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Differential Detection of Rhinoviruses and Enteroviruses RNA Sequences Associated With Classical Immunofluorescence Assay Detection of Respiratory Virus Antigens in Nasopharyngeal Swabs From Infants With Bronchiolitis

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To define the role of enteroviruses and human rhinoviruses as etiological agents in childhood bronchiolitis, clinical aspirates from 84 infants admitted to hospital with symptoms of obstructive bronchiolitis were tested by picornavirus RT-PCR assay, adenovirus PCR assay and classical immunofluorescence antigen detection of common respiratory viral agents. Respiratory syncytial viruses (A&B) were detectable in 45 of 84 (53.6%) nasopharyngeal aspirates from infants with bronchiolitis, whereas coronaviruses, influenza viruses, and parainfluenza viruses were not detectable in the same samples. Adenoviruses were detectable by PCR in 11 of 84 (13.1%) nasopharyngeal swabs. By using a picornavirus RT-PCR assay followed by a differential molecular hybridisation, rhinovirus and enterovirus RNA sequences were detected in 16 of 84 (19%) and in 10 of 84 (11.9%) of the nasopharyngeal swabs tested. Positive human rhinovirus or enterovirus RT-PCR assay, however, was the only evidence of respiratory infection in 8 of 84 (9.5%) and in 7 of 84 (8.33%) of the studied patients. Respiratory syncytial viruses, human rhinoviruses, adenoviruses, and enteroviruses occur in dual infections detected in 18 of 84 (21.4%) respiratory samples tested. The median duration of stay in hospital was not significantly different between the patients demonstrating a single viral infection and those with a dual viral infection (6.22 ± 2.07 vs. 5.04 ± 0.95 days; $P > 0.05$). In summary, combination of molecular and classical detection assays of common viruses can be used to demonstrate enterovirus and human rhinovirus respiratory infection in childhood bronchiolitis, and provides an improved approach to obtain new insights into concomitant viral respiratory tract infection in infants. ***J. Med. Virol. 61: 341–346, 2000.*** © 2000 Wiley-Liss, Inc.

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INTRODUCTION

Human rhinoviruses and enteroviruses are the clinically important human pathogens in the picornavirus genus. Human rhinoviruses are responsible for the majority of all common colds each year during winter [Arruda and Hayden, 1993; Arruda et al., 1997]. In infants, however, human rhinovirus infection can be also associated with symptoms of pneumonia, exacerbation of asthma and bronchiolitis [McMillan et al., 1993; Nicholson et al., 1993]. Although in recent years knowledge of respiratory syncytial viruses and others agents causing these diseases has expanded, the role of human rhinoviruses in childhood lower respiratory tract infections and particularly in obstructive bronchiolitis is poorly defined.

The two subgroups of respiratory syncytial virus (A and B), influenza viruses, parainfluenza viruses and adenoviruses are the viral agents involved most commonly in obstructive bronchiolitis in infants [Cubie et al., 1992; Freymuth et al., 1995, 1997]. Classical im-

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munofluorescence assay of viral antigens in nasal aspirates is largely used for the viral diagnosis of acute respiratory tract infection by these viral agents. By contrast, conventional laboratory diagnosis of human rhinoviruses infection is based on isolation in cell cultures and confirmation of acid sensitivity. Therefore, the diagnosis of human rhinoviruses respiratory tract infections is limited by the poor sensitivity of cell culture assays and serologic diagnosis is virtually impossible because of the large number of serotypes [Kellner et al., 1988; Eugene-Ruellan et al., 1998; Hyypia et al., 1998]. Recently, the Polymerase Chain Reaction (PCR) has been used for the identification of both human rhinoviruses and enteroviruses in clinical samples [Atmar et al., 1993; Johnston et al., 1993; Halonen et al., 1995]. The relevance of these molecular assays for the detection of human rhinoviruses in nasopharyngeal aspirates from infants with respiratory tract infection was demonstrated. Unfortunately, the majority of these studies did not examine a large number of clinical samples taken from infants with lower respiratory tract infection or bronchiolitis [Gama et al., 1989; Ireland et al., 1993; Gilbert et al., 1996; Andeweg et al., 1999].

In the present study, a picornavirus one-step RT-PCR assay for a rapid and sensitive detection of both human rhinovirus and enterovirus sequences in nasopharyngeal aspirates was developed. This new assay includes an optimal RNA extraction method and an amplicon identification by probe hybridisation to discriminate between rhinovirus and enterovirus RNA sequences. To investigate the role of enteroviruses and human rhinoviruses as etiological agent in bronchiolitis, 84 nasopharyngeal aspirates were tested by picornavirus RT-PCR assay, classical immunofluorescence antigens detection of respiratory syncytial viruses, influenza viruses, parainfluenza viruses, coronaviruses, and adenovirus PCR assay. Combined detection assays of respiratory viruses established an association between enterovirus and human rhinovirus infections and bronchiolitis, and defined the prevalence of concomitant viral respiratory tract infection in infants with bronchiolitis. Clinical and physiopathological consequences of multiple virus infections of respiratory tract in childhood obstructive bronchiolitis are discussed.

PATIENTS AND METHODS

Patient Samples

Nasopharyngeal aspirates were collected from 84 infants (male, n = 57; mean age = 6month/female, n = 27; mean age = 9.8 month) with symptoms of obstructive bronchiolitis. Patients with rhinitis, chronic allergic rhinitis, upper respiratory tract infections, otitis, genetic pathology or with a chronic immunodepression were excluded from this study. All the immunocompetent infants enrolled in this multicentre study (University Hospital of Lille, General hospitals of Lens, Valenciennes, Calais, and Dunkerque) were hospitalised with symptoms of bronchiolitis in departments of pediatry between November 1997 and February 1998. The nasopharyngeal aspirates were collected with a disposable mucus extractor on the 1st to 10th days after the onset of the symptoms. Sterile nasopharyngeal swabs were then transferred into two separate viral transport medium tubes (0.5% bovine serum albumin, antibiotics 1500 U of penicillin, 1 ng of streptomycin, MEM, 4.76 mg of HEPES, in 2 ml of tryptose phosphate broth). One tube was used for immunofluorescence detection assay of viral antigens and the second was immediately frozen and stored at −80°C before RT-PCR and PCR assays [Freymuth et al., 1997; Hyypia et al., 1998].

Immunofluorescence Assay

One tube with nasal aspirates resuspended in 2 ml of viral transport medium was used for immunofluorescence detection assay of viral antigens. The cells were separated by centrifugation, washed in PBS and load on microscope slides before being fixed with acetone. This test used 4 different FITC-conjugated monoclonal antibody reagents to detect respiratory syncytial viruses A&B, influenza viruses, parainfluenza viruses and coronaviruses, respectively (Argen Biosoft, France). All incubations were carried out at 37°C for 30 min and the slides were washes twice in PBS [Freymuth et al., 1997].

Nucleic Acids Extraction

DNA and total RNA were extracted simultaneously from 200 μl of nasal aspirate in viral transport medium using a rapid extraction protocol on silica column system (High pure viral nucleic acid kit) according to the manufacturer's recommendations (Boehringer Mannheim Gmbh, Biochemica). Nucleic acids were recovered after elution of the column in a final volume of 50 μl of DEPC-treated sterile water. Nucleic acids concentration in each sample was estimated by spectrophotometric measurement (optical density) at 260 nm.

Picornavirus RT-PCR Assay

A rapid one-tube RT-PCR assay (Access RT-PCR system) was carried out as described by the manufacturer (Promega, France). Briefly, each RT-PCR assay was carried out in a total volume of 50 μl, using 0.25 μg nucleic acids extracted from nasal aspirate in the presence of 200 μM deoxynucleotide triphosphates, 25 mM $MgSO_4$, 2.5 IU Tfl DNA Taq polymerase, 20 IU AMV reverse transcriptase, 10 μl of a special 5× buffer (AMV/ Tfl Taq polymerase) and 30 pmol of each primers (OL26: 5′ GCACTTCTGTTTCCCC 3′; OL27: 5′ CGGACACCCAAAGTAG 3′) that recognise nucleotide sequences localised in the 5′ uncoding nucleotide sequence (5′NC) of human rhinovirus and enterovirus genomes. Amplification cycle was undertaken in an MJ research thermocycler (Watertown, USA) according to the recommendations of the AMV-RT/Taq DNA-TfL provider (Promega , France) using 40 PCR cycles (temperature of 55°C for primers annealing phase). Picornavirus RT-PCR products (380 bp) were subjected to agarose gel electrophoresis (2%) with ethidium bro-

mide staining. RNA-free negative controls containing only the RT-PCR reaction mix were included in each assay. Genomic viral RNA extracted from 10^4 pfu of coxsackievirus B3 or human rhinovirus 9 strain (ATCC) purified by ClCs isopycnic ultracentrifugation, was used as a positive control and diluted to test the sensitivity of amplification and detection procedures [Andréoletti et al., 1996].

Southern-Blotting Analysis of Picornavirus RT-PCR Products

Southern-blotting procedure was carried out as described previously by Leparc et al. [1993]. The filters were pre-hybridised at 60°C for 2 hr into the hybridisation buffer (5× SSC-0.1% *N*-lauroylsarcosine-0.02% SDS-5% blocking solution (provided by Boehringer). The biotin-labelled probe corresponding to a part of the human rhinovirus 5′NC region was then added to obtain a final concentration of 15 pmol/ml (probe: 5′ bGGCAGCCACGCAGGCT 3′). Hybridisation was performed overnight at 42.6°C. The biotin labelled hybrids were detected using streptavidin conjugated to alkaline phosphatase (Boehringer Mannheim, Gmbh). The filters were then covered with chemiluminescent substrate (CSPD, provided by Boehringer) and exposed on X-ray films (Amersham, Les Ullis, France) [Leparc et al., 1993].

Adenovirus PCR Assay

Each PCR assay was carried out in a total volume of 50 μl, using 0.25 μg nucleic acids extracted from nasal aspirate in the presence of 200 μM deoxynucleotide triphosphates, 1.5 mM $MgCl_2$, 2.5 IU Taq polymerase and 50 pmol of each primers (P1: 5′GCCGAGAAGGGCGTCCGCAGGTA 3′; P2: 5′ TACGCCAACTCCGCCCACGCGCT 3′) that recognise nucleotide sequences localised in adenovirus hexon gene, in an MJ research thermocycler (Watertown, USA) as described previously by Freymuth et al. [1997]. PCR products (161 pb) were subjected to agarose gel electrophoresis (2%) with ethidium bromide staining. DNA-free negative controls containing only the PCR reaction mix and positive adenovirus controls obtained by DNA extraction from adenovirus 2 strain (ATCC strain) were included. Southern-blotting analysis of adenovirus PCR products was carried out as described above by using a biotin-labelled probe (5′ bCACCAGCCGCACCGCGGCGTCATCGA 3′) [Freymuth et al., 1997].

Statistical Analysis

The results were analysed by the chi-square Fisher's exact test or by the Mann–Whitney test (Epi Info 6/CDC-OMS statistical analysis software).

RESULTS

Validity of Picornavirus RNA Detection Assay

The sensitivity of human rhinovirus and enterovirus genomic detection assay was assessed by limit detection of the signal in serial 10-fold dilutions of a known human rhinovirus 9 or coxsackievirus B3-RNA copy number added to 200 μl sterile viral transport medium. Our one-step RT-PCR procedure was able to detect 700 genomic RNA copies of known human rhinovirus 9 or coxsackievirus B3 per 200 μl of viral transport medium after agarose gel electrophoresis (Fig. 1A). Southern-blotting hybridisation of cDNA amplified products with a specific human rhinovirus probe gave a 10-fold higher sensitivity than electrophoresis (Fig. 1B).

The specificity of the primers used in this RT-PCR assay was confirmed by the presence of amplification products with RNA extracted from poliovirus 3, coxsackieviruses B3 and B5, and echovirus 11 and by the absence of amplified cDNA with RNA extracted from herpes viruses and adenovirus 2-infected and non-infected MRC5 cells [Gama et al., 1989] (Fig. 1C). An internal-probe hybridisation was used to identify human rhinoviruses-specific oligonucleotide sequence within the amplification product. The specificity of this differential Southern-blotting hybridisation procedure was demonstrated by the absence of a positive signal for non-human rhinoviruses picornaviruses cDNA amplicons (Fig. 1D).

Detection of Respiratory Viruses in Nasal Aspirates From Patients With Bronchiolitis

Using a picornavirus RT-PCR assay followed by differential southern-blotting hybridisation, Enteroviruses or human rhinoviruses RNA sequences were detected in 10 of 84 (11.9%) and in 16 of 84 (19%) nasopharyngeal aspirates from infants with bronchiolitis, respectively (Table I). No statistical correlation could be drawn between the gender group and the prevalence of human rhinoviruses or enteroviruses respiratory tract infections in infants with bronchiolitis (14 and 25.9% for male and female infants positive for human rhinovirus PCR respectively, $P = 0.145$; 15.8 and 3.7% for male and female infants positive for enterovirus PCR respectively, $P = 0.103$; chi-square Fisher's exact test).

To investigate the etiological role of picornavirus in childhood bronchiolitis, the detection of enterovirus and human rhinovirus RNA sequences was associated with classical detection of common respiratory viral agents and with PCR detection of adenoviruses in nasopharyngeal samples (Table I). By immunofluorescence detection assay of viral antigens and Picornavirus RT-PCR assay, respiratory syncytial viruses were the only respiratory viral agents detectable in 21 of 57 (36.8%) and in 8 of 27 (29.6%) nasopharyngeal aspirates taken from male and female child with obstructive bronchiolitis, respectively. No viral antigens of influenza viruses, parainfluenza viruses and coronaviruses, however, were detectable by immunofluorescence detection assay in the same samples (Table I). Adenovirus PCR assay was positive in 11 of 84 (13.1%) nasopharyngeal aspirates and all revealed evidence of simultaneous dual respiratory tract infection (Tables I and II). Interestingly, positive human rhinovirus and enterovirus RT-PCR results were obtained in 8 of 84 (9.5%) and in 7 of 84 (8.3%) nasopha-

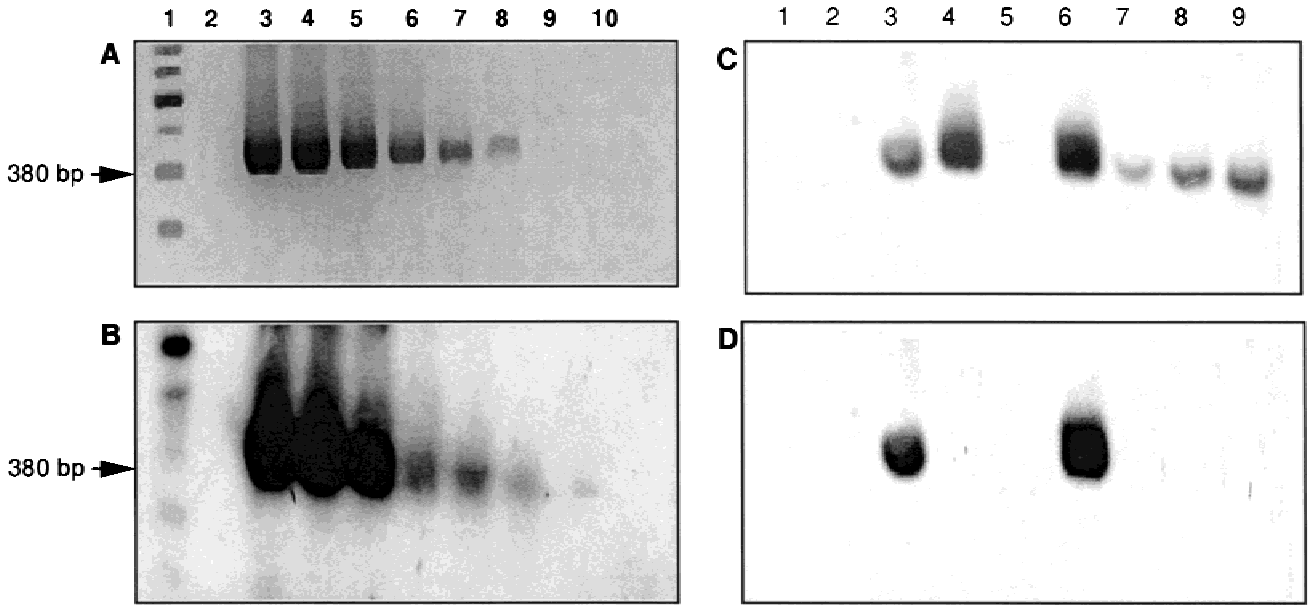

Fig. 1. Sensitivity and specificity of picornavirus RT-PCR assay followed by a specific human rhinovirus (HRV) southern-blotting hybridisation procedure. (**A**) Sensitivity of picornavirus RT-PCR assay after agarose gel electrophoresis; the signals were obtained from the RT-PCR products derived from the amplification of control positive HRV 9-RNA extracted from 10-fold dilutions of a known RNA copy number in 200 μl of sterile viral transport medium, corresponding to 7.10^7 (**lane 3**) to 7.10^1 (**lane 10**) positive RNA copies. The PCR product was detected in as few as 700 positive RNA copies (**lane 8**). (**B**) Southern-blotting hybridisation of cDNA amplified products with a specific HRV-probe; comparatively to the agarose gel electrophoresis detection (A), Southern-blotting gave a ten-fold higher sensitivity (70 HRV 9-RNA copies, **lane 9**). (**C**) Specificity of picornavirus RT-PCR assay; no signal was observed with RNA extracted from herpes simplex-1 or adenovirus 2-infected cell cultures (**lanes 2** and **5**); a positive signal was observed with RNA extracted from cell cultures infected by poliovirus 1, coxsackieviruses B3&B5, ECHO 11 (**lanes 4**, **7**–**9**) , and HRV9&11 strains (ATCC strains) (**lanes** 3 and **6**); no signal was seen with RNA extracted from mock-infected Vero cells (**lane 1**). (**D**) Differential Southern-blotting hybridisation of cDNA amplified products with a specific HRV-probe; comparatively to the agarose gel electrophoresis detection (C), only the HRV-cDNA amplified products were detectable (lanes 3 and 6).

TABLE I. Results of Molecular and IFA Virus Detection Assays in Nasopharyngeal Aspirates From Infants with Bronchiolitis*

	Positive detection in nasopharyngeal aspirates	
Virus detection assays	No. (%) of patients with single virus infection (n = 44)	No. (%) of patients with dual virus infection (n = 38)
PCR assay		
HRVs	8 (18.1)	8 (21)
EVs	7 (15.9)	3 (7.9)
ADVs	0	11 (28.9)
IF assay (IFA)		
RSVs	29 (65.9)	16 (42.1)
IV A & B	0	0
PIV I, II, III	0	0
Coronaviruses	0	0

IV A & B, Influenza Viruses A & B; PIV I, II, III, Parainfluenza Viruses I, II, III; RSVs, Respiratory syncytial viruses A & B; ADVs, Adenoviruses; HRVs, Human rhinoviruses; EVs, Enteroviruses.

ryngeal aspirates samples, and were the only evidence of respiratory viral infection in 15 patients with obstructive bronchiolitis (Table I). The combined immunofluorescence detection and PCR assays showed no viral agent detection in 2 of 84 (2.4%) patients admitted to hospital with symptoms of obstructive bronchiolitis.

Detection of Dual Respiratory Viral Infections in Infants With Bronchiolitis

Eighteen (21.4%) dual virus infections were identified out of 84 cases of obstructive bronchiolitis by using classical immunofluorescence assay of viral antigens associated with molecular detection of human rhinovirus, enterovirus and adenovirus genomic sequences. Table II describes the composition of mixed viral infections detected. The patients with a dual virus respiratory infection, presented no additional severity symptoms of obstructive bronchiolitis (similar medium percentage of O_2 saturation in arterial blood) than the patients with single virus infection (data not shown). The median duration of stay in hospital was not significantly different between the patients with a single

TABLE II. Analysis of 18 Mixed Virus Infections Detected in Nasopharyngeal Aspirates From 84 Infants With Bronchiolitis*

Virus(es) identified in mixed infections	No. of occurrences in mixed infections	No. of occurrences with			
		RSV	ADVs	HRVs	EVs
RSVs	16	—	9	6	1
ADVs	11	9	—	1	1
HRVs	7	6	1	—	0
EVs	2	1	1	0	—

*RSVs, Respiratory syncytial viruses A & B; ADVs, Adenoviruses; HRVs, Human rhinoviruses; EVs, Enteroviruses.

viral infection (respiratory syncytial virus, human rhinovirus, enterovirus) and those with a dual viral infection (6.22 ± 2.07 vs. 5.04 ± 0.95 days; $P > 0.05$; Mann–Whitney test).

DISCUSSION

Previous reports demonstrated the applicability of RT-PCR to rapid detection of human rhinoviruses in nasal and throat swabs and nasopharyngeal aspirates specimens. Because human rhinoviruses have been detected in a significant number of culture-negative specimens, it has been suggested that RT-PCR could replace the laborious and time-consuming traditional tissue culture isolation of human rhinoviruses from respiratory samples [Mori and Clewley, 1994; Santti et al., 1997; Hyypia et al., 1998]. In the present study, we developed an human rhinoviruses RT-PCR assay that includes an optimal RNA extraction method and amplicon identification with probe hybridisation to discriminate between the rhinoviruses and enteroviruses RNA genomic sequences.

In this prospective study, detection of viral respiratory infection in nasopharyngeal aspirates was carried out by picornavirus RT-PCR assay associated with classical immunofluorescence detection assays of respiratory syncytial viruses, influenza viruses, parainfluenza viruses, coronaviruses, and an adenovirus PCR assay. Combination of virus detection assays permitted the identification of viral respiratory tract infection in 97.6% of 84 patients with bronchiolitis demonstrating the relevance of this viral detection approach. Diagnosis of virus respiratory tract infection in bronchiolitis was clearly achieved in patients with a single positive virus detection assay in their nasopharyngeal samples (Table I). As previously described, respiratory syncytial virus was the first etiological agent link to symptoms of bronchiolitis in male and female infants [Maletsky et al., 1971; Freymuth et al., 1997]. Surprisingly, coronaviruses, influenza viruses and parainfluenza viruses considered as major infectious etiological agents in childhood bronchiolitis were not detectable in the samples examined, suggesting absence of epidemic outbreaks involving these viruses during our clinical study [Eugene-Ruellan et al., 1998].

The picornavirus RT-PCR assay yielded evidence of a low prevalence of enteroviruses and human rhinoviruses respiratory tract infection in children admitted to hospital with obstructive bronchiolitis (Table I). These molecular results are in agreement with the data published previously by Portnoy et al. [1965] and Kellner et al. [1988], that demonstrated by cell culture assays the low prevalence of human rhinovirus respiratory infection in childhood bronchiolitis. RT-PCR assay provides a larger percentage of positive human rhinovirus detection, however, than the positive human rhinovirus culture medium rates (6.7% in infants with bronchiolitis) reported in a previous French study [Freymuth et al., 1986].

Looking for an association of human rhinoviruses with bronchiolitis, the results presented above demonstrated evidence that 21.4% of immunocompetent infants had a dual respiratory tract infection. Previous serological studies reported that 30% of patients with respiratory syncytial virus also had serological evidence of another simultaneous viral infection. No significant differences in the severity of disease and in the median duration of stay in hospital was found between the infants with dual infections and those with a single virus. Similarly, Portnoy et al. [1965] and Maletzky et al. [1971] did not observe differences in the duration of hospitalisation between the patients infected with one and those infected by more than one respiratory virus. In reports published previously on virus respiratory tract infections, classical immunofluorescence and culture assays identified mixed respiratory in less than 5% of samples examined [Waner, 1994]. Interestingly, the sensitivity of picornavirus RT-PCR and adenovirus PCR assays combined to classical immunofluorescence assay detection of common respiratory viruses permitted the detection of dual respiratory infection in 21.4% of the patients studied. As reported previously, respiratory syncytial virus occurred in 88.8% (16/18) of the mixed infection diagnosed, respiratory syncytial virus and adenovirus or respiratory syncytial virus and human rhinovirus being the most common identified virus pair (Table II) [Waner, 1994]. In concomitant respiratory infections, virus detection by PCR or RT-PCR could be linked to latent or defective pathogens. Therefore, respiratory virus detection by immunofluorescence detection assay could correspond to a replicative viral infection that should be considered as linked to the clinical illness. In cases of mixed infection, however, picornaviruses and adenoviruses could serve as a clinical illness promotion factor functioning additively or synergistically in bronchiolitis [Waner, 1994]. Therefore, human rhinoviruses or enteroviruses preexisting asymptomatic airway infections could enhance the risk of respiratory syncytial virus bronchiolitis in

infants. Further clinical studies, including examination of respiratory specimens from symptomatic and asymptomatic subjects by quantitative RT-PCR assay detection of common respiratory viral pathogens, will be necessary to investigate the physiopathological and clinical consequences of mixed infections in childhood bronchiolitis.

In summary, this study demonstrates the relevance and the usefulness of a picornavirus RT-PCR assay associated with classical detection of common viral pathogens for the etiological diagnosis of bronchiolitis in infants. This diagnosis procedure could be relevant for the management of the patients with bronchiolitis and for the choose of the appropriate antiviral agent and the monitoring of its efficiency.

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