed recently in our laboratory to detect the three human coronavirus 229E, OC43, and NL63 and the procedure was described in details by Vabret et al. [2005]. Lastly, DNA nucleic acids were re-extracted from 200 µl of all samples by use of Qiamp® DNA Blood Mini Kit (QIAgen). Adenovirus sequences were searched using a commercial PCR assay (Adenovirus Consensus®, Argene, Vanithes, France) according to a previously described procedure [Vabret et al., 2004].

Costs of the Methods

The costs of the conventional methods and m-PCR were obtained by adding the costs of the time-works required by technicians to perform the techniques to the costs of reagents used in these techniques. Costs of the

devices used for m-PCR were not taken into account in the calculation.

Statistical Analysis

Significant differences between the viruses detected in the in-patient and out-patient groups of children were determined by the chi-square test.

RESULTS

The first part of the study compared the efficiency of conventional and of molecular methods for the detection of respiratory viruses in the in-patient group of children, and the second part compared the prevalence of each species of viruses between the in-patient and out-patient groups.

Comparison of Conventional and Molecular Methods in Children Admitted to Hospital (In-Patients)

The comparison of conventional and molecular methods for the detection of respiratory viruses was carried out on 263 children suffering from an acute lower respiratory infection and admitted to pediatrics departments (Table I). Three of the specimens could not be processed by the conventional methods.

DFA. The first step of the conventional methods was a DFA, which made it possible to detect seven viruses: influenza viruses A and B, PIVs 1, 2, 3, RSV, and adenovirus. One hundred fifty (57.6%) out of the 260 nasal aspirates were then found DFA-positive. There were 138 (92%) samples containing RSV, 11 (7.3%) influenza A virus, and 1 PIV type 3.

Conventional methods. Viral isolation in HuH7 cell line was attempted as the second part of the conventional methods on the DFA-negative samples. This method allowed the detection of six additional viruses: influenza virus C, PIV 4, rhinovirus, enterovirus, coronavirus 229E, coronavirus OC43. Forty-three additional viruses were thus detected and more specimens, 188 (72.3%), were found to contain a virus by use of DFA together with HuH7 cell culture. According to the viral species, the HuH7 cell culture improved the detection of some viruses which were missed by the DFA: influenza A virus in seven cases, RSV in five cases, and adenovirus in five cases. It also allowed the detection of viruses for which there is a lack of specific and reliable serologic reagents: rhinovirus in 23 cases, enterovirus in 2 cases. Overall, there were 143 (76%) specimens containing RSV, 23 (12.2%) rhinovirus, and 18 (9.5%) influenza A virus, using conventional techniques.

TABLE I. Comparison of DFA[a], DFA and HuH7 Cell Culture, and Multiplex PCR (m-PCR) in 263 Children Admitted to Hospital for an Acute Respiratory Syndrome From November 1st, 2003 to March 30th, 2004

	DFA[a]	DFA and HuH7 cell culture	m-PCR
No of detectable viruses	7	13	15
No. of nasal aspirates	260	260	263
No. (%) of positive nasal aspirates	150 (57.6)	188 (72.3)	242 (92)
No. of virus detected	150	193	317
No. of samples containing			
Two or more viruses	0	5	52
No. of specific virus detected[b]			
RSV	138	143	158
hMPV	—	—	17
Influenza A virus	11	18	18
Influenza B virus	0	0	1
Influenza C virus	—	0	1
PIV 1	0	0	3
PIV 2	0	0	1
PIV 3	1	2	3
PIV 4	—	0	2
Rhinovirus	—	23	91
Enterovirus	—	2	6
HCoV 229E	—	0	5
HCoV OC43	—	0	3
HCoV NL63	—	—	3
Adenovirus	0	5	5[c]
Delay in diagnosis	2 h.	5 d.	2 d.
Cost by sample (€)[d]			
Reagents	8.05	27.70	12.84
Time work	4.06	20.88	10.85
Total	12.11	48.58	23.69

[a]Direct immunofluorescence assay.
[b]RSV, respiratory syncytial virus; hMPV, human metapneumovirus; PIV, parainfluenza virus; HCoV, human coronavirus.
[c]Detection by the Adenovirus consensus® PCR (Argene, France).
[d]Cost is based on the four multiplex RT-PCR (devices not included).

Molecular methods. The molecular methods included the four m-PCR and an adenovirus PCR. They included the detection of the human metapneumovirus and the coronavirus NL63 to the 13 viruses, which were potentially detectable by the conventional methods. These molecular methods carried out directly on nucleic acids extracted from nasal aspirates resulted in more positive samples, 242 (92%), than those found by conventional methods; they identified 35% and 20% more viral infections than could be found by DFA or DFA and culture, respectively. The m-PCRs 1, 2, 3, and 4 found 124 additional viruses which had not been detected by conventional methods: RSV (n = 15), human metapneumovirus (n = 17), influenza B and C virus (n = 2), PIV 1, 2, 3, 4 (n = 8), rhinovirus (n = 68), enterovirus (n = 4), coronavirus 229E (n = 5), coronavirus OC43 (n = 3), and coronavirus NL63 (n = 3). There was no discrepancy between the results of m-PCR3 and m-PCR4 for the detection of coronavirus 229E and OC43. Interestingly, there was no benefit linked to the use of molecular methods for the detection of influenza A virus and adenovirus. Using molecular methods, there were 158 (65.2%) specimens containing RSV, 17 (7%) hMPV, 20 (8.2%) influenza virus, 9 (3.7%) PIV, 91 (37.6%) rhinovirus, 6 (2.4%) enterovirus, 11 (4.5%) coronavirus, and 5 (2%) adenovirus, among the 242 positive specimens.

Advantages and limitations of the methods. The advantages and limitations of the three methods, DFA, DFA, and culture, m-PCR were shown in Table I. The main advantages of the DFA were rapidity and low cost: 12.11€ each sample. But the lack of reliable serologic reagents for rhinovirus, enterovirus, hMPV, and coronavirus made this method less attractive. The addition of HuH7 culture to the DFA method enabled the detection of rhinovirus, enterovirus, and coronavirus, but, conversely, had many disadvantages compared to DFA and culture: a delay in diagnosis which amounted to 5 days, a technique which was more complex and time-consuming, a cost which reached 48.5€ each sample. The m-PCR had the advantages of a shorter delay in specific diagnosis, a lower cost (23.69€) than DFA and culture, while also searching for all respiratory viruses identified up to now. They were able to detect 92% of positive specimens, thus exceeding the diagnostic efficiency of DFA or of DFA and culture, which detected 57.6%- and 72.3%-positive samples, respectively. However 52 (21.4%) of the PCR-positive samples contained at least two viruses.

Prevalence of viruses Between the In-Patient and Out-Patient Groups of Children

The second part of the results compared the prevalence of each species of virus between the in-patient and out-patient groups of children attending the emergency unit of the hospital and suffering from an acute respiratory syndrome. Nasal aspirates from 411 (91.5%) children were found positive by the PCR (Table II). RSV, rhinovirus, and influenza virus were the most frequent viruses detected in this population, representing 43.6%, 31.8%, and 8.8% of the virus found, respectively, followed by human metapneumovirus (4.4%), coronavirus (3.4%), PIV (3.2%), adenovirus (2.3%), and enterovirus (2.1%). After clinical examination, and without considering the results of virological tests, the children were separated in children admitted to hospital (in-patients) and children allowed to return home (out-patients). Between the two groups of children, there was no difference in sex, but the mean age in the in-patient group was slightly lower: 5 months versus 9 months in the out-patient group. The m-PCR and the adenovirus PCR found nearly as many positive nasal aspirates in the out-patient group: 169 (90.8%) as in the in-patient group: 242 (92%). The distribution of viruses was also different according to the two groups of children. Interestingly, influenza viruses were detected relatively more frequently in the out-patient group of children than in the in-patient group: 14% versus 6.2% (P = 0.012). On the other hand, RSV represented the

TABLE II. Prevalence of Respiratory Viruses in the In-Patient and Out-Patient Groups of Children Attending Hospital for Acute Respiratory Infections From November 1st, 2003 to March 30th, 2004

	Total	Out-patients	In-patients	
No. of children	449	186	263	
Sex F/M (p)	205/244 (n.s.)	80/125 (n.s.)	106/138 (n.s.)	
Age (mean in months)	7.3 (± 5.4)	9.3 (± 6.3)	5.3 (± 4.5)	$P < 0.01$[a]
No.(%) of positive nasal aspirates	411 (91.5)	169 (90.8)	242 (92)	
No.(%) of virus[b]				
RSV	226 (43.6)	68	158	$P < 0.01$[a]
Rhinovirus	165 (31.8)	73	91	ns
Influenza A, B, C	46 (8.8)	28	20	0.012[a]
hMPV	23 (4.4)	6	17	ns
PIV 1, 2, 3, 4	17 (3.2)	8	9	ns
HCoV 229E, OC43, NL63	18 (3.4)	7	11	ns
Adenovirus[c]	12 (2.3)	7	5	ns
Enterovirus	11 (2.1)	5	6	ns
Total no. of virus	518	202	317	

[a]Chi-square test.
[b]RSV, human respiratory syncytial virus; hMPV, human metapneumovirus; PIV, parainfluenza virus; HCoV, human coronavirus.
[c]Detection by the Adenovirus consensus[®] PCR (Argene, France).

majority (61%) of viruses detected in children who had been admitted to hospital and 36% of the viruses detected in the out-patient group ($P < 0.01$). Such differences were not so striking with the other viruses. For example, rhinoviruses were detected in a slightly higher percentage of cases in the out-patient group (36.5%), than in the in-patient group (28.5%). There was also a tendency for hMPV to be detected more frequently in the in-patient group of children.

DISCUSSION

The superiority of single PCR over conventional methods for the diagnosis of acute respiratory infections in children admitted to hospital has long been established [Freymuth et al., 1997; Eugene-Ruellan et al., 1998; Weinberg et al., 2004; Rovida et al., 2005]. The m-PCR appeared more interesting as they were able to detect several respiratory viruses simultaneously. A major objective of this study was to investigate whether this molecular approach detected more efficiently conventional viruses: influenza virus, PIV, adenovirus, and RSV than the traditional DFA, with or without isolation in cell culture. It also aimed at studying how this molecular method could include the detection of unconventional viruses: rhinovirus, human metapneumovirus, or coronavirus. The present study confirmed the superiority of the four m-PCR over conventional methods for the diagnosis of acute respiratory infections in children admitted to hospital. In comparison with the DFA, there were 43 (28.6%) additional viruses detected by both DFA and culture, and 167 additional viruses by the multiplex PCRs. Many studies have shown that m-PCR were more efficient than conventional methods [Fan et al., 1998; Grondahl et al., 1999; Hindiyeh et al., 2001; Kehl et al., 2001; Liolios et al., 2001; Gruteke et al., 2004; Puppe et al., 2004; Syrmis et al., 2004]. All these methods were designed to detect only a small number of species of respiratory viruses. Conversely Coiras et al. [2003, 2004] developed two m-PCR methods which could detect 14 respiratory viruses. The first one was able to detect six viruses (influenza A, B, and C viruses, RSV-A, -B, and adenovirus) more efficiently than conventional methods: 46 additional viruses were detected by the multiplex PCR, 34 of which were confirmed by individual PCR. In 2004, the researchers described a second m-PCR, which detected eight other respiratory viruses: PIV types 1, 2, 3, 4, coronavirus OC43 and 229E, rhinovirus and enterovirus, and showed that all samples found to be positive by DFA and/or cell culture (40/201) were confirmed by the multiplex, which also detected 63 additional viruses. Diagnosis of respiratory virus infections could also be improved thanks to the development of real-time PCR methods. Templeton et al. [2004] described two real-time m-PCR methods for the detection of seven respiratory viruses: influenza A and B viruses, RSV, PIV types 1, 2, 3, 4. These real-time m-PCR were found to be more sensitive than cell culture; there were 20 additional viruses to the 67 viruses detected by viral culture: 5 influenza A virus, 8 RSV, 7 PIV; 17 of those tested by secondary PCR were all confirmed. Finally Kuypers et al. [2006] compared real-time m-PCR with fluorescent-antibody assays for diagnosis of respiratory virus infections in 1,138 specimens from children with respiratory illnesses. A least 1 virus was detected in 436 (38.3%) specimens by DFA and in 608 (53.4%) specimens by PCR ($P < 0.001$). In the present study, there were 150 viruses detected by DFA, 43 (28.6%) additional viruses detected by both DFA and culture, and 124 additional viruses detected by the multiplex PCR over conventional methods. The m-PCR 1, 2, 3, and 4 found viruses which had been missed by conventional methods: RSV (n = 15), influenza B and C virus (n = 2), PIV 1, 2, 3, 4 (n = 8), rhinovirus (n = 68), enterovirus (n = 4), coronavirus 229E (n = 5), coronavirus OC43 (n = 3), and 20 viruses which could not be detected by conventional methods: human metapneumovirus (n = 17), and coronavirus NL63 (n = 3). Interestingly, there was no benefit linked to the use of molecular methods for the detection of influenza A virus and adenovirus.

Many respiratory viruses could be detected in children suffering from an acute respiratory syndrome and attending the emergency unit of hospitals. In the present study, nasal aspirates from 411 (91.5%) children were found positive by the PCR; and RSV, rhinovirus, influenza virus, and human metapneumovirus were the most frequent viruses detected in this population, representing 43.6%, 31.8%, 8.8%, and 4.4% of the virus found, respectively. The prevalence of RSV, influenza virus and human metapneumovirus infections was comparable to that described in many other studies using multiplex PCR [Echevarria et al., 1998; Fan et al., 1998; Osiowy, 1998; Grondahl et al., 1999; Aguilar et al., 2000; Coiras et al., 2003; Coiras et al., 2004; Templeton et al., 2004; Bellau-Pujol et al., 2005; Rovida et al., 2005]. An intriguing point was the frequency of rhinovirus detection in those patients when PCR methods were used. The clinical significance of a positive rhinovirus PCR assay has been questioned since rhinovirus RNA has been detected in asymptomatic children [Nokso-Koivisto et al., 2002], and has been shown to persist in 50% of the cases 2 weeks after an acute infection [Jartti et al., 2004]. However it has been shown that rhinovirus was the single pathogen identified in some acute respiratory infections of children, after eliminating cases of bacterial or dual viral infections [Guittet et al., 2003]. In that study, rhinovirus were detected in upper respiratory infections, bronchiolitis or bronchitis, pneumonia, and acute attack of asthma, in 25.6%, 25.6%, 6.2%, and 5.7% of the cases, respectively. Gruteke et al. [2004] designed a study where a m-PCR included primers for the detection of rhinovirus. They performed the molecular test on the specimens only if DFA for RSV was negative. They found that rhinovirus was the single most frequently detected virus after RSV. One other intriguing observation was that 52 (21.4%) of the PCR-positive samples from the in-patient group of children contained at least two viruses, making the interpretation of the results sometimes more complicated. In the

present study, m-PCR were found to improve significantly the detection of respiratory viruses in clinical specimens over conventional methods. It has been shown recently that most of the respiratory viruses not detected by DFA had low copy numbers of viral nucleic acid present. The mean number of virus copies/ml in specimens that were positive by PCR and also positive by DFA was more than 3 logs higher that the number in specimens that were negative by DFA [Kuypers et al., 2006], which suggested that quantification of respiratory viruses could provide important information about the role of these pathogens when two or more viruses had been detected. It could also be helpful to better understand if co-infections could be responsible for a more acute disease or an exacerbation of symptoms.

One other important point from our study was that most children attending to hospital would not been admitted if they had influenza infection while most of the RSV-infected children would have been admitted. A similar study has been conducted by Tsai et al. [2001] in Taiwan between 1997 and 1999 on 2,295 viral infections detected among 6,986 children, who reported the same findings that RSV was the most prevalent virus detected among admitted to hospital suffering from a bronchiolitis, whereas influenza virus was isolated virus was most frequently in the out-patient group. Further investigations are in progress in our hospital to evaluate if the possibility of using molecular or conventional rapid viral diagnosis tests in pediatrics emergency could provide useful information to assist the clinicians in admitting children to hospitals or making therapeutic decisions.

However, as it was suggested by Gruteke et al. [2004] and Rovida et al. [2005], we consider that in a clinical virological laboratory, where the speed, low cost, and high sensitivity of the methods were required, there is to day only one possibility: the combined use of DFA and m-PCR. DFA can be used for detection of conventional viruses, particularly RSV and influenza viruses A and B, and m-PCR for unconventional viruses, especially hMPV and rhinovirus. However if a single procedure should be used for the diagnosis of acute respiratory infections, m-PCR should be preferred over the conventional approach because they are characterized by greater sensitivity, relative rapidity, and lower cost for detecting viruses.

REFERENCES

Aguilar JC, Pérez-Brenä MP, Garcia ML, Cruz N, Erdman DD, Echevarria JE. 2000. Detection and identification of human parainfluenza viruses 1, 2, 3, and 4 in clinical samples of pediatric patients by multiplex reverse transcription-PCR. J Clin Microbiol 38:1191–1195.

Bellau-Pujol S, Vabret A, Legrand L, Dina J, Gouarin S, Petitjean-Lecherbonnier J, Pozzetto B, Ginevra C, Freymuth F. 2005. Development of three multiplex RT-PCR assays for the detection of 12 respiratory RNA viruses. J Virol Methods 126:53–63.

Coiras MT, Pérez-Brenä P, Garcia ML, Casas I. 2003. Simultaneous detection of influenza A, B, and C viruses, respiratory syncytial virus, and adenoviruses in clinical samples by multiplex reverse transcription nested-PCR assay. J Med Virol 69:132–144.

Coiras MT, Aguilar JC, Garcia ML, Casas I, Perez-Brena P. 2004. Simultaneous detection of fourteen respiratory viruses in clinical specimens by two multiplex reverse transcription nested-PCR assays. J Med Virol 72:484–495.

Donofrio JC, Coonrod JD, Davidson JN, Betts RF. 1992. Detection of influenza A and B in respiratory secretions with the polymerase chain reaction. PCR Methods Appl 1:263–268.

Echevarria JE, Erdman DD, Swierkosz EM, Holloway BP, Anderson LJ. 1998. Simultaneous detection and identification of human parainfluenza viruses 1, 2, and 3 from clinical samples by multiplex PCR. J Clin Microbiol 36:1388–1391.

Eugene-Ruellan G, Freymuth F, Bahloul C, Badrane H, Vabret A, Tordo N. 1998. Detection of respiratory syncytial virus A and B and parainfluenza virus 3 sequences in respiratory tracts of infants by a single PCR with primers targeted to the L-polymerase gene and differential hybridization. J Clin Microbiol 36:796–801.

Fan J, Henrickson KJ, Savatski LL. 1998. Rapid simultaneous diagnosis of infections with respiratory syncytial viruses A and B, influenza viruses A and B and human parainfluenza virus types 1, 2, and 3 by multiplex quantitative reverse transcription-polymerase chain reaction-enzyme hybridisation assay (Hexaplex). Clin Infect Dis 26:1397–1402.

Frank AL, Couch RB, Griffis CA, Baxter BD. 1979. Comparison of different tissue for isolation and quantification of influenza and parainfluenza viruses. J Clin Microbiol 10:32–36.

Freymuth F, Quibriac M, Petitjean J, Daon F, Amiel ML. 1987. Les virus responsables d'infections respiratoires en pédiatrie. Bilan de 3 480 aspirations nasales réalisées chez l'enfant en une période de six ans. Ann Pediatr 34:493–501.

Freymuth F, Vabret A, Galateau-Salle F, Ferey J, Eugene G, Petitjean J, Gennetay E, Brouard J, Jokik M, Duhamel JF, Guillois B. 1997. Detection of respiratory syncytial virus, parainfluenzavirus 3, adenovirus and rhinovirus sequences in respiratory tract of infants by polymerase chain reaction and hybridization. Clin Diagn Virol 8:31–40.

Freymuth F, Vabret A, Rozenberg F, Dina J, Petitjean J, Gouarin S, Legrand L, Corbet S, Brouard J, Lebon P. 2005. Replication of respiratory viruses, particulary influenza virus, rhinovirus and coronavirus in HUH7 hepatocarcinoma cell line. J Med Virol 77:295–301.

Gilbert LL, Dakhama A, Bone BM, Thomas EE, Hegele RG. 1996. Diagnosis of viral respiratory tract infections in children by using a reverse transcription-PCR panel. J Clin Microbiol 34:140–143.

Grondahl B, Puppe W, Hoppe A, Kuhne I, Weigl JA, Schmitt HJ. 1999. Rapid identification of nine microorganisms causing acute respiratory tract infections by single-tube multiplex reverse transcription-PCR: Feasibility study. J Clin Microbiol 37:1–7.

Gruteke P, Glas AS, Dierdorp M, Vreede WB, Pilon JW, Bruisten SM. 2004. Practical implementation of a multiplex PCR for acute respiratory tract infections in children. J Clin Microbiol 42:5596–5603.

Guittet V, Brouard J, Vabret A, Lafay F, Guillois B, Duhamel JF, Freymuth F. 2003. Rhinovirus and acute respiratory infections in hospitalized children. Retrospective study 1998–2000. Arch Pediatr 10:417–423.

Hierholzer JC, Castells E, Banks GG, Bryan JA, McEwen CT. 1993. Sensitivity of NCI-H292 human lung mucoepidermoid cells for respiratory and other human viruses. J Clin Microbiol 31:1504–1510.

Hindiyeh M, Hillyard DR, Carroll KC. 2001. Evaluation of the Prodesse Hexaplex multiplex PCR assay for direct detection of seven respiratory viruses in clinical specimens. Am J Clin Pathol 116:218–224.

Huang YT, Turchek BM. 2000. Mink lung cells and mixed mink lung and A549 cells for rapid detection of influenza virus and other respiratory viruses. J Clin Microbiol 38:422–423.

Jartti T, Lehtinen P, Vuorinen T, Koskenvuo M, Ruuskanen O. 2004. Persistence of rhinovirus and enterovirus RNA after acute respiratory illness in children. J Med Virol 72:695–699.

Kehl SC, Henrickson KJ, Hua W, Fan J. 2001. Evaluation of the Hexaplex assay for detection of respiratory viruses in children. J Clin Microbiol 39:1696–1701.

Kuypers J, Wright N, Ferrenberg J, Huang ML, Cent A, Corey L, Morrow R. 2006. Comparison of real-time PCR assays with fluorescent-antibody assays for diagnosis of respiratory virus infections in children. J Clin Microbiol 44:2382–2388.

Liolios L, Jenney A, Spelman D, Kotsimbos T, Catton M, Wesselingh S. 2001. Comparison of a multiplex reverse transcription-PCR-enzyme hybridization assay with conventional viral culture and immunofluorescence techniques for the detection of seven viral respiratory pathogens. J Clin Microbiol 39:2779–2783.

Mullins JA, Erdman DD, Weinberg GA, Edwards K, Hall CB, Walker FJ, Iwane M, Anderson LJ. 2004. Human metapneumovirus infection among children hospitalized with acute respiratory illness. Emerg Infect Dis 10:700–705.

Nokso-Koivisto J, Kinnari TJ, Lindahl P, Hovi T, Pitkaranta A. 2002. Human picornavirus and coronavirus RNA in nasopharynx of children without concurrent respiratory symptoms. J Med Virol 66:417–420.

Osiowy C. 1998. Direct detection of respiratory syncytial virus, parainfluenza virus, and adenovirus in clinical respiratory specimens by a multiplex reverse transcription-PCR assay. J Clin Microbiol 36:3149–3154.

Puppe W, Weigl JA, Aron G, Grondahl B, Schmitt HJ, Niesters HG, Groen J. 2004. Evaluation of a multiplex reverse transcriptase PCR ELISA for the detection of nine respiratory tract pathogens. J Clin Virol 30:165–174.

Rovida F, Percivalle E, Zavattoni M, Torsellini M, Sarasini A, Campanini G, Paolucci S, Baldanti F, Revello G, Gerna G. 2005. Monoclonal antibodies versus reverse transcription-PCR for detection of respiratory viruses in a patient population with respiratory tract infections admitted to hospital. J Med Virol 75:336–347.

Shih SR, Tsao KC, Ning HC, Huang YC, Lin TY. 1999. Diagnosis of respiratory tract viruses in 24 hr by immunofluorescent staining of shell vial cultures containing Madin-Darby Canine Kidney (MDCK) cells. J Virol Methods 81:77–81.

Syrmis MW, Whiley DM, Thomas M, Mackay IM, Williamson J, Siebert DJ, Nissen MD, Sloots TP. 2004. A sensitive, specific, and cost-effective multiplex reverse transcriptase-PCR assay for the detection of seven common respiratory viruses in respiratory samples. J Mol Diagn 6:125–131.

Templeton KE, Scheltinga SA, Beersma MF, Kroes AC, Claas EC. 2004. Rapid and sensitive method using multiplex real-time PCR for diagnosis of infections by influenza A and influenza B viruses, respiratory syncytial virus, and parainfluenza viruses 1, 2, 3, and 4. J Clin Microbiol 42:1564–1569.

Tsai HP, Kuo PH, Liu CC, Wang JR. 2001. Respiratory viral infections among pediatric inpatients and outpatients in Taiwan from 1997 to 1999. J Clin Microbiol 39:111–118.

Vabret A, Gouarin S, Joannes M, Barranger C, Petitjean J, Corbet S, Brouard J, Lafay F, Duhamel JF, Guillois B, Freymuth F. 2004. Development of a PCR- and hybridization-based assay (PCR Adenovirus Consensus[®]) for the detection and species identification of adenoviruses in respiratory specimens. J Clin Virol 31:116–122.

Vabret A, Mourez Th, Dina J, Van Der Hoek, Gouarin S, Petitjean J, Brouard J, Freymuth F. 2005. Human Coronavirus NL63, France. Emerg Infect Dis J 11:1225–1229.

Weinberg GA, Erdman DD, Edwards KM, Hall CB, Walker FJ, Griffin MR, Schwartz B, the New Vaccine Surveillance Network Study Group. 2004. Superiority of reverse-transcription polymerase chain reaction to conventional viral culture in the diagnosis of acute respiratory tract infections in children. J Infect Dis 189:706–710.

Woods GL, Young A. 1988. Use of A-549 cells in a clinical virology laboratory. J Clin Microbiol 26:1026–1028.