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The use of a multiplex real-time PCR assay for diagnosing acute respiratory viral infections in children attending an emergency unit

C. Mengelle[a,*,1], J.M. Mansuy[a,1], A. Pierre[a], I. Claudet[b], E. Grouteau[b], P. Micheau[b], K. Sauné[a,c], J. Izopet[a,c]

[a] Department of Virology, Toulouse University Hospital, Toulouse, France
[b] Children Emergency Unit, Toulouse University Hospital, Toulouse, France
[c] Department of Physiopathology, Toulouse Purpan, Unité Inserm U563, Toulouse, France

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ABSTRACT

Background: The use of a multiplex molecular technique to identify the etiological pathogen of respiratory viral infections might be a support as clinical signs are not characteristic.
Objectives: The aim of the study was to evaluate a multiplex molecular real-time assay for the routine diagnosis of respiratory viruses, to analyze the symptoms associated with the pathogens detected and to determine the spread of virus during the period.
Study design: Respiratory samples were collected from children presenting with respiratory symptoms and attending the emergency unit during the 2010–2011 winter seasons. Samples were tested with the multiplex RespiFinder® 15 assay (PathoFinder™) which potentially detects 15 viruses.
Results: 857 (88.7%) of the 966 samples collected from 914 children were positive for one (683 samples) or multiple viruses (174 samples). The most prevalent were the respiratory syncytial virus (39.5%) and the rhinovirus (24.4%). Influenza viruses were detected in 139 (14.4%) samples. Adenovirus was detected in 93 (9.6%) samples, coronaviruses in 88 (9.1%), metapneumovirus in 51 (5.3%) and parainfluenzae in 47 (4.9%). Rhinovirus (40%) was the most prevalent pathogen in upper respiratory tract infections while respiratory syncytial virus (49.9%) was the most prevalent in lower respiratory tract infections. Co-infections were associated with severe respiratory symptoms.
Conclusion: The multiplex assay detected clinically important viruses in a single genomic test and thus will be useful for detecting several viruses causing respiratory tract disorders.

1. Background

Acute respiratory infections (ARIs) are more prevalent than any other form of infectious disease in children. They range from mild upper respiratory tract problems to serious lower respiratory infections such as bronchiolitis and pneumonia. Viruses are the main pathogens and they account for many emergency hospital admissions [1,2]. Clinical signs and symptoms overlap between different viruses, but also between viruses and bacteria, making etiological diagnosis based on clinical presentation alone difficult and sometimes leading to overuse of antibiotics.

Techniques involving culture, fluorescent detection of antigens or immunochromatography have been replaced by nucleic acid tests (NATs). Due to numerous viruses that might be involved, many monoplex nucleic acid tests are necessary to identify the pathogen(s) responsible for a respiratory disorder. This strategy is thus expensive and time consuming. The use of multiplex assays should significantly reduce hands-on time and cost, and rapidly provide reliable results. The multiplex ligation-dependent probe amplification technology (MPLA)-RespiFinder® Respiratory assay [3] recently became commercially available. This assay is approved for *in vitro* diagnosis in Europe and Canada and can detect up to 15 respiratory viruses.

2. Objectives

This prospective study was done to evaluate this multiplex technique for use in clinical diagnosis. All the samples taken from

Abbreviations: ARI, acute respiratory infections; NAT, nucleic acid tests; MPLA, multiplex ligation-dependent probe amplification; IV, influenza viruses; PiV, parainfluenza viruses; RSV, respiratory syncytial viruses; RV, rhinovirus; CoV, coronaviruses; MPV, human metapneumovirus; ADV, adenovirus.

* Corresponding author at: Department of Virology, Federative Institute of Biology, 330 Avenue de Grande Bretagne, TSA 40031, 31059 Toulouse Cedex 09, France. Tel.: +33 5 67 69 04 18; fax: +33 5 67 69 04 25.
E-mail address: mengelle.c@chu-toulouse.fr (C. Mengelle).
[1] Both first authors equally contributed to this work.

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children attending the emergency unit of the Toulouse University Hospital suffering from ARIs were collected prospectively and analyzed. Clinical data related to the viruses detected were also analyzed, as was the spread of seasonal respiratory viruses for the winter following the influenza A H1N1pdm09 epidemic (October 2010 to March 2011).

3. Study design

3.1. Samples

Nasopharyngeal swabs (Virocult® Kitnia, Labarthe Inard, France), aspirates or nasal washes were prospectively collected from children under 15 with symptoms of ARIs (see below) who attended the emergency unit of the Toulouse University Hospital between October 1, 2010 and March 31, 2011 and sent to the Virology Department for analysis.

Paediatricians completed a specific questionnaire related to the symptoms, including fever and upper respiratory manifestations (rhinitis, pharyngitis, otitis, sore throat, cough) and presence of symptoms of lower respiratory infections (bronchiolitis, pneumonia, acute flu and flu syndrome). Whether or not a child had been vaccinated against influenza was also recorded.

3.2. Detection of respiratory viruses

3.2.1. Nucleic acid extraction

The collected samples were diluted in 1 ml Minimum Essential Medium (Gibco® – Life Technologies, Rockville, MD, USA) and nucleic acids were extracted with the MagNA Pure 96™ instrument using the MagNA Pure 96 DNA and viral NA small volume kit® (Roche Diagnostics, Meylan, France) according to the manufacturer's instructions (extracted volume: 200 μL, elution volume: 100 μL).

3.2.2. Multiplex PCR method

Extracts were analyzed using the RespiFinder® 15 assay (PathoFinder™, Maastricht, Netherlands), a multiplex ligation-dependent probe amplification (MLPA) technology [3]. This assay can detect 15 viruses: influenza viruses (IV) types A and B, parainfluenza viruses 1 to 4 (PiV), respiratory syncytial viruses A and B (RSV), rhinovirus (RV), coronaviruses 229E, OC43 and NL63 (CoV), human metapneumovirus (MPV) and adenovirus (ADV). The test also includes a probe for detecting the avian Influenza virus A H5N1. An internal control for PCR inhibitors detection was included in each test. A positive Flu A sample and a negative sample were used as controls.

3.2.3. Influenza A virus subtyping

Samples that were positive for influenza A were subtyped with the RealTime Ready Inf A/H1N1 Detection Set (Roche Diagnostics, Meylan, France) on the Light Cycler 480™ system (Roche Diagnostics, Meylan, France).

3.3. Statistical analysis

Data were analyzed using Stata™ v9.0 software (StataCorp, Texas, USA). Qualitative variables were analyzed with the Chi-squared test. p values of less than 0.05 were considered significant. Logistic regression was used to determine the odds ratios (OR) of age and co-infections linked to severe respiratory symptoms.

4. Results

4.1. Patient population and clinical features

A total of 914 children, of whom 509/914 (55.7%) were male were enrolled in the study between October 1, 2010 and March 31, 2011 and provided 966 samples. The mean age was 1.6 ± 2.6 years and the median age was 7.3 months [range: 0.2–186], but 572/914 (62.6%) children were under 1 year old.

The 914 children in the study group included 232/914 (25.4%) with upper respiratory tract infections (243 samples) defined as rhino-pharyngitis or sore throat, with or without otitis media. 682 children (723 samples) suffered from lower respiratory infections defined as bronchiolitis (338/914; 37%), pneumonia or broncho-pneumonia (109/914; 11.9%), acute asthma (78/914; 8.5%) and flu or flu-like syndrome (158/914; 17.3%). 76 (8.3%) children were suffering from a chronic ($n = 10$) or a congenital disorder ($n = 66$): respiratory, haematological, neurological, cardiac disorders. 25/914 (2.7%) children had been vaccinated in the fall against flu, and 16/25 (64%) of them were suffering from a chronic or congenital disorder.

4.2. Virus detected with the MLPA in single or multiple infections (Fig. 1)

At least one virus was detected in 857/966 specimens (88.7%). The most prevalent pathogen was RSV, detected in 382/966 samples (39.5%) of which 219/382 (57.3%) were type A. Three samples contained both A and B. RV was detected in 236/966 (24.4%) samples and IV in 139/966 (14.4%) samples which were mainly type A (95/139 samples; 68.3%). A subsample of 37 IVA-positive samples was subtyped: 33/37 (89.2%) were influenza AH1N1pdm09, the others were seasonal H3N2 IV.

ADV was detected in 93 (9.6%) samples and CoV in 88 (9.1%) samples (CoV NL63, OC43 and 229E in 67, 16 and 5 samples respectively). MPV were detected in 51 samples (5.3%) and PiV in 47 (4.9%) samples (type 1 in 7 (14.9%) samples, type 2 in 3 (6.4%) samples, type 3 in 19 (40.4%) samples and type 4 in 18 (38.3%) samples).

More than one virus was detected in 174 (18%) samples. Of the multiple infections, 32.6% involved rhinovirus, 25.3% RSV, 26.9% IV, 48.4% ADV, 14% MPV, 57.1% CoV and 37% PIV. Two viruses were involved in 162 (16.8%) samples (Table 1), three viruses in 10 samples (1%) and four viruses in 2 samples (0.2%).

4.3. Viruses detected in upper respiratory infections

185/243 (76.1%) samples collected among children with upper respiratory tract infections were positive. RV was the virus most frequently detected in 74/185 (40%) samples, followed by RSV in 49/185 (26.5%) samples (Fig. 2a).

4.4. Viruses detected in lower respiratory infections

672/723 (92.9%) samples collected among children with lower respiratory tract infections were positive. RSV was the most frequently detected in 335/672 (49.9%) samples. 195/335 (58.2%) were type A while 139/335 (41.5%) were type B. The second most involved virus was RV in 162/672 (24.1%) samples (Fig. 2b).

Viruses were detected in 336/360 (93.3%) samples collected among children with bronchiolitis and in 96/112 (85.7%) samples collected among children with pneumonia, RSV being the most frequently detected. Almost all cases of acute asthma (80/85; 94.1%) were positive for at least one virus, with RV being the most frequent.

The 156 patients suffering from flu or a flu-like syndrome provided 166 samples, of these 106 (64.6%) were IV positive (69 IVA and 37 IVB). Thirty of the 758 remaining patients were IV positive