Viruses are the major cause of pediatric respiratory tract infection and yet many suspected cases of illness remain uncharacterized. This study aimed to determine the distribution of several respiratory viruses in children diagnosed as having influenza-like illness, over the winter period of 2005–2008. Molecular assays including conventional and real time PCR protocols, were employed to screen respiratory specimens, collected by clinicians of the Influenza sentinel system and of outpatient pediatric clinics, for identification of several respiratory viruses. Of 1,272 specimens tested, 814 (64%) were positive for at least one virus and included 387 influenza viruses, 160 rhinoviruses, 155 respiratory syncytial viruses, 95 adenoviruses, 81 bocaviruses, 47 parainfluenza viruses, 44 metapneumoviruses, and 30 coronaviruses. Simultaneous presence of two or three viruses was observed in 173 of the above positive cases, 21% of which included influenza virus and rhinovirus. The majority of positive cases occurred during January and February. Influenza virus predominated in children older than 1 year old, with type B being the dominant type for the first season and subtypes A/H3N2 and A/H1N1 the following two winter seasons, respectively. Respiratory syncytial virus prevailed in children younger than 2 years old, with subtypes A and B alternating from year to year. This is the most comprehensive study of the epidemiology of respiratory viruses in Greece, indicating influenza, rhinovirus and respiratory syncytial virus as major contributors to influenza-like illness in children.

**KEY WORDS:** epidemiology; children; polymerase chain reaction; coinfection; Greece

**INTRODUCTION**

Respiratory viruses are a major cause of influenza-like illness in children and adults, leading to substantial morbidity and mortality each year [Kaye et al., 2006; Jansen et al., 2007; Camps et al., 2008; Johnson et al., 2009; Singleton et al., 2010]. Especially in young children (<1 year old) and elderly people (>65 years old) complications may occur, even though influenza-like illness is most often self-limited and restrained to the upper respiratory tract [Hall, 1987; Flamaing et al., 2003; Cilla et al., 2008].

**Abbreviations:** HRSV, human respiratory syncytial virus; HMPV, human metapneumovirus; HRV, human rhinovirus; HAdV, human adenovirus; HPIV, human parainfluenza virus; HBV, human bocavirus; HCov, human coronavirus; RT-PCR, reverse transcription PCR; rt-PCR, real time PCR.

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Influenza-like illness case definitions vary among different surveillance programs around the world, however in most cases, it is characterized by sudden onset of symptoms such as high fever (>38°C) and cough in the absence of other diagnosis [Aguilera et al., 2003; Nichol, 2006]. Other symptoms including myalgia, headache, chills and fatigue can only be used as optional inclusion criteria. In influenza-like illness cases in children, influenza virus has been the most commonly detected virus, followed by other respiratory viruses such as human respiratory syncytial virus (HRSV) and human metapneumovirus (HMPV) [Zambon et al., 2001; Talavera and Mezquita, 2007]. Symptoms caused by these viruses are similar, making it difficult for clinicians to associate clinical features of influenza-like illness with the presence of a specific virus. In addition, viral identification could be very useful in terms of patient management, in the prevention of unjustified use of specific antiviral drugs and/or antibiotics, as well as in the implementation of appropriate public health measures, which may contain the spread of highly contagious viruses within a hospital setting. The introduction of molecular methods has revealed that a wide spectrum of well-characterized respiratory viruses such as human rhinovirus (HRV), human parainfluenza virus 1, 2, and 3 (HPIV 1, 2, and 3) and human adenovirus (HAdV) as well as recently identified viruses, such as human bocavirus (HBoV) and human coronavirus (HCoV) can contribute to influenza-like illness [Furuse et al., 2010].

There is only a limited number of studies concerning respiratory tract infections in Greece, but they are dedicated mainly to one specific virus at a time [Tsolia et al., 2003; Xepapadaki et al., 2004; Papa et al., 2007; Gioula et al., 2010]. This study focused on screening for a wider panel of respiratory viruses contributing to influenza-like illness with the analysis of more than 1,200 specimens over a 3-year prospective study, thus revealing their incidence in children with influenza-like illness. Furthermore, the distribution of the detected viruses according to season and age group, their potential association to secondary respiratory symptoms and their pattern of coinfection were investigated.

MATERIALS AND METHODS

Patients

Pediatric patients (n = 1,272, age 0–18 years old) diagnosed with influenza-like illness over the winter seasons (November to May) 2005/2006 (n = 392), 2006/2007 (n = 377) and 2007/2008 (n = 503) were enrolled in the study. Clinicians were instructed to collect specimens within 3 days of a sudden onset of high temperature (≥38°C) and cough. Secondary symptoms included sore throat, coryza, myalgia, fatigue, headache, abdominal pain, and respiratory hindrance. Specimens were collected, on a certain day every week, by physicians in healthcare units and collaborating hospitals, providing services for nearly 70% of the whole Greek population. These included, sentinel-collected samples (n = 405) referred by the Hellenic Centre for Disease Control and Prevention (HCDCP) as well as, non-sentinel specimens (n = 867) from the outpatient clinics of pediatric hospitals of Southern Greece, including Athens. The majority of specimens were nose and throat swabs collected into a GLY-medium (Mediaproducts BV, Groningen, The Netherlands) using a Dacron swab (DeltaLab, Barcelona, Spain), as well as nasopharyngeal aspirates and bronchoalveolar lavages.

Clinical referral symptoms were recorded. All specimens were transported on ice to the National Influenza Reference Laboratory of Southern Greece and stored at −80°C until further analysis.

Nucleic Acid Extraction

Total nucleic acid (NA) was extracted from respiratory specimens using either the manual kit QIAamp Viral RNA Mini kit (Qiagen, Hilden, Germany) or the automated method NucliSens-EasyMag (BioMérieux, Marcy l’Etoile, France) according to the manufacturer’s instructions. Efficiency of these two NA extraction methods was identical (data not shown). To detect presence of inhibitors during NA extraction, a method including an internal amplification control was used [Nolan et al., 2006].

Molecular Methods for Detection of Respiratory Viruses

Nine different protocols for NA amplification were employed for viral detection (Table I), including reverse transcription PCR (RT-PCR), multiplex RT-PCR, multiplex nested RT-PCR, real-time PCR (rt-PCR) as well as multiplex real time RT-PCR (rRT-PCR). When necessary, cDNA synthesis was performed by reverse-transcription using the Moloney Murine Leukemia Virus Reverse Transcriptase (M-MLV RT, Promega, Fitchburg, WI) and random hexamer priming [Kuroiwa et al., 2004].

As positive controls in each assay, clinical samples found positive with culture and/or direct immunofluorescence, as well as samples derived from external quality assessment programs such as the Public Health Laboratory Centre of Hong Kong in collaboration with WHO and the European Quality Control for Molecular Diagnostics (QCMD), were used. In the case of HBoV and HMPV, where no positive sample was initially available, sequencing of the PCR products was employed to confirm the result. Specificity of virus detection was evaluated by sequencing, whereas cross reactivity was tested using clinical samples positive by culture or molecular methods for other respiratory or unrelated viruses.

All assays were optimized for MgCl₂, dNTPs, primer and probe concentrations as well as, annealing temperature and extension time, in order to achieve maximum sensitivity and specificity for each viral
target (Table I) [Elnifro et al., 2000; Gunson et al., 2006]. To determine the limit of detection (LOD), an indication of the analytical sensitivity of the PCR during the optimization process, plasmids expressing the gene target of interest were constructed using the TOPO-TA Cloning kit (Invitrogen, Carlsbad, CA) [Kuypers et al., 2004]. Each plasmid was 10-fold serially diluted and used for generation of standard curves for DNA quantification. Negative controls of nuclease-free water were included in every assay, as well as a low copy number positive control to ensure consistency in sensitivity.

**Statistical Analysis**

Univariate statistical analysis was done by Fisher’s exact and results were reported in terms of Relative Risk (RR) and 95% confidence intervals (95%CI).

**RESULTS**

A total of 1,272 samples from males (n = 708, 55.7%) and females (n = 564, 44.3%), were analyzed (mean age 4.5 years old, median age 4 years old, age range 20 days to 18 years old). Viral presence was confirmed in 193 (49.2%), 276 (73.2%), 345 (68.6%) cases in the three respective winter seasons (Table II). More specifically, 387 cases (47.5%) were positive for influenza virus, 160 (19.7%) for HRV, 155 (19%) for HRSV, 95 (11.7%) for HAdV, 81 (10%) for HBoV, 47 (5.8%) for HPIV, 44 (5.4%) for HMPV and 30 (3.7%) for HCoV. Detection rates observed between different age groups were 62.7% (age group 0 to <1), 64.8% (age group 1 to <5), 61.3% (age group 5 to <11) and 67.6% (age group 11–18) (Fig. 1). No significant difference with respect to individual or total viral detection rates was observed between the respective age groups of sentinel and non-sentinel patients (data not shown). All viruses were found participating in 173 (21.3%) mixed infections, of which 161 were dual and 12 triple infections (Table III). Individual virus detection rate varied over the surveillance periods and according to age.

Influenza virus was the most frequently detected viral agent in individuals older than 5 years old (RR: 2.082, 95%CI: 1.772–2.447) with activity peaking during January and February of each year. Influenza virus type B was the dominant recovered type for the first season (83.6%), while subtypes A/H3N2 (78.1%) and A/H1N1 (70.8%) dominated the following two winter seasons, respectively. HRV was the second virus detected most frequently and its appearance was more predominant at the beginning and the end during the first, second, and third winter periods. No significant difference in detection rates with reference to age was detected among the three different sampling periods. Simultaneous detection with other viruses occurred in 55.6% of HRV positive cases.

| Virus      | PCR protocol                  | Concentration | For/Rev annealing Tm (°C) | LOD (copies/reaction) | Refs.          |
|------------|-------------------------------|---------------|---------------------------|-----------------------|----------------|
| HRSV       | Multiplex nested RT-PCR       | 2.5<sup>a</sup> | 0.1/0.1<sup>a</sup> | 52                   | Stockton et al. [1998] |
| HRSV A     | 1.5<sup>b</sup>              | 0.2/0.2<sup>b</sup> | — | 52                   | —              |
| HRSV B     | 3.0/3.0<sup>b</sup>          | — | — | 52                   | —              |
| HRV        | Nested RT-PCR                | 3.5<sup>a</sup> | 1/1.2<sup>a</sup> | 50                   | Steininger et al. [2001] |
| HMPV       | RT-PCR                       | 3<sup>b</sup>  | 1/1<sup>b</sup> | —                    | —              |
| HPIV 1     | Multiplex RT-PCR             | 3<sup>b</sup>  | 0.6/1<sup>b</sup> | —                    | Banerjee et al. [2007] |
| HPIV 2     | 0.8/0.8                      | — | — | —                    | Erdman et al. [2003] |
| HPIV 3     | 0.4/0.6                      | — | — | —                    | —              |
| HAdV       | rt-PCR                       | 3<sup>a</sup>  | 0.5/0.3<sup>c</sup> | 200<sup>c</sup> | Heim et al. [2003] |
| HBoV       | rt-PCR                       | 5.5<sup>a</sup> | 0.6/0.9<sup>c</sup> | 100<sup>c</sup> | —              |
| HCoV 229E  | Multiplex rRT-PCR            | 5<sup>a</sup>  | 0.6/0.9<sup>c</sup> | 200<sup>c</sup> | —              |
| HCoV OC43  | 0.9/0.9<sup>d</sup>          | — | 60<sup>d</sup> | 60<sup>d</sup> | —              |
| Influenza A| Multiplex rRT-PCR            | 3<sup>a</sup>  | 0.6/0.6<sup>c</sup> | 300<sup>c</sup> | —              |
| Influenza B| 0.9/0.9                      | 300<sup>a</sup> | — | 50<sup>a</sup> | —              |
| A/H1N1     | Multiplex rRT-PCR            | 4<sup>a</sup>  | 0.6/0.6<sup>c</sup> | 200<sup>c</sup> | —              |
| A/H3N2     | 0.9/0.9<sup>a</sup>          | — | 300<sup>a</sup> | 50<sup>a</sup> | —              |

LOD, limit of detection; HRSV, human respiratory syncytial virus; HRV, human rhinovirus; HMPV, human metapneumovirus; HPIV, human parainfluenza virus; HAdV, human adenovirus; HBoV, human bocavirus; HCoV, human coronavirus; RT-PCR, reverse transcription PCR; r-PCR, real-time PCR.

<sup>a</sup>First PCR round.
<sup>b</sup>Second PCR round.
<sup>c</sup>Labeled at the 5’ end with FAM and terminally quenched at the 3’ end with Black Hole Quencher-1.
<sup>d</sup>Labeled at the 5’ end with CY3 and terminally quenched at the 3’ end with Black Hole Quencher-2.
<sup>e</sup>Labeled at the 5’ end with CY5 and terminally quenched at the 3’ end with Black Hole Quencher-2.
<sup>f</sup>Provided by the National Institute of Public Health and the Environment, Laboratory for Infectious Diseases and Perinatal Screening, Bilthoven, Netherlands to laboratory members of the formerly European Influenza Surveillance Scheme (EISS) network.
The majority of identified HRSV strains (91%) were recovered from preschool patients (RR: 4.912, 95%CI: 2.872–8.402). Especially for those younger than 1 year old, HRSV was the predominant isolated virus (33.5%). It circulated mostly during January and February of each year although the dominant subtype fluctuated between A (89.3%) over the first, B (70%) over the second and A (89.9%) over the third surveillance period.

HAdV was present in 95 (11.7%) cases of the total samples analyzed. Positive samples were identified during the whole surveillance period with activity peaking during January. Preschool children seemed to be infected more frequently than the older ones (RR: 2.408, 95%CI: 1.425–4.069), with the virus prevalence being more intense among the age group of 2–5 years old (61.1%), an age group which was also prone to mixed infections with HAdV (82.6%).

HBoV was detected in 10% of infected cases, 88.9% of which concerned preschool children (RR: 3.902, 95%CI: 1.970–7.726). HBoV seemed to follow a seasonal peak, coinciding with HRV, in early winter and late spring of every year. Simultaneous detection with other viruses occurred in 61.7% of HBoV positive cases.

HMPV and HPIV were detected at low rates during the entire surveillance period. HMPV was recovered from 44 samples and revealed a season peak during February and March. A higher rate of HMPV detection (82%) was observed in children younger than 5 years old (RR: 2.195, 95%CI: 1.029–4.680). On the other hand, HPIV caused approximately 6% of the positive cases and showed no variation throughout the study period. HPIV types 1 and 3 were observed during the first year (14.2% and 85.8%, respectively), types 2 and 3 during the second (18.2% and 81.8%, respectively) and types 1, 2, and 3 during the third year of study (4.6%, 54.5%, and 40.9%, respectively).

HCoV was the least detected virus with 3.7% of the clinical cases belonging to subtypes 229E or OC43. These two types were evenly distributed throughout the first year (41.7% 229E, 58.3% OC43); however, OC43 predominated during the second (100%) and 229E during the third year (81.8%).

Mixed, compared to single infections, showed a tendency to occur in patients younger than 5 years, which was statistically significant (RR: 1.430, 95%CI: 1.213–1.686).

### Table II. Distribution of Respiratory Viruses Including Mixed Infections Over the Three Winter Sampling Periods

| Virus   | 2005–2006 | 2006–2007 | 2007–2008 |
|---------|-----------|-----------|-----------|
|         | Nov.–Dec. (n = 86) | Jan.–Feb. (n = 218) | Mar.–May (n = 88) | Nov.–Dec. (n = 25) | Jan.–Feb. (n = 304) | Mar.–May (n = 48) | Nov.–Dec. (n = 39) | Jan.–Feb. (n = 324) | Mar.–May (n = 140) |
| Influenza | 11 (12.8) | 41 (18.8) | 17 (19.3) | 3 (12.0) | 157 (51.6) | 21 (43.8) | 1 (2.6) | 103 (31.8) | 33 (23.6) |
| HRSV     | 3 (3.5) | 19 (8.7) | 6 (6.8) | 2 (8.0) | 41 (13.5) | 5 (10.4) | 3 (7.7) | 55 (17.0) | 21 (15.0) |
| HRV      | 7 (8.1) | 5 (2.3) | 5 (5.7) | 11 (44.0) | 51 (16.8) | 3 (6.3) | 7 (17.9) | 42 (13.0) | 29 (20.7) |
| HAdV     | 11 (12.8) | 26 (11.9) | 11 (12.5) | 3 (12.0) | 9 (3.0) | 2 (4.2) | 7 (17.9) | 19 (5.9) | 7 (5.0) |
| HMPV     | 0 (0.0) | 8 (3.7) | 4 (4.5) | 0 (0.0) | 3 (1.0) | 0 (0.0) | 0 (0.0) | 3 (0.0) | 23 (7.1) |
| HPIV     | 7 (8.1) | 6 (2.8) | 1 (1.1) | 2 (8.0) | 9 (3.0) | 0 (0.0) | 7 (17.9) | 11 (3.4) | 4 (2.9) |
| HBoV     | 11 (12.8) | 11 (5.0) | 6 (6.8) | 3 (12.0) | 14 (4.6) | 0 (0.0) | 2 (5.1) | 23 (7.1) | 11 (7.9) |
| HCoV     | 1 (1.2) | 7 (3.2) | 4 (4.5) | 1 (4.0) | 4 (1.3) | 2 (4.2) | 0 (0.0) | 7 (2.2) | 4 (2.9) |

HRSV, human respiratory syncytial virus; HRV, human rhinovirus; HAdV, human adenovirus; HMPV, human metapneumovirus; HPIV, human parainfluenza virus; HBoV, human bocavirus; HCoV, human coronavirus.

aTotal number of samples analyzed.
1.037–1.972). Most of the mixed infections included HRV (51.5%) in combination with another respiratory virus, while in 40.5% influenza was the second virus detected (Table III).

The most frequent secondary symptoms among all cases positive for respiratory viruses were coryza (84.5%), sore throat (70.3%), fatigue (69.7%), headache (62.4%), and myalgia (49.7%) (Table IV). Furthermore, additional associations have been observed: 31% of patients who were infected with HAdV suffered from diarrhea and/or emesis and/or abdominal pain (RR: 3.312, 95%CI: 2.238–4.901), in 20% of the HBoV positive cases, patients presented with diarrhea (RR: 1.856, 95%CI: 1.112–3.096), 31.6% of HCoV positive patients suffered from emesis (RR: 2.473, 95%CI: 1.179–5.189) and 27.7% of HRV positive patients felt abdominal pain (RR: 1.584, 95%CI: 1.162–2.159). No difference in the severity of symptoms was observed between mixed and single infections.

**DISCUSSION**

This study examined the epidemiology of respiratory viruses in children of southern Greece with influenza-like illness over a period of three consecutive years. The application of molecular diagnostic methods allowed for the identification of a wide range of viral pathogens implicated in nearly 65% of these cases. Comparable detection rates have been reported in studies evaluating similar spectrum of respiratory viruses, although those studies referred to different sampling period and population makeup [Miller et al., 2007; Zhang et al., 2009].

### TABLE III. Subtypes of Respiratory Viruses Among Mixed Infections Tested

| Virus       | A/H3N2 | Influenza A/H1N1 | Influenza A/H3N2 | HRV in combination with another respiratory virus | HAdV | HMPV | HPIV1 | HPIV2 | HPIV3 | HBoV | HCoV 229E |
|-------------|--------|------------------|------------------|-----------------------------------------------|------|------|-------|-------|-------|------|---------|
| HRSV        | 5      | 6                | 2                |                                               |      |      |       |       |       |      |         |
| HRSVB       | 0      | 3                | 2                |                                               |      |      |       |       |       |      |         |
| HRV         | 12     | 19               | 5                |                                               | 13   | 2    |       |       |       |      |         |
| HAdV        | 0      | 0                | 5                |                                               | 14   | 0    | 7     |       |       |      |         |
| HMPV        | 1      | 1                | 1                |                                               | 4    | 0    | 5     | 0     |       |      |         |
| HPIV1       | 1      | 0                | 0                |                                               | 0    | 0    | 0     | 0     |       |      |         |
| HPIV2       | 1      | 1                | 0                |                                               | 2    | 0    | 0     | 2     | 0     |      |         |
| HPIV3       | 0      | 2                | 4                |                                               | 0    | 1    | 5     | 7     | 0     | 0    |         |
| HBoV        | 2      | 2                | 3                |                                               | 10   | 3    | 15    | 11    | 1     | 0    |         |
| HCoV 229E   | 3      | 0                | 3                |                                               | 0    | 0    | 2     | 0     | 0     | 0    | 2      |
| HCoV OC43   | 0      | 2                | 1                |                                               | 1    | 1    | 0     | 2     | 5     | 1    | 0      |

HRSV, human respiratory syncytial virus; HRV, human rhinovirus; HAdV, human adenovirus; HMPV, human metapneumovirus; HPIV, human parainfluenza virus; HBoV, human bocavirus; HCoV, human coronavirus.

### TABLE IV. Secondary Symptoms With Reference to Virus Detected

| Symptomsa | Influenza | HRSV | HRV | HAdV | HMPV | HPIV | HBoV | HCoV |
|-----------|-----------|------|-----|------|------|------|------|------|
| Coryza    | 87.3      | 76.0 | 78.7| 92.3 | 81.3 | 77.8 | 70.0 | 89.5 |
| Sore throat | 73.0     | 44.0 | 72.3| 92.3 | 62.5 | 66.7 | 60.0 | 68.4 |
| Fatigue   | 73.0      | 52.0 | 66.0| 92.3 | 68.8 | 77.8 | 50.0 | 57.9 |
| Headache  | 70.6      | 48.0 | 55.3| 53.9 | 37.5 | 55.6 | 40.0 | 52.6 |
| Myalgia   | 56.9      | 32.0 | 42.6| 46.2 | 31.3 | 66.7 | 40.0 | 26.3 |
| Conjunctivitis | 18.6 | 12.0 | 14.9| 15.4| 12.5 | 11.1 | 0.0  | 26.3 |
| Emesis    | 16.7      | 12.0 | 19.1| 30.8b| 12.5 | 0.0  | 10.0 | 31.6b|
| Abdominal pain | 17.6 | 20.0 | 27.7a| 30.8b| 18.8 | 0.0  | 10.0 | 15.8 |
| Respiratory hindrance | 7.8 | 16.0 | 8.5 | 15.4 | 12.5 | 11.1 | 0.0  | 0.0  |
| Diarrhea  | 9.8       | 4.0  | 8.5 | 30.8b| 16.7 | 11.1 | 20.0b| 5.3  |
| Clues of pneumonia | 2.5 | 12.0 | 2.1 | 15.4 | 18.8 | 11.1 | 10.0 | 5.3  |

HRSV, human respiratory syncytial virus; HRV, human rhinovirus; HAdV, human adenovirus; HMPV, human metapneumovirus; HPIV, human parainfluenza virus; HBoV, human bocavirus; HCoV, human coronavirus.

aComplete clinical data available for 85% of positive cases.

bRR: 3.312, 95%CI: 2.238–4.901.
cRR 2.420, 95%CI 1.461–4.009.
dRR 2.420, 95%CI 1.123–5.128.
eRR: 1.584, 95%CI: 1.162–2.159.
Influenza was the most prevalent virus detected accounting for nearly half of all viral infections, demonstrating a type and subtype variability from year to year reflecting the predominating virus subtype in the Greek population. HRV was the second more frequently detected virus (19.7%), highlighting it as an important contributor to influenza-like illness, an observation also made by others [Kelly and Birch, 2004; Druce et al., 2005], although this virus has been usually incriminated in common cold cases in the absence of high fever [Bellei et al., 2008]. HRSV presented similar detection rates to HRV, with subtypes A and B alternating from year to year [Deng et al., 2006]. Its circulation was simultaneous with influenza virus, a phenomenon reported before [Canducci et al., 2008; Fabbiani et al., 2009]. The proportion of positive HAdV samples in Greek pediatric influenza-like illness cases was slightly higher than other studies showed [Echavarria et al., 2006]. HBoV was the fifth most frequently detected virus accounting for 10% of the laboratory-confirmed infections, in line with previous reports [Manning et al., 2006; Lau et al., 2007]. HMPV cases (5.4%) were concordant with previous reports from Northern Greece [Gioula et al., 2010], while detection of types 229E and OC43 of HCoV, rarely associated with severe respiratory infections in children, amounted to approximately 4.0% of positive cases. A higher prevalence of HCoVs has been reported in infants with acute respiratory disease [Canducci et al., 2008], however NL63 and HKU1 HCoV subtypes were also included in that study.

Data on seasonal occurrence indicated that the majority of viral infections occurred between January and February, with the exception of HRV and HBoV infections which appeared typically at the beginning and the end of every observation period. HRV predominant circulation, before and after the influenza virus peak, has been associated with children returning to school and with weather conditions favoring HRV expansion [Monto, 2002, 2004], while its high prevalence might have moderated or prevented other viruses from establishing themselves [Greer et al., 2009]. On the other hand, it is expected that physicians screening for influenza-like illness would be more likely to swab influenza positive patients during its peak season. HBoV prevalence during the early winter and late spring has been described before [Arnold et al., 2006; Calvo et al., 2010], however a recent review has indicated that there is no obvious regular seasonal occurrence of the virus [Allander, 2008].

Total viral detection rates were similar among the four age groups, however, for some viruses, variability in prevalence with age was evident. Influenza virus predominated in children older than 5 years while HRSV, HMPV and HBoV prevailed in preschool children, an observation also made by others [Manning et al., 2006; Fabbiani et al., 2009]. There is a complexity in factors that could explain the observed age patterns and may relate to the host, the socio-economical environment, as well as viral genetic variation. Infant immune system immaturity and/or presence of maternal antibodies were identified as major host determinants [Crowe and Williams, 2003]. Presence of siblings, poor living conditions and crowding in schools, are all factors which facilitate the spread of respiratory viruses [Groothuis et al., 2011]. Moreover, viral genetic variation may contribute to the escape of antigenically distinct variants from previously acquired immunity or protective vaccination [Carrat and Flahault, 2007; Kirchberger et al., 2007].

In 21.3% of cases, combinations of two or three viruses were observed mainly including influenza virus and HRV (21%). However, it is difficult to clearly determine the contribution of HRV to infection, due to the fact that the virus is also detected in asymptomatic control patients [Peltola et al., 2008; Fry et al., 2011]. No potential relative contribution of HRV in mixed infections, with regards to the severity of referral symptoms, was observed, although a similar analysis with regards to the severity of disease progression was not feasible, due to lack of clinical information. Casalegno et al. [2010] studying co-circulation of HRV and pandemic A(H1N1) influenza virus indicated that HRV epidemics may interfere with the spread of the pandemic strain. Similar analysis in this study did not reach statistical significance, although patterns of HRV and seasonal influenza virus were analogous to those reported by Linde et al. [2009] for HRV and the pandemic strain. Undoubtedly, there can be no direct comparison between these studies, because the pattern of spread of pandemic and of seasonal influenza strains was different.

This study was conducted using high fever and cough as major inclusion criteria which only allowed us to observe differences between secondary clinical characteristics of viral infection. Interestingly, 20% of the infected HBoV patients presented diarrhea, suggesting that HBoV-related disease may extend beyond the respiratory to the gastrointestinal tract, defining it also, as a possible enteric pathogen [Kahn, 2007; Neske et al., 2007; Pozo et al., 2007]. Moreover, as emesis was present in one third of influenza-like illness HCoV-positive patients, it might be a useful potential indicator of HCoV infection in children. It is also worth mentioning that HAdV disease came along with significantly high proportion of emesis, diarrhea, and abdominal pain, which may reveal the existence of HAdV species affecting the enteric tract, although these types in respiratory specimens have rarely been published [Echavarria et al., 2006]. Finally, although mixed viral infections have been associated with more severe cases of respiratory infections in infants [Richard et al., 2008; Semple et al., 2005], our study was not designed to reveal such differences in disease severity, if any.

This study, carried over the winter period of three consecutive years, provides useful epidemiological data about respiratory virus circulation, establishing the major contribution of influenza virus, HRV and
HRSV to influenza-like illness in Greek children. To our knowledge this is the most comprehensive study to date of the etiologic agents associated with influenza-like illness in Greece. These results demonstrate that a wide range of respiratory pathogens are circulating in Greece and this fact needs to be considered by clinicians when treating patients reporting with influenza-like illness.

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