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# Epidemiological and Clinical Study of Viral Respiratory Tract Infections in Children From Italy

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Impact of recently discovered viruses on epidemiology of acute respiratory tract infections (ARTI) is still unclear. We studied the impact of recently discovered human metapneumovirus (hMPV), human bocavirus (HBoV), and new coronaviruses (HCoV-NL63 and HKU1) on the global epidemiology of ARTI. From October 2006 to April 2007, 237 pediatric patients affected by ARTI were enrolled in our study. Specimens were tested for respiratory viruses by polymerase chain reaction. One hundred twenty-four out of 237 samples (52.3%) were positive for one or more viruses. Picornaviruses were the most prevalent viruses (n = 61, 43.6%), followed by respiratory syncytial virus (n = 34, 24.3%) and Adenovirus (n = 25, 17.9%); hMPV (n = 9, 6.4%) was the fourth most common virus detected. HBoV and HCoV showed a low prevalence (respectively 2.9% and 2.1%). RSV was the prevalent agent of LRTI (38%). Viruses were identified in more than 50% of the studied ARTI, providing useful information on clinical features and epidemiology of specific agents affecting children in cold months. Although routine surveillance of respiratory viruses does not seem cost-effective, continuous monitoring of ARTI etiology could be a useful tool for planning resources for the development of new vaccines and antiviral agents. ***J. Med. Virol. 81:750–756, 2009.*** © 2009 Wiley-Liss, Inc.

**KEY WORDS:** respiratory tract infection; metapneumovirus; bocavirus; respiratory syncytial virus; coronavirus

## INTRODUCTION

Acute respiratory tract infections (ARTI) are associated with significant morbidity worldwide, especially among young children. Viruses are a leading cause of ARTI, presenting a relevant epidemiological variability depending on climate and regions [Armstrong et al., 1999]. However, etiology is still undefined in a significant proportion of ARTI [Monto, 1994; Henrickson et al., 2004]. During the past few years, new respiratory viruses have been discovered [Kahn, 2007]; human metapneumovirus (hMPV) is considered to be one of the most interesting and it has been reported worldwide [Van den Hoogen et al., 2001; Debiaggi et al., 2006; Kahn, 2006; Sarasini et al., 2006; Boivin et al., 2007]. It is associated with a large spectrum of clinical manifestations that range from mild upper respiratory tract disease to severe bronchiolitis and pneumonia [Williams et al., 2004, 2006]. It has been reported that the epidemiological and clinical characteristics of hMPV closely resemble those of respiratory syncytial virus (RSV) [Boivin et al., 2002; Van den Hoogen et al., 2004]. Furthermore, other recently identified pathogens, such as human bocavirus (HBoV), human coronavirus NL63 (HCoV-NL63), and HKU1 (HCoV-HKU1), have been associated with respiratory tract disease [Van Der Hoek et al., 2004; Allander et al., 2005; Woo et al., 2005a,b; Kesebir et al., 2006; Pyrc et al., 2007]. The impact of hMPV, HBoV, and new coronaviruses on the global epidemiology of ARTI is still unclear. In fact, most reports have been retrospective [Peret et al., 2002; Stockton et al., 2002] or predominantly focused on hospitalized children and children affected by lower respiratory tract infections [Maggi et al., 2003; Wilkesmann et al., 2006; Wolf et al., 2006]. Moreover, human rhinoviruses and enteroviruses, previously identified in childhood upper respiratory tract infections, have

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recently been suspected to be major etiological agents of bronchiolitis and pneumonia in infants [Papadopoulos et al., 2002; Jartti et al., 2004; Jacques et al., 2006].

On the basis of these reports, epidemiological and clinical features of ARTI in children need to be highlighted and ongoing investigations are necessary. Our study was aimed at prospectively evaluating the clinical syndromes of a pediatric population affected by ARTI and comparing the clinical features of different virus infections.

## MATERIALS AND METHODS

### Study Population

From October 2006 to April 2007, we prospectively enrolled children aged 0–15 years affected by ARTI who had presented to the Clinic of Infectious Diseases of the University of Siena, which is the reference outpatient and inpatient unit for clinical and microbiological examination of pediatric patients in the Siena area. At enrolment, informed consent was obtained from all children's parents. Patients were followed as outpatients or were hospitalized on the basis of the clinical severity, as determined by the judgment of the attending physician. On the basis of clinical features, laboratory, and radiological findings, ARTI were classified into two main categories: (1) lower respiratory tract infections (LRTI), diagnosed in the presence of signs of lower airway involvement (tachypnea, dyspnea, wheezing, or rales) and/or a positive chest X-ray; (2) upper respiratory tract infections (URTI), diagnosed when rhinitis, pharyngitis, and/or otitis media were present in the absence of LRTI signs.

A respiratory specimen (nasopharyngeal swab or aspirate) was collected from each child, together with demographic and clinical data. Only specimens obtained from hospitalized children within 48 hr after hospital admission were analyzed in order to avoid inclusion of hospital-acquired infections. Collected samples were stored at −80°C until processed. Respiratory specimens were tested for Paramyxoviridae family, genus Metapneumovirus (hMPV), genus Paramyxovirus (PIV1 to 3), and genus Pneumovirus (RSV); Parvoviridae family, genus Bocavirus (HBoV); Coronaviridae family, genus Coronavirus (HCoV); Orthomyxoviridae family, genus Influenzavirus A–B virus (Infl A–B); Adenoviridae family, genus Mastadenovirus, species adenovirus (ADV) and viruses belonging to the Picornaviridae family (genus rhinovirus and enterovirus, PIC) by polymerase chain reaction (PCR) or reverse transcriptase-PCR (RT-PCR).

### RNA and DNA Extraction

Isolation of viral RNA and DNA was performed, respectively, by using a ZR Viral RNA Kit and a ZR Viral DNA Kit (Zymo Research, Analytical Control Spa, Milan, Italy) from 200 μl of samples. RNA and DNA were eluted in 10 μl of sterile water.

### PCR Assays for Respiratory Viruses

The nucleic acid extracts were tested by PCR and nested PCR for hMPV, human coronavirus, RSV, rhinoviruses and enteroviruses, influenza virus A and B, PIV1-3, human bocavirus and adenovirus. Briefly, for reverse transcription and PCR of RNA viruses, a commercial kit (Qiagen One step RT-PCR kit, Qiagen, Milan, Italy) was used, while for DNA viruses, PCR (HotStartTaq, Qiagen) was performed as described by the manufacturer. A subsequent nested PCR was carried out using 5 μl of the first reaction product, which were then added to the reaction mixture to a final volume of 50 μl. The primer pairs, the amplified fragment of the target gene and the thermal profiles for relevant viruses are reported in Table I. Previously clinical or cultured material was used as a positive control and to validate the molecular assays.

In order to identify the type of the detected HCoV, the amplicons were sequenced with a Sequenase kit (Amersham–Pharmacia, Uppsala, Sweden), using the infrared-labeled primer 5′-ATGGGTTGGGA(CT)-TATCC(ACT)AA(AG)TGTGA-3′ on a LiCOR automated sequencer (LiCOR 4200 $IR^2$ Sequence Analyzer).

### Statistical Analysis

Statistical analysis was performed with SPSS 13.0 Software. Categorical variables between groups were compared with the $\chi^2$-test or, when appropriate, the Fisher exact test; comparisons were based on the nonparametric Kruskal–Wallis test and the Mann–Whitney *U*-test for continuous variables. A two-tailed *P*-value of $<0.05$ was considered to be statistically significant.

## RESULTS

### Prevalence of Respiratory Viruses Among Children With ARTI

From October 2006 to April 2007, 237 children (50.4% males, median age 2.5 years, range 0–13 years) with ARTI were enrolled in this study. A total of 207 nasopharyngeal swabs and 30 nasopharyngeal aspirates were obtained: 146 samples (61.1%) were collected from outpatients and 91 (38.4%) from inpatients. One hundred twenty-four out of 237 samples (52.3%) were positive for one or more respiratory viruses by molecular methods; specimens from hospitalized children were positive more often than those from outpatients (60.4% vs. 47.3%, $P = 0.06$). Among the positive samples, a single infection was demonstrated in 109 cases (87.9%); 2 viruses were identified in 14 specimens (11.3%), and 3 viruses in 1 sample (0.8%), as shown in Table II. A total of 140 respiratory viruses were identified. Enteroviruses and rhinoviruses (PIC) were the most prevalent viruses (n = 61, 43.6%), followed by RSV (n = 34, 24.3%), and ADV (n = 25, 17.9%); hMPV (n = 9, 6.4%) was the fourth most common virus detected. HBoV and HCoV showed a low percentage in our population (respectively

TABLE I. PCR Protocol for Each Analyzed Virus

| Virus | Target gene | Thermal profile | Primers pair |
|---|---|---|---|
| hMPV | Nucleoprotein gene (55–530) | 45°C 30′, 94°C 2′ (×1)<br>94°C1′, 54°C 30″, 72°C1′ (×40)<br>94°C 30″, 50°C 20″, 72°C 20″ (×40) | META REV 5′-ttggtgtgtctggtgctga-3′<br>META FOR 5′-atgtctcttcaagggattca-3′<br>META REV 5′-ttggtgtgtctggtgctga-3′ |
| HBoV | Non coding region (2301–2700) | 94°C 5′ (×1)<br>94°C 40″, 48°C 40″, 72°C 1′ (×45), 72°C 2′ (×1) | BOCA FOR 5′- cccaagaaacgtcgtctaac-3′<br>BOCA REV 5′-gtgttgactgaatacagtgt-3′ |
| HCoV | Replicase polyprotein gene (14241–14681) | 50°C 30′, 94°C 15′ (×1), 94°C50″, 54°C30″, 72°C1′ (×40), 72°C10′ (×1) | hCoV1 5′-atgggttggga(ct)tatcc(act)aa(ag)tgtga-3′<br>hCoV2 5′-ccatcatcaga(agt)agaatcatcat(agt)-3′<br>hCoV3 5′-ccatcatcactca(ag)aatcatcat(agt)-3′ |
| | | 94°C 2′ (×1), 94°C 30″, 53°C 30″, 72°C 45″ (×40), 72°C 5′ (×1) | hCoV4 5′-gcatcaccacta(gc)t(act)gt(ag)ccacc-3′<br>hCoV5 5′-gcatcaccagaa(gc)t(act)gt(ag)ccacc-3′<br>hCoV6 5′-gcatcaccggatgatgttccacc-3′ |
| RSV | Fusion protein gene (383–948) | 37°C 30′, 94°C 4′ (×1)<br>94°C1′, 48°C50″, 72°C1′ (×5), 94°C1′, 52°C 50″, 72°C1′ (×35)<br>94°C45″, 54°C 35″, 72°C 40″ (×35) | SR FOR 5′-tgtaacattaagcaagaaaagg-3′<br>SR REV 5′-aacaaggtgtatctatcacacc-3′<br>SR FOR nest 5′-ttaaccagcaaagtgttaga-3′<br>SR REV nest 5′-tttgttataggcatatcattg-3′ |
| PIC | Non coding 5′ (67–561) | 37°C 30′, 94°C 4′ (×1), 94°C1′, 48°C50″, 72°C1′ (×5), 94°C1′, 52°C 50″, 72°C1′ (×35)<br>94°C45″, 52°C 35″, 72°C 40″ (×40) | ENT FOR 5′-accttttgtacgcctgtt-3′<br>ENT REV 5′-cacggacacccaaagta-3′<br>ENT FOR nest 5′-aagcacttctgtttccc-3′<br>ENT REV nest 5′-attcaggggccggagga-3′ |
| Infl A | Matrix protein gene (71–710) | 45°C 30′, 94°C 2′ (×1), 94°C1′, 48°C50″, 72°C1′ (×5), 94°C1′, 52°C 50″, 72°C1′ (×35)<br>94°C45″, 52°C 35″, 72°C 40″ (×40) | IA FOR 5′-ccgtcaggccccctcaaagc-3′<br>IA REV 5′-gaccagcactggagctaggg-3′<br>IA FOR nest 5′-ggctaaagacaagaccaatcct-3′<br>IA REV nest 5′-gccagaaccattgtgttcac-3′ |
| PIV 1-3 | Large protein gene (10665–11142) | 37°C 30′, 94°C 4′ (×1), 94°C1′, 54°C20″, 72°C30″ (×40)<br>94°C 45″, 54°C 20″, 72°C 20″ (×40) | PIV1 FOR 5′-aaatactgtctcaactggagat-3′<br>PIV1 REV 5′-tgattgtctccttgtaccat-3′<br>PIV2 FOR 5′-gcgagataacatctcatccc-3′<br>PIV2 REV 5′-tgattatctccttgaaccat-3′<br>PIV3 FOR 5′-ggaagtacaatctatgtagg-3′<br>PIV3 REV 5′-tacgcctattctaacagctgc-3′<br>PIV1-3 REV nest 5′-cagctgctagatgtattgcac-3′<br>PIV2 REV nest 5′-agatcgaaagcatcagttgcg-3′ |
| ADV | Hexon (14–319) | 80°C 30″, 94°C 4′ (×1), 51°C 30″, 72°C 30″94°C1′ (×25), 55°C 2′, 72°C5′ (×1)<br>80°C 30″, 94°C 2′ (×1), 52°C 30″, 72°C 30″94°C1′ (×30), 55°C 2′, 72°C5′ (×1) | ADV FOR 5′-cgatgatgccgcagtggtctta-3′<br>ADV REV 5′-gcacgccgcggatgtcaaagta-3′<br>ADV FOR nest 5′-gcgccaccgagacgtacttca-3′<br>ADV REV nest 5′-cggtatcctcgcggtccacag-3′ |

2.9% and 2.1%). The rate of influenza infections was less than 3% and no PIV1-3 were detected in our population during this period.

RSV infection led to hospitalization in 85.3% of cases, a significantly higher percentage than other viruses ($P < 0.01$, data not shown). However, an analysis of the clinical outcome of inpatients and days of hospitalization did not show a statistical difference among the potential viral etiologies. The percentage of co-infections was significantly higher for hMPV (5/9 cases, 55.6%) compared to RSV (4/34 cases, 11.8% $P = 0.01$) and PIC (12/61 cases, 21.3% $P = 0.03$) (Table II). However, PIC, which were the viruses most frequently involved in co-infections, were not related to clinical severity, since they were associated neither with higher rates of hospitalization nor with prevalent involvement of the lower respiratory tract.

TABLE II. Co-Infections in Positive Samples

| No. of cases | Viruses detected | Diagnosis |
|---|---|---|
| 4 | PIC-ADV | 1 bronchitis, 3 URTI |
| 3 | PIC-RSV | 1 pneumonia, 1 bronchitis, 1 URTI |
| 2 | PIC-HCoV | 2 URTI |
| 2 | PIC-hMPV | 2 URTI |
| 2 | hMPV-ADV | 2 URTI |
| 1 | HBoV-RSV | 1 URTI |
| 1 | PIC-hMPV-ADV | 1 pneumonia |

Only four cases of influenza A were diagnosed during this period. This low number of cases reflects the seasonal trend of flu in 2006–2007, when influenza activity was low in Italy, with a peak incidence of 7.69/1,000 people/week (Istituto Superiore di Sanità, FluNet).

## Seasonal and Age Distribution

The monthly distribution of each virus is shown in Figure 1a. The rate of RSV infections increased during late autumn and peaked in January. The prevalence of hMPV and ADV peaked in April, when a decrease in RSV infections occurred. HBoV was detected only in late

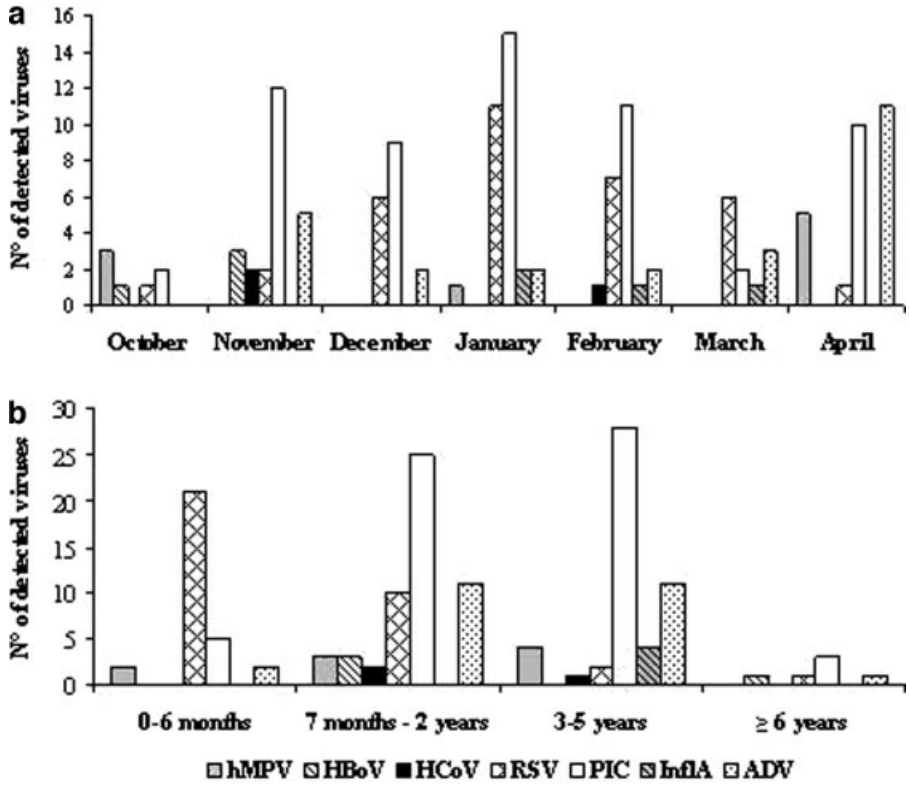


Fig. 1. Monthly (**a**) and age group (**b**) distribution of detected respiratory viruses.

autumn. PIC were detected rather constantly during the study period.

The distribution of viral agents over the different age groups is presented in Figure 1b. RSV infections were much more frequent in children younger than 6 months than older ones [21/38 (55.3%) vs. 13/199 (6.5%), $P < 0.01$]. The median age of RSV-infected children was 5 months (IQR 1-19), significantly lower than that of children infected by other viruses ($P < 0.01$). No case of hMPV infection was observed in children over the age of 5 years.

## Clinical Features

An analysis of the clinical features shows that 166 (70%) patients were affected by URTI and 71 (30%) by LRTI (Table III). Viral detection was carried out in 63.4% of LRTI and in 47.6% of URTI ($P = 0.037$). In patients where RSV was detected, an LRTI was diagnosed in 79.4% (27/34) of cases, a significantly higher proportion than that observed for other viruses (hMPV 22.2%, PIC 27.9%, InflA 0%, HCoV 0%, ADV 12%, HBoV 25%, $P < 0.01$ for all comparisons except for RSV vs. HBoV $P = 0.048$). In particular, RSV was the most important agent responsible for bronchiolitis. Interestingly, PIC were the second most frequent viruses detected in LRTI and they accounted for 9/33 (27.3%) cases of pneumonia. Picornaviruses were also the most prevalent viruses causing URTI. Human MPV was mainly responsible for URTI, although it caused 6.1% of total pneumonia cases. HBoV was generally detected in URTI, although it was responsible for pneumonia in one patient that required hospitalization [Terrosi et al., 2007]. We sequenced the three strains of coronaviruses detected by RT-PCR in order to correlate the severity of the pathology with the specific strain.

TABLE III. Etiologic Agents and Related Clinical Diagnosis

| | | Etiologically confirmed number of cases (% of the total) | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | Total cases | hMPV | HBoV | HCoV | RSV | PIC | Infl A | ADV |
| URTI | 166 | 7 (4.2) | 3 (1.8) | 3 (1.8) | 7 (4.2) | 44 (26.5) | 4 (2.4) | 22 (13.3) |
| LRTI | 71 | 2 (2.8) | 1 (1.4) | | 27 (38) | 17 (23.9) | | 3 (4.2) |
| Croup | 2 | | | | | 1 (50) | | |
| Bronchitis | 15 | | | | 2 (13.3) | 5 (33.3) | | 2 (13.3) |
| Bronchiolitis | 21 | | | | 16 (76.2) | 2 (9.5) | | |
| Pneumonia | 33 | 2 (6.1) | 1 (3) | | 9 (27.3) | 9 (27.3) | | 1 (3.0) |

Two were typed as OC43 and one was typed as NL63. All were detected in children with a mild respiratory tract infection; none of these children developed croup or bronchiolitis. Adenoviruses mostly caused a moderate to severe pharyngitis, but they were also detected in two children with bronchiolitis and one with pneumonia, accounting for about 4% of LRTI.

## DISCUSSION

The epidemiological and clinical features of ARTI can change according to seasonal and/or geographical differences and different etiologic agents [Williams et al., 2004; Choi et al., 2006]. Our study highlighted the particular characteristics of ARTI in Italian children from late autumn to the early spring months, evaluating every kind of respiratory illness, regardless of the presence or absence of fever, clinical severity or required hospitalization. The pediatric population analyzed in this study could be considered representative of the total pediatric population seeking medical care for ARTI, since we studied patients coming to the reference unit for pediatric infectious diseases. On this basis, we could clearly estimate the prevalence of each virus in community-acquired respiratory tract infections during this period of time.

PIC and RSV accounted for about 70% of the etiological agents in our population. RSV led to LRTI and hospitalization in a significantly higher percentage than other viruses, especially in children younger than 6 months of age. These data confirm the primary clinical relevance of this virus in pediatric patients. Interestingly, about 40% of PIC infections required admission to hospital and about 24% led to LRTI. These viruses are usually related to mild URTI and, for this reason, previous Italian studies rarely included them in their analysis [Maggi et al., 2003, 2007; Sarasini et al., 2006]. Our results underline the need for PIC investigation during viral diagnosis of respiratory tract diseases in light of recently published data regarding the association of some rhinoviruses with severe respiratory tract infections in children [Jacques et al., 2006; Renwick et al., 2007; Brownlee and Turner, 2008].

Among the emerging respiratory viruses, hMPV is one of the most frequently reported in recent international studies [Van den Hoogen et al., 2001; Boivin et al., 2007].

In Italy, it has been variously associated with URTI or LRTI, affecting from 3% to 13% of children with ARTI [Bosis et al., 2005; Gerna et al., 2005; Sarasini et al., 2006]. Our data appear to be in agreement with previous reports, revealing hMPV in 3.8% (9/237) of all samples. A wide variability in the hMPV circulation rate in the course of some years has been demonstrated [Maggi et al., 2003; Gerna et al., 2005]. Long sectional studies during the seasons and over the years seem to be essential to assess whether the alternation of high and low incidences could be a real epidemiological trend. Although the prevalence of hMPV was quite low in our population, hospitalization for pneumonia was required in over 20% of hMPV infections. In our study, no hMPV was detected in children older than 5 years, according to serological surveys, demonstrating that hMPV infection is acquired early in life [Van den Hoogen et al., 2001]. Observations that claim a clinical similarity between hMPV and RSV infection have mainly been based on cohorts of hospitalized children [Foulongne et al., 2006; Wilkesmann et al., 2006]. We analyzed a population of both inpatients and outpatients and we could therefore highlight that hMPV led to significantly lower rate of hospitalization and LRTI compared to RSV.

Only two studies have investigated the occurrence of HBoV infection in ARTI in Italy and they found a prevalence of 4–9% [Gerna et al., 2007; Maggi et al., 2007]. In our population, HBoV was detected at a rate of 1.7% (4/237 samples), lower than previously reported. However, the results are not comparable because of the different study design. In fact, none of the previous studies was prospective; moreover, one of these included only hospitalized children and showed differences in the rate of HBoV detection during a period of years [Maggi et al., 2007]. There is a growing consensus on the relationship between HBoV infections and ARTI, although high rates of co-infections with other respiratory viruses have been reported [Boivin et al., 2007]. In our population, HBoV was detected as the only virus in three out of four children and in one of these it caused wheezing pneumonia that required hospitalization, as previously described [Terrosi et al., 2007]. However, since the causal role of HBoV in LRTI still needs to be defined, a quantitative estimate of the viral load could be useful in clarifying its role in causing ARTI.

Our results show a low prevalence (1.3%, 3/237 samples) of human coronaviruses, in agreement with previous Italian studies [Esposito et al., 2006; Sarasini et al., 2006; Pierangeli et al., 2007]. Neither bronchiolitis nor pneumonia were detected in children infected by HCoV, indicating that these viruses can be most frequently associated with upper respiratory tract syndrome.

Simultaneous infections by different viruses have been well documented [Choi et al., 2006; Kaida et al., 2007]. In this study, co-infections with hMPV and HCoV were more frequently found in children. The clinical picture induced by co-infections was not more severe than that observed in patients with only one viral infection. In fact, only 2 out of 15 co-infections induced pneumonia; one of which was a triple infection (PIC-hMPV-ADV). Thus, further studies are needed to clarify the pathogenesis and interactions of co-infectants.

In conclusion, we were able to identify potential pathogens from more than 50% of studied ARTI, providing useful information on the clinical features and epidemiology of specific agents affecting children in cold months. In fact, because of the similar spectrum of clinical symptoms caused by different viruses in ARTI, ranging from mild upper respiratory symptoms in outpatients to severe pneumonia requiring hospitalization, viral infections do not appear to be easily distinguishable by clinical signs alone. A wide range of prospective studies, employing control populations, may

be helpful in evaluating the causal roles of these organisms and the risk factors that may correlate with more severe disease. Although routine surveillance of respiratory viruses might not seem to be cost-effective, continuous monitoring of ARTI etiology could be a useful means of planning the resources necessary for the development of new vaccines and antiviral agents.

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