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

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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.

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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,
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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 [Freyymuth 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 MgCl₂), 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

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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.

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

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

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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) |
| No. (%) of positive nasal aspirates | 411 (91.5) | 169 (90.8) | 242 (92) |
| No. (%) of virus | | | |
| RSV | 226 (43.6) | 68 | 158 |
| Rhinovirus | 165 (31.8) | 73 | 91 |
| Influenza A, B, C | 46 (8.8) | 28 | 20 |
| hMPV | 23 (4.4) | 6 | 17 |
| PIV 1, 2, 3, 4 | 17 (3.2) | 8 | 9 |
| HCoV 229E, OC43, NL63 | 18 (3.4) | 7 | 11 |
| Adenovirus | 12 (2.3) | 7 | 5 |
| Enterovirus | 11 (2.1) | 5 | 6 |
| Total no. of virus | 518 | 202 | 317 |

*aChi-square test.

bRSV, human respiratory syncytial virus; hMPV, human metapneumovirus; PIV, parainfluenza virus; HCoV, human coronavirus.

cDetection 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 than 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.

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