Abstract

Besides the influenza virus (IV), several other viruses are responsible for influenza-like illness (ILI). Although human parechoviruses (HPeVs) and enteroviruses (EVs) may impact on ILI, limited data on their epidemiological characteristics are available. During seven consecutive winter seasons (from 2010–2011 to 2016–2017), within the framework of an influenza surveillance system (InFluNet), 593 respiratory swabs were collected from children ≤5 years of age with ILIs. Molecular detection showed that 58.3% of swabs were positive for at least one of the viruses under study: 46% for IV, 13% for EV and 5.4% for HPeV. A single virus was identified in 51.3% of samples while more than one virus was detected in 7% of the samples. The risk of contracting IV was higher than the risk associated with EV, which in turn was higher than the risk of contracting HPeV. The risk of developing an IV infection was twofold greater in children >3 years than in those ≤3 years, who had higher risk of EV/HPeV infection. The frequency of EV/HPeV-positive swabs increased significantly during the 2016–2017 winter season compared to the previous six seasons. Sixteen EV genotypes were identified belonging to species A and B. HPeV-1 was the most frequently detected genotype, followed by -6 and -3. In this study, IV was mainly responsible for ILI, however EV and HPeV were also involved and particularly affected children ≤3 years of age. Influenza surveillance samples could provide us with valuable insight into the epidemiological features of viruses involved in ILI.

INTRODUCTION

Influenza-like illness (ILI) is one of the principal causes of mortality and morbidity in children under five years of age, globally [1]. The etiology of respiratory infections can be largely associated with viruses, of which influenza virus (IV) is the main contributor. In temperate regions, IVs (Orthomyxoviridae family) spread during winter time with an estimated attack rate of 10–35% in unvaccinated children [2]. IV infection can lead to hospitalization and death, especially among high-risk groups such as children under 2 years of age, pregnant women, the elderly and people suffering from specific medical conditions [3]. IV types A and B co-circulate at different levels during each season: H3N2 and H1N1 are currently the main IV A subtypes in circulation.

Although IV is the main contributor to ILI cases, the outbreaks may involve a variety of pathogens [4–6]; among these, human parechovirus (HPeV) and enterovirus (EV) are known to cause similar respiratory diseases to ILI [7–9]. HPeVs and EVs (Picornaviridae family) are spread worldwide [10], and children up to 5 years of age are the members of the population most affected by these viruses [11]. HPeV/EV infection generally results in mild self-limited illness, yet it may occasionally cause severe diseases (such as meningitis, encephalitis and acute flaccid paralysis) and even death [12, 13]. According to updated taxonomy, 19
different HPeV types have been identified, EV strains are divided into four species (A–D) which include more than 100 serotypes [11, 14]. Thanks to molecular characterization and phylogenetic analysis, an increasing number of EV and HPeV variants has been documented in the last few years [15–17]. Since 2014, the occurrence of EV-D68 outbreaks causing severe respiratory illness and death in children has raised concerns on the health threat posed by this emerging virus [18, 19]. In Italy, no data on EV and HPeV epidemiology and their contribution to ILI have been reported so far.

The objectives of this study were: (i) to evaluate the frequency and the risk of infection with IV, EV and HPeV in children up to 5 years with ILI in Lombardy (Northern Italy) during seven consecutive winter seasons (from 2010–2011 to 2016–2017); (ii) to perform the molecular characterization of circulating IVs, EVs and HPeVs in this population.

RESULTS

Characteristics of ILI cases

During the study period (from the 2010–2011 to the 2016–2017 season), 593 respiratory samples were collected from 593 children ≤5 years of age affected with ILI. The distribution of ILI cases by gender, age group, season and period of ILI onset is reported in Table 1. Overall, 56% (332/593; 95% confidence interval [CI]: 51.5–59.5%) of the children enrolled in the study were males. The median age was 3 years (Q1–Q3: 2–4 years); 60.9% (361/593; 95% CI: 56.9–64.7%) of the children were >3 years (Table 1). The distribution of ILI cases by season ranged between 10.5% in the 2010–2011 season and 21.1% in the 2015–2016 season (Table 1). Altogether, 50.8% (301/593; 95% CI: 46.7–54.8%) of ILI cases occurred in the second half of the influenza season (from February to April) (Table 1), while 65.1% (386/593; 95% CI: 69.5–76.7%) were reported in January and February alone.

Detection of IVs, EVs and HPeVs

Overall, 346 out of 593 (58.3%; 95% CI: 54.3–64.2%) samples tested positive for at least one of the viruses under study: 46% (95% CI: 42–50%) of ILI cases tested positive for IV, 13% (95% CI: 10.5–15.9%) for EV, and 5.4% (95% CI: 3.8–7.5%) for HPeV (Table 1). A single virus was identified in 51.3% (95% CI: 47.2–55.3%) of samples while more than one virus was detected in 7% (95% CI: 5.3–9.4%) of swabs (Table 1). Among the samples that tested positive for more than one virus, IV and EV were co-detected in 64.3% (95% CI: 49.2–77%) of cases; IV and HPeV in 30.9% (95% CI: 19.1–46%), EV+HPeV and IV+E+HPeV in one case (2.4%; 95% CI: 0.4–12.3%), respectively.

EV or HPeV were identified in 73 out of 320 IV-negative samples (22.8%; 95% CI: 12.5–17.7%); none of the viruses under study were detected in 41.7% of the swabs (95% CI: 37.7–45.7%).

The risk of infection with IV was approximately 6- and 15-fold higher compared to the risk of infection with EV (odds ratio [OR]: 5.7; 95% CI: 4.3–7.6) and HPeV (OR: 14.9; 95% CI: 10.2–22.3), respectively. The risk of infection with EV was 2.6-fold higher than that of HPeV (OR: 2.6; 95% CI: 1.7–4.1) and the risk of contracting a single infection was nearly 14-fold higher than for multiple infections (OR: 13.8; 95% CI: 9.7–19.8).

The median age of the children who tested positive was significantly higher than those who tested negative (3.5 years vs 3 years; P<0.001). The median age of both EV- (2.7 years, 2–4 years) and HPeV-positive (2 years, 1.7–3 years) children was significantly lower (P<0.01) than that of IV-positive children (4 years, 3–4.3 years) (Table 1). No difference was observed between the median age of children with single-virus- or multiple-virus-positive samples (4 years vs 4 years; P=0.3) (Table 1).

Most (75.1%; 95% CI: 69.6–79.8%) IV-positive samples were detected in children >3 years who had a 1.9-fold higher risk of infection with IV (95% CI: 1.4–2.7) than children ≤3 years (Table 2). Approximately half (50.6%; 95% CI: 39.7–61.5%) of the EV-positive children were ≤3 years with a 1.7-fold higher risk of infection (95% CI: 1.1–2.8) than children >3 years; the risk of infection with HPeV was 4.8-fold (95% CI: 2.3–10.4) higher among children ≤3 years, where 62.5% (20/32) of HPeV-positive cases occurred (Table 2).

As reported in Table 2, no significant differences were observed between genders regarding the risk of infection with the viruses under study, even if most of the positive samples came from males (Table 1). The risk of infection with more than one of the viruses under study did not increase according to age group, gender and period of ILI onset (Table 2).

According to the month of ILI onset, the risk of infection with EV and HPeV in the period spanning from November to January was significantly higher than that from February to April (EV=OR: 1.8; 95% CI: 1.1–2.9; HPeV=OR: 2.6; 95% CI: 1.2–6.0), while no difference in the risk of infection with IV was observed in both periods considered (OR: 1.1; 95% CI: 0.8–1.5) (Table 2).

As shown in Table 1, the proportion of IV-positive samples ranged between 38.7% (95% CI: 27.6–51.1%) in the 2010–2011 season and 54.8% (95% CI: 43.4–65.7%) in the 2012–2013 season (P<0.001). The highest proportion of EV- and HPeV-positive samples was recorded in the 2016–2017 season (28.9%; 95% CI: 20–40 and 13.2%; 95% CI: 7.3–22.6%, respectively) (Table 1), which differed considerably from the others due to the fact that influenza activity started 6 weeks earlier (Fig. 1): the majority (76.3%; 95% CI: 65.6–84.5%) of the ILI cases identified during the 2016–2017 season occurred between November and January.

Fig. 1 shows the weekly distribution of IV/EV/HPeV-positive and -negative respiratory samples and the incidence of ILI per 1000 children ≤5 years of age during the 2010–2011
Table 1. Characteristics of study children ≤5 years with ILI in Lombardy from the 2010–2011 to the 2016–2017 season and virological results

<table>
<thead>
<tr>
<th>No. of children</th>
<th>Total</th>
<th>IV detection</th>
<th>EV detection</th>
<th>HPeV detection</th>
<th>Single-virus detection</th>
<th>Multiple-virus detection</th>
<th>No virus detection</th>
</tr>
</thead>
<tbody>
<tr>
<td>% (95 % CI)</td>
<td>100 %</td>
<td>46 % (42–50 %)</td>
<td>13 % (10.5–15.9 %)</td>
<td>54 % (38.8–75 %)</td>
<td>51.3 % (47.2–55.3 %)</td>
<td>7 % (5.3–9.4 %)</td>
<td>41.7 % (37.7–45.7 %)</td>
</tr>
<tr>
<td>No. of males</td>
<td>332</td>
<td>151</td>
<td>46</td>
<td>23</td>
<td>174</td>
<td>25</td>
<td>133</td>
</tr>
<tr>
<td>% (95 % CI)</td>
<td>56 % (51.5–59.5 %)</td>
<td>55.3 % (49.4–61.1 %)</td>
<td>59.7 % (48.6–70 %)</td>
<td>71.9 % (54.6–84.4 %)</td>
<td>57.2 % (51.6–62.7 %)</td>
<td>59.5 % (44.5–73 %)</td>
<td>53.8 % (47.6–60 %)</td>
</tr>
<tr>
<td>Median age (years)</td>
<td>3</td>
<td>4</td>
<td>2.7</td>
<td>2</td>
<td>4</td>
<td>4</td>
<td>3</td>
</tr>
<tr>
<td>[Q1-Q3] (years)</td>
<td>[2–4]</td>
<td>[3–4.3]</td>
<td>[2–4]</td>
<td>[1.7–3]</td>
<td>[2–4.2]</td>
<td>[2–4.1]</td>
<td>[1.8–4]</td>
</tr>
<tr>
<td>Age group no. % (95 % CI)</td>
<td>≤3 years</td>
<td>232 (39.1 % (33.4–43.1 %)</td>
<td>68 (24.9 % (20.1–30.4 %)</td>
<td>39 (50.6 % (39.7–61.5 %)</td>
<td>20 (62.5 % (45.3–77.1 %)</td>
<td>95 (31.3 % (26.3–36.7 %)</td>
<td>18 (42.9 % (29.1–57.8 %)</td>
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<td></td>
<td>&gt;3 years</td>
<td>361 (60.9 % (56.9–64.7 %)</td>
<td>205 (75.1 % (69.6–79.8 %)</td>
<td>38 (49.4 % (38.4–60.3 %)</td>
<td>12 (37.5 % (23–54.7 %)</td>
<td>209 (68.7 % (63.3–73.7 %)</td>
<td>24 (57.1 % (42.2–71 %)</td>
</tr>
<tr>
<td>Season no. % (95 % CI)</td>
<td>2010–2011</td>
<td>62 (10.5 % (8.2–13.2 %)</td>
<td>24 (38.7 % (27.6–51.1 %)</td>
<td>7 (11.3 % (5.6–21.5 %)</td>
<td>1 (0.3 % (0.3–8.6 %)</td>
<td>24 (38.7 % (27.6–51.5 %)</td>
<td>4 (6.4 % (2.5–15.5 %)</td>
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<td>2011–2012</td>
<td>82 (13.8 % (11.3–16.8 %)</td>
<td>37 (45.1 % (34.8–55.9 %)</td>
<td>8 (9.8 % (5–18.1 %)</td>
<td>4 (4.9 % (1.9–11.9 %)</td>
<td>43 (32.4 % (41.8–62.9 %)</td>
<td>3 (3.7 % (1.2–10.2 %)</td>
</tr>
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<td></td>
<td>2012–2013</td>
<td>73 (12.3 % (9.9–15.2 %)</td>
<td>40 (54.8 % (43.4–65.7 %)</td>
<td>3 (4.1 % (1.4–11.4 %)</td>
<td>3 (4.1 % (1.4–11.4 %)</td>
<td>36 (49.3 % (38.1–60.5 %)</td>
<td>5 (6.8 % (3–15 %)</td>
</tr>
<tr>
<td></td>
<td>2013–2014</td>
<td>95 (16 % (13.3–19.2 %)</td>
<td>43 (45.3 % (35.6–55.3 %)</td>
<td>9 (9.3 % (5.7–17 %)</td>
<td>3 (3.2 % (1–8.9 %)</td>
<td>48 (50.3 % (40.6–60.4 %)</td>
<td>4 (4.2 % (1.6–10.3 %)</td>
</tr>
<tr>
<td></td>
<td>2014–2015</td>
<td>80 (13.5 % (11–16.5 %)</td>
<td>39 (48.8 % (38.1–59.5 %)</td>
<td>10 (12.5 % (7–21.5 %)</td>
<td>4 (5 % (2–12.2 %)</td>
<td>44 (55 % (44.1–65.4 %)</td>
<td>4 (5 % (2–12.2 %)</td>
</tr>
<tr>
<td></td>
<td>2015–2016</td>
<td>125 (21.1 % (18–24.5 %)</td>
<td>57 (45.6 % (37.1–54.3 %)</td>
<td>18 (14.4 % (9.3–21.6 %)</td>
<td>7 (5.6 % (2.7–11.1 %)</td>
<td>70 (56 % (47.2–64.4 %)</td>
<td>6 (4.8 % (2.2–10.1 %)</td>
</tr>
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<td></td>
<td>2016–2017</td>
<td>76 (12.8 % (10.4–15.7 %)</td>
<td>33 (43.4 % (33–54.6 %)</td>
<td>22 (28.9 % (20–40 %)</td>
<td>10 (13.2 % (7.3–22.6 %)</td>
<td>39 (51.3 % (40.3–62.2 %)</td>
<td>16 (21.1 % (13.4–31.5 %)</td>
</tr>
<tr>
<td>Month of ILI onset no. % (95 % CI)</td>
<td>November–January</td>
<td>292 (49.2 % (45.2–53.3 %)</td>
<td>126 (46.2 % (40.3–52.1 %)</td>
<td>49 (63.6 % (52.5–73.5 %)</td>
<td>23 (71.9 % (54.6–84.4 %)</td>
<td>145 (47.7 % (42.1–53.3 %)</td>
<td>26 (61.9 % (46.8–75 %)</td>
</tr>
<tr>
<td></td>
<td>February–April</td>
<td>301 (50.8 % (46.7–54.8 %)</td>
<td>147 (53.8 % (48–60 %)</td>
<td>28 (36.4 % (26.5–47.5 %)</td>
<td>9 (28.1 % (15.6–45.6 %)</td>
<td>159 (52.3 % (46.7–57.8 %)</td>
<td>16 (38.1 % (25–53.2 %)</td>
</tr>
</tbody>
</table>
and 2016–2017 seasons in Lombardy. The number of respiratory samples collected from ILI cases as well as IV detections reflect the weekly ILI consultation rates; ILI incidence and IV detection peaked between mid-January and mid-February (between week 4 and 6) in almost all of the seasons with the exception of 2016–2017 when they peaked in December (week 52) (Fig. 1). EV circulated throughout the winter months with no specific trend from 2010–2011 to 2014–2015, however an epidemic trend was observed in 2015–2016 [peak in week 8 (end of February); curve width: 9 weeks] and in 2016–2017 [peak in week 52 (end of December); curve width: 12 weeks]. No specific trend for HPeV was observed (Fig. 1).

**Molecular characterization of IVs, EVs and HPeVs**

**IVs**
Overall, 42.9 % (117/273; 95 % CI: 37.1–48.8 %) of IVs were subtyped as A(H3N2), 23.8 % (65/273; 95 % CI: 18.8–28.8 %) as A(H1N1)pdm09 and 33.3 % (91/273; 95 % CI: 28–39.1 %) as IV type B. Fig. 2 shows the IV type/subtype distribution according to season.

Nearly 30 % (80/273 : 29.3 %) of IV-positive samples were sequenced.

A(H3N2) IVs were detected in all of the seasons and predominated during the 2011–2012, 2013–2014 and 2016–2017 seasons (83.8 , 69.8 and 100 % of IV-positive samples, respectively). A(H3N2) variants were identified during the 2011–2012 and 2014–2015 seasons; all A(H3N2) viruses sequenced in these two seasons were characterized by several amino acid substitutions in their HA antigenic sites resulting in new viral variants different to those in circulation in the previous seasons. During 2011–2012, all A(H3N2) HA sequences belonged to clade A/Victoria/208/2009: approximately one-third fell into the genetic group 6 and two-thirds into group 3 (divided into subgroups 3A, 3B

<table>
<thead>
<tr>
<th>Age group</th>
<th>IV</th>
<th>EV</th>
<th>HPeV</th>
<th>Single virus</th>
<th>Multiple virus</th>
</tr>
</thead>
<tbody>
<tr>
<td>≤3 years</td>
<td>1</td>
<td>1.7 [1.1–2.8]</td>
<td>4.8 [2.3–10.4]</td>
<td>1</td>
<td>1.2 [0.6–2.2]</td>
</tr>
<tr>
<td>&gt;3 years</td>
<td>1.9 [1.4–2.7]</td>
<td>1</td>
<td>1</td>
<td>1.4 [1.0–1.9]</td>
<td>1</td>
</tr>
<tr>
<td>Gender</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Male</td>
<td>1 [0.8–1.4]</td>
<td>1.2 [0.7–1.9]</td>
<td>2.1 [0.9–4.8]</td>
<td>1.1 [0.8–1.5]</td>
<td>1.2 [0.6–2.2]</td>
</tr>
<tr>
<td>Female</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>Month of ILI onset</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>November–January</td>
<td>1</td>
<td>1.8 [1.1–2.9]</td>
<td>2.6 [1.2–6]</td>
<td>1</td>
<td>1.6 [0.8–3.1]</td>
</tr>
<tr>
<td>February–April</td>
<td>1.1 [0.8–1.5]</td>
<td>1</td>
<td>1</td>
<td>1.1 [0.8–1.5]</td>
<td>1</td>
</tr>
</tbody>
</table>

**Table 2.** Common OR and exact confidence limits (in square brackets) computed for risk of infection from IV, EV, HPeV and from single virus or more than one virus (multiple virus). Statistically significant results are indicated in bold.

**Fig. 1.** Weekly distribution of IV/EV/HPeV-positive and -negative respiratory samples and incidence of ILI per 1000 children ≤5 years from the 2010–2011 to the 2016–2017 season, Lombardy.
and 3C). All A(H3N2) strains sequenced in the 2014–2015 season belonged to the 3C genetic group.

A(H1N1)pdm09 IVs circulated to a greater extent during the 2010–2011 and 2014–2015 seasons (58.3 and 61.5 % of IV-positive samples, respectively); A(H1N1)pdm09 which circulated during the last seven seasons were similar to the A/California/7/2009 strain (>95.9 % identity), the novel H1N1 virus that was introduced into the human population during the 2009 pandemic.

Type B IVs were the predominant IVs circulating during the 2012–2013 and 2015–2016 seasons (72.5 and 78.9 % of IV-positive samples, respectively).

**EVs**

No EV-D68 was detected. A total of 16 out of 77 (20.8 %) EV-positive samples were successfully sequenced. These EV strains were divided into two species: A (6/16: 37.5 %) and B (10/16: 62.5 %). Fig. 3 shows the phylogenetic trees of EV species A and B. Overall, nine different genotypes were identified: four (44.4 %) belonged to species A (coxackievirus A-2, -4, -5 and -6) and five (55.6 %) to species B (coxackievirus A-9, B-2 and B-5, echovirus-11 and -13).

**HPeVs**

Twenty out of 32 (62.5 %) HPeV-positive samples were sequenced. The phylogenetic tree is shown in Fig. 4. HPeV-1 was the most frequently detected genotype (10/20: 50 %), followed by genotype 6 (8/20: 40 %) and 3 (2/20: 10 %). The overall similarity of the 20 HPeV nucleotide sequences ranged between 70.9 and 100 %. The similarity values among strains belonging to the same genotype were 87–99.5 %, 98.7 % and 93.5–100 % for HPeV-1, HPeV-3 and HPeV-6, respectively.

**DISCUSSION**

This study based on the influenza syndromic surveillance sentinel network evaluated the circulation of EV and HPeV beyond IV among children less than 5 years of age during seven consecutive winter seasons (from 2010–2011 to 2016–2017), demonstrating that IV was the main cause of ILI although EV and HPeV can also contribute to ILI incidence in children, particularly those ≤3 years of age.

Overall, 58 % of the study samples tested positive for at least one of the viruses under study (IV/EV/HPeV). This frequency is similar to the percentages observed in other studies in which the percentages of respiratory virus detection in children <5 years with ILI ranged between 51.6 and 68.1 % [7, 20, 21]. Other studies focused on children hospitalized for acute respiratory infections (ARI) [22] or on children/adult outpatients with ILI have reported virus detection percentages ranging from 32.4 to 42.7 % [23, 24], while a recent study by Taylor et al. has shown that at least one respiratory virus was identified in 79.6 % of ILI episodes in children under 10 years of age [6]. This wide heterogeneity of results among studies may depend on several factors such as the panel of respiratory viruses investigated, the methodology used, the target population and the study setting.

Although a single virus was identified in the majority of our ILI cases, multiple viruses were detected in 7 % of swabs similarly to the results reported in other Italian studies in which co-infection rates in children with ARI ranged from 4.8 to 5.2 % [22, 25]. In our study, two-thirds of co-infections were caused by IV and EV, and the remaining one-third by IV and HPeV. Lekana-Douki et al. have reported that 43.1 % of EV-positive samples among children with ILI were co-infections with IVs and other respiratory viruses [7]; Harvala et al. have demonstrated that at least one other virus was detected in two-thirds of HPeV-positive respiratory specimens collected from children <5 years of age [9].

In our study, IV was the most common virus to ILI which was detected in 46 % of the cases. This result is similar to those of other authors showing that between 24.7 [26] and 48.8 % [21] of ILI cases in children were caused by an IV
Fig. 3. Phylogenetic trees of EV species A (a) and B (b) of the VP1 nucleotide sequences of EV strains obtained from this study (N=16) (in bold) and reference strains (N=133). Poliovirus NCBI reference strain (accession number NC_002058) was placed as the root.
infection. Children >3 years had a twofold increased risk of infection from IV than children ≤3 years, which was in good agreement with the results obtained in other studies [6, 21, 24, 26] in which the rate of IV-positive ILI cases in children increased with age. In our ILI series, the risk of infection from IV was approximately 6- to 15-fold higher than the risk of infection from EV and HPeV, respectively. Moreover, the frequency of IV-positive samples varied from season to season ranging from 38.7 % in the 2010–2011 post-pandemic season – characterized by the predominant circulation of A(H1N1)pdm09 IVs, which greatly affected the pediatric population during the 2009 pandemic – to 54.8 % during the 2012–2013 season, which was predominated by IV type B, which may be due to the fact that IV type B had not circulated much in the study area since 2007–2008, therefore there were numerous susceptible
Fig. 4. HPeV phylogenetic tree of the VP3/VP1 nucleotide sequences of HPeV strains obtained from this study (N=20) (in bold) and reference strains (N=18).

children under 5 years of age. A similar situation was observed during the 2014–2015 season when IV was identified in 48.8 % of ILI cases; the 2014–2015 season was characterized by the co-circulation of A(H1N1)pdm09 and A(H3N2) IVs; this latter was a drifted variant of previously circulating A(H3N2) [27], whereas A(H1N1)pdm09 had not been circulating during the three previous winter seasons.

IV detection in our ILI series showed a characteristic epidemic trend peaking in the second part of January/beginning of February in all of the seasons under study but in the 2016–2017 winter season, when a peak was reached at the end of December, more than one month earlier than usual.

In our study, EV was the second most frequently identified virus; there was more than a twofold risk of infection from EV compared to HPeV. EV was detected in 13 % of ILI cases, with a frequency ranging from 4.1 % in 2012–2013 up to 28.9 % during the 2016–2017 winter season, which was two times higher than the frequency reported in the 2015–2016 season.

Our results are consistent with findings obtained in other similar studies on the molecular detection of EV in respiratory samples collected from children with ILI, in which the frequency of EV identification ranged from 5 to 21.6 % [7, 28, 29]. Contrastingly, most studies aimed at evaluating respiratory virus circulation have used commercial multiplex-PCR assays which do not enable EVs to be distinguished from rhinoviruses (RVs); these studies have reported higher EV/RV detection rates (19.8–41.5 %) in ILI outpatients [6, 23], probably due to the significant contribution of RVs in respiratory tract infections [22, 29].

In temperate climates, EVs have a characteristic summer–fall seasonal trend with peaks in June–October [30], although outbreaks can also occur during winter [31]. In our study, the risk of contracting an EV infection in the period between November and January was almost twofold greater than from February to April. EVs circulated throughout the winter season with no specific trend up to 2014–2015, while they exhibited an epidemic-like trend in the last two seasons, with an increased EV detection rate (nearly 30 %) among ILI cases in 2016–2017. These results are in good agreement with the results obtained in the ‘ECDC rapid risk assessment’ which reported increased numbers of EV detections since April 2016, with a widespread transmission of different EV strains throughout that year [32]. As reported by CDC [30, 33], although predominant serotypes change over time, the analysis of long-term surveillance data identified a core group of EVs that consistently appear among those most commonly reported. In our ILI series, several EV genotypes were identified belonging to species A (coxackievirus A-2, -4, -5 and -6) and B (coxackievirus A-9, B-2 and B-5, echovirus-11 and -13). However, due to the limited number of sequenced EVs, no seasonal trends of the circulating genotypes were observed.

Since 2014, EV-D68 infections in children with severe respiratory and/or neurological diseases have risen in the USA [18, 19] and in Europe [34, 35], thus creating considerable concern regarding this emerging virus. No EV-D68 was identified in our ILI series.

In our study, HPeV was identified in 5.4 % of cases. Data obtained from other studies [7, 9, 36] have shown frequencies of HPeV detection in children with respiratory infections ranging from 0.6 to 3 %. To date, limited data are available on the circulation of this emerging virus in respiratory samples since most studies on HPeV infection are focused on neurological diseases in very young children [12]. In this study HPeV-1 was the most frequently detected genotype, followed by -6 and -3, as reported by other authors [9, 36]. While HPeV-1 and -6 mainly cause respiratory diseases, HPeV-3 is known to cause severe diseases such as sepsis-like illness and meningitis [37–39].

The early winter (November–December) and late summer (July–August) seasonality of HPeV infections in temperate climates has been established [9, 40, 41]. In our study, most of the HPeV infections were detected in the first part of each season (November–January), with a more than twofold risk of infection from HPeV observed in February–April. Due to the limited number of HPeV detections, no seasonal trends of this virus were observed, which seems to circulate mainly in the winter months. Interestingly, the frequency of HPeV-positive swabs in 2016–2017 ILI cases was significantly higher (13.2 %) than that reported in the previous six seasons (1.6–5.6 %), thus suggesting an increased circulation of this pathogen in children ≤ 5 years during the last season. The high rate of EV and HPeV detections in the 2016–2017 season may reflect the early activity of IVs in the study area and the consequent increase in sampling in November–January.

It is well known that the highest incidence of EV infection is observed in infants and young children [9, 30]; in fact the risk of infection from EV and HPeV was from twofold to fivefold higher among children ≤ 3 years.

EV and HPeV were identified in 23 % of IV-negative swab samples. Overall, nearly 42 % of our swabs remained without etiology. It has been reported that diagnosis remains negative in 20.4–45.4 % of respiratory infections in children [6, 21, 25, 29]. The proportion of negative swabs in our study may be due to the presence of other pathogens – such as unknown viruses or bacteria, which were not investigated. The samples were deemed to be of sufficient quality since all of the swabs tested positive for RNase P, which assures specimen quality. Other viruses – such as RV and respiratory syncytial virus (RSV) – are known to play a major role in pediatric respiratory infections, as reported in several studies [22, 25, 26, 42].

Finally, although EV and HPeV are known to cause respiratory infections, they are not included in any surveillance programmes in Italy; the availability of respiratory specimens collected from ILI cases during the national influenza surveillance scheme may enable us to evaluate the effects
and epidemiological and virological features of these neglected viruses. A catch-all approach to the surveillance of respiratory viruses might be more cost-effective than the single pathogen path, particularly for cases in which residual samples from existing influenza surveillance testing can be used [43]. Gaining insight into these viruses will enable us to develop specific antiviral drugs and vaccines, with substantial beneficial effects on human health.

A limitation of this study is that samples were restricted to the influenza season, thus the prevalence of EV and HPeV refer to this period of the year only. However, since in temperate regions the incidence of ILI is negligible during inter-epidemics, the prevalence of EV and HPeV observed in this study ought to give a fair approximation of the contribution of these viruses to ILI.

In conclusion, this study showed a significant circulation of EV and HPeV in addition to IV in northern Italy during seven consecutive winter seasons. To the best of our knowledge, this is the first study evaluating EV and HPeV circulation among young children in Italy during several consecutive seasons. The availability of influenza surveillance system samples may enable us to gain valuable knowledge on the effects and epidemiological features of EV and HPeV and other pathogens in ILI. It is therefore essential to implement molecular methods and increase viral sequencing and typing in order to obtain more accurate information on the viruses involved in the occurrence of ILI.

METHODS

Influenza Surveillance Network and ILI case definition

The Italian Influenza Surveillance Network (Influent) is coordinated by the Italian Ministry of Health and is based on the participation of sentinel physicians (pediatricians and general practitioners) who survey approximately 2% of the general population seeking care in ambulatory facilities due to ILI occurrence. Sentinel physicians report data on the number of ILI cases and collect respiratory samples - nasal/throat swabs in viral transport medium (Virolcult, MWE Medical Wire, UK) - for virological evaluations at reference laboratories on a weekly basis. Virological surveillance begins in week 46 (mid-November) and ends in week 17 (end of April) of the following year [44].

The standard case definition of ILI is: sudden onset of fever (>38°C) or feverishness, one or more respiratory symptoms (cough, sore throat and/or shortness of breath) and one or more systemic symptoms (myalgia, headache and/or malaise) [45].

The ILI cases that occurred in children ≤5 years of age which were recognized by pediatricians operating within the Influent in Lombardy (a region in Northern Italy accounting for nearly 10 million inhabitants out of 60 million at national level) during seven consecutive winter seasons (from 2010–2011 to 2016–2017) were included in this study.

Data and samples result from a surveillance system that received regulatory approvals. All the patients and their parents or legal guardians received oral information and gave consent for swab and data collection. Data were collected for surveillance purpose and are totally anonymous.

This study was carried out in agreement with national regulations on data collection, storage and reporting, and patients’ confidentiality. The study accomplished the Helsinki Declaration of 1975, rev. 2000 [46].

Detection of IV, EV and HPeV

RNA was extracted from 200 µl of each respiratory tract sample and eluted in 100 µl of elution buffer using the Invisorb Spin Virus RNA Mini kit (Stratec Biomedical AG, Germany), according to the manufacturer’s instructions. In order to check extraction performance, a one-step real-time retro-transcription (RT) PCR assay targeting human ribonuclease P gene (RNase P) was carried out (TaqMan RNase P Assay, ABY dye/QSYprobe, Thermo Fisher Scientific, USA); samples showing a RNase P cycle threshold (Ct) value <40 were considered suitable to be tested for virus identification.

A one-step real-time RT-PCR assay was performed to simultaneously detect type A and B IVs using specific primer/probe sets targeting the matrix (M) gene and the nucleoprotein (NP) gene, respectively [47]. IV A positive samples were further subtyped by a one-step real-time RT-PCR assay using specific primer/probe sets for the hemaglutinin (HA) gene to discriminate between A(H1N1)pdm09 and A(H3N2) IVs [48].

A one-step real-time multiplex RT-PCR assay was carried out in order to simultaneously detect EV and HPeV genome using specific primer/probe sets targeting the 5’ untranslated region (5’ UTR) [49]. The reaction mixture contained 1 µM of each EV primer, 0.4 µM of each HPeV primer and 0.2 µM of each probe and was carried out with the following thermal profile: 50°C×30 min, 95°C×15 min, and 50 cycles at 95°C×15 s, 58°C×30 s, and 72°C×10 s. The EV-positive samples were then tested using a one-step real-time RT-PCR assay specific for 5’ UTR of EV-D68 [50].

All real-time RT-PCR assays were set up using 5 µl of RNA in a final reaction volume of 25 µl of AgPath-ID one-step RT-PCR reagents (Thermo Fisher Scientific, USA). Real-time assays were performed in a 7300 real-time PCR system (Thermo Fisher Scientific, USA). A sample was considered positive when its Ct value was <40.

Molecular characterization of IVs, EVs and HPeVs

All nucleotide sequences were obtained from clinical samples directly. The molecular characterization of a number of IVs was carried out by means of the nucleotide sequence analysis of the globular head region of the HA (HA1 subunit) specific for A(H1N1)pdm09 (nucleotide, nt. 64–1058) [51], A(H3N2) (nt. 174–1058) [52] and B IV (nt. 12–1169) [53].
The molecular characterization of EV and HPeV was carried out using specific primers targeting the gene encoding for the viral protein (VP) 1 of EV (nt. 2602–2977) [54] and the VP3/VP1 junction region of HPeV (nt. 2159–2458) [9].

Amplicons were purified using a commercial purification kit (NucleoSpin Gel and PCR clean-up kit, Macherey-Nagel, Germany) and sequenced according to the Sanger method. A(H1N1)pdm09, A(H3N2) and B IVs sequenced in this study were combined with reference strains retrieved from the Global Initiative on Sharing All Influenza Data (GISAID) EpiFlu database (platform.gisaid.org/epi3/). A multiple sequence alignment was conducted using the ClustalW program, implemented in the biological sequence alignment editor BioEdit [55]. EV/HPeV type identification was achieved firstly by carrying out nt sequence similarity searches using the Basic Local Alignment Search Tool (BLAST) from the National Center for Biotechnology Information (NCBI) website [56]. The EV and HPeV strains identified in this study were further aligned with reference sequences obtained from the GenBank database on the NCBI website [57] and summarized in Tables S1 and S2 (available in the online Supplementary Material) for EV and HPeV reference sequences, respectively. The phylogenetic analysis was conducted using the MEGA package, version 6.0 [58]. The best substitution model was selected by analysis of each sequence dataset (EV species A, EV species B and HPeV, respectively) with the models tool available in MEGA version 6.0 [58]. The phylogenetic tree of EV species A was constructed by the neighbour-joining method and the Kimura 2-parameter (K2) model using a discrete Gamma distribution (+G) with five rate categories, while the EV species B phylogenetic tree by the maximum likelihood method and the general time reversible (GTR) model using a discrete Gamma distribution (+G) with five rate categories and assuming that a certain fraction of sites are evolutionarily invariant (+I). The HPeV phylogenetic tree was constructed by the maximum likelihood method and the Tamura 3-parameter (T92) model using a discrete Gamma distribution (+G) with five rate categories and invariant sites (+I). To estimate the robustness of the phylogenetic trees a bootstrap analysis was performed and bootstrap values >70 % were considered significant. All sequences obtained in this study were deposited in the GenBank nucleotide database under the accession numbers MF919543–58 (EV) and MF919559–78 (HPeV).

Statistical analysis

The statistical analysis was performed using Open Source Epidemiologic Statistics for Public Health OpenEpi, version 3.03 [59]. The frequency of positive samples was expressed as a crude proportion with corresponding 95 % CI calculated with the Mid-P exact test assuming a normal distribution. Proportions between groups were compared using the Mid-P exact test based on binomial distribution. Lower (Q₁) and upper (Q₃) quartiles were computed. The risk of infection was expressed as the number of individuals with a laboratory-confirmed infection out of the total number of individuals with ILI and a specific characteristic. OR and exact confidence limits were computed. The two-sample t-test was used for continuous variables. A P-value <0.05 was considered significant (two-tailed test).

References


