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Surveillance of enteroviruses from paediatric patients attended at a tertiary hospital in Catalonia from 2014 to 2017

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ABSTRACT

Background: Enterovirus (EV) infections are usually asymptomatic or mild, but symptomatic infections can evolve to severe complications. Outbreaks of EV-A71 and EV-D68 have been recently reported worldwide, sometimes related to severe clinical outcomes.

Objective: To describe EV genetic diversity and the clinical outcomes from paediatric patients attended at a tertiary university hospital in Barcelona (Catalonia, Spain) from 2014 to 2017.

Study design: Specimens were collected from paediatric (< 17 years old) cases with suspicion of respiratory tract infection or EV infection. EV laboratory-confirmation was performed by specific real-time multiplex RT-PCR assay. Partial viral VP1 protein was sequenced for genetic characterisation by phylogenetic analyses.

Results: A total of 376 (7%) from 5703 cases were EV laboratory-confirmed. Phylogenetic analyses of VP1 (210; 81%) sequences distinguished up to 27 different EV types distributed within EV-A (82; 40%), EV-B (90; 42%), EV-C (5; 2%), and EV-D (33; 15%), in addition to 50 (19%) rhinoviruses. The most predominant were EV-A71 (37; 45%) and EV-D68 (32; 99%). EV-A71 was highly related to neurological complications (25/39, 63%), of which 20/39 were rhombencephalitis, and most EV-D68 (28/32, 88%) were associated with lower respiratory tract infections (LRTI), and exceptionally one (3%) with acute flaccid paralysis.

Conclusions: EV-A71 and EV-D68 were the most detected EV in respiratory specimens. EV-A71 was highly related to neurological disease and EV-D68 was often associated with LRTI. However, both potential relatedness to neurological diseases makes the monitoring of EV circulation obligatory.

1. Background

Enteroviruses (EVs) are lineal, single-stranded, positive-sense RNA viruses, belonging to the *Picornaviridae* family. More than 100 different types of human enterovirus (EV) have been described, distributed in 4 species (EV-A, -B, -C and -D), which together with rhinoviruses (RVs) and non-human EVs compose the genus *Enterovirus* [1,2].

EV infections are usually asymptomatic or mild, but infections can sometimes evolve to severe diseases. EVs have been implicated in a wide range of diseases, including hand-foot-mouth disease (HFMD), herpangina, respiratory syndrome, meningitis, encephalitis and acute flaccid paralysis (AFP), among others [3]. EV infections can occur at any age, but the paediatric population is the most susceptible, especially children < 5 years old, who do not have specific immune response to a particular type of EV [4].

EVs circulate around the world all over the year. In temperate countries, EV infections usually follow a seasonal pattern reporting the highest incidence during the late summer and early autumn [5,6]. However, a second peak can be also reported between spring and early summer, as occurred in our geographical area [7]. The circulation of the different EV types is variable from one year to another. Furthermore, while in some regions an EV might be endemic, in others it might be the aetiological agent of limited sporadic outbreaks [8]. The EV surveillance has been reinforced from 2014 and on due to EV-D68 and EV-A71 outbreaks in North America and Europe, respectively [9,10].

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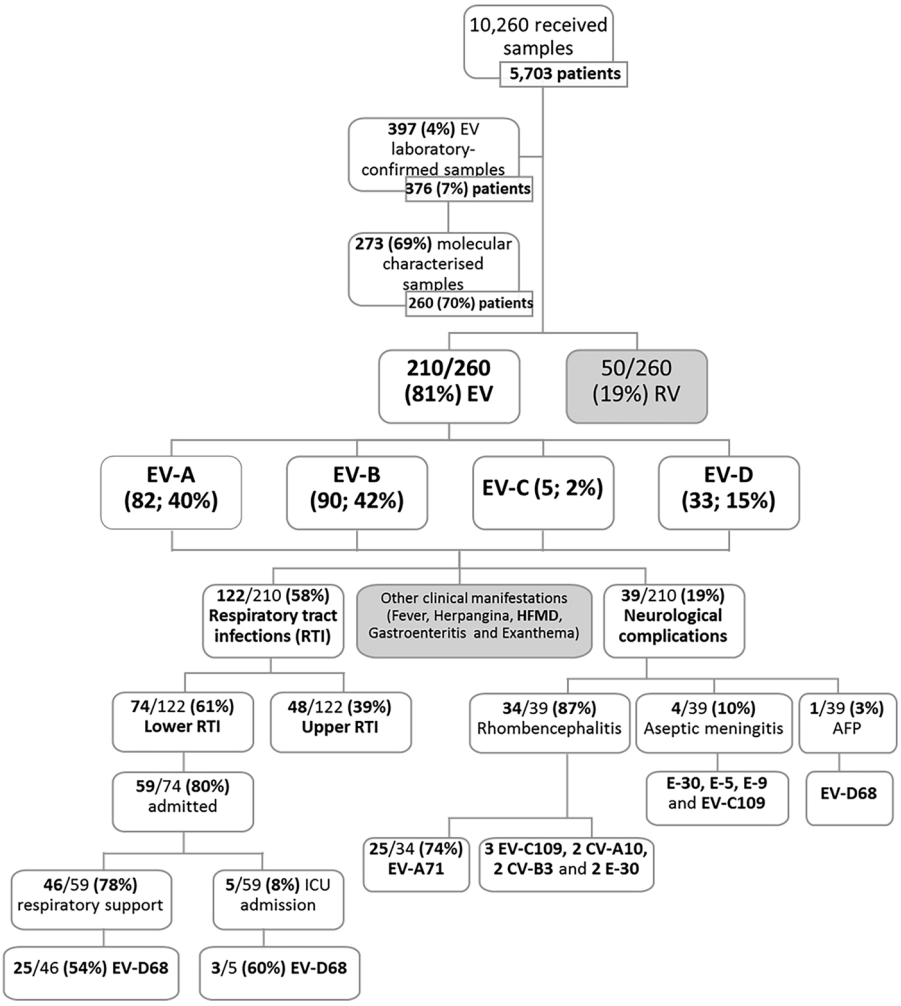

Fig. 1. Overview flow-chart of the complete study, distinguishing the different studied populations (number; percentage). EV refers to enterovirus; E to echovirus; CV to coxsackievirus; ICU to intensive care unit; and AFP to acute flaccid paralysis.

2. Objectives

The main objective of this study is to describe the seasonality, the prevalence, the genetic diversity and the clinical features related to respiratory EV from the paediatric cases attended at a tertiary hospital in Barcelona (Catalonia, Spain) during the 2014–2017 seasons.

3. Study design

This is a descriptive, observational, retrospective and longitudinal study performed at the Respiratory Viruses Unit of the Microbiology Department and the Paediatric Department at the Vall d'Hebron University Hospital.

3.1. Patients and samples

From October 2014 (week 40/2014) to May 2017 (week 20/2017), upper (nasopharyngeal aspirates or swabs) and lower (bronchoalveolar lavages, bronchoaspirates and tracheal aspirates) respiratory tract specimens were collected for respiratory viruses' laboratory-confirmation from paediatric patients (< 17 years old) with suspicion of acute respiratory tract infection (RTI) or enterovirus infection who were

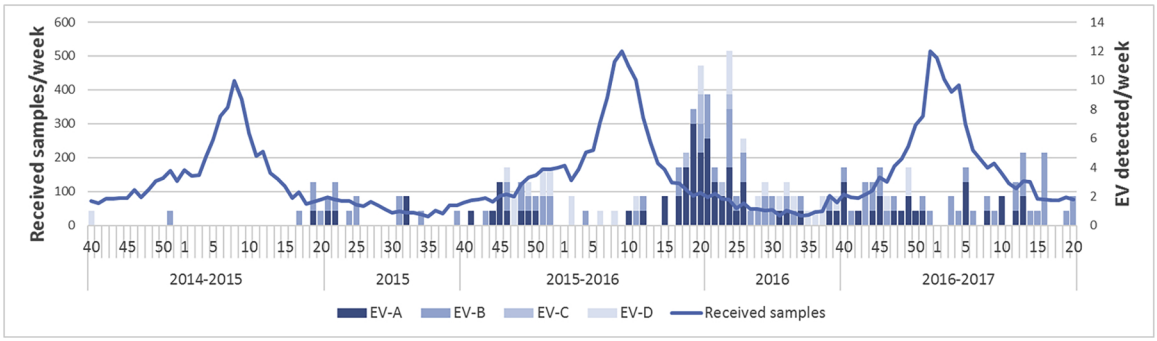

Fig. 2. Weekly enterovirus (EV) distribution by specie from week 40/2014 to week 20/2017.

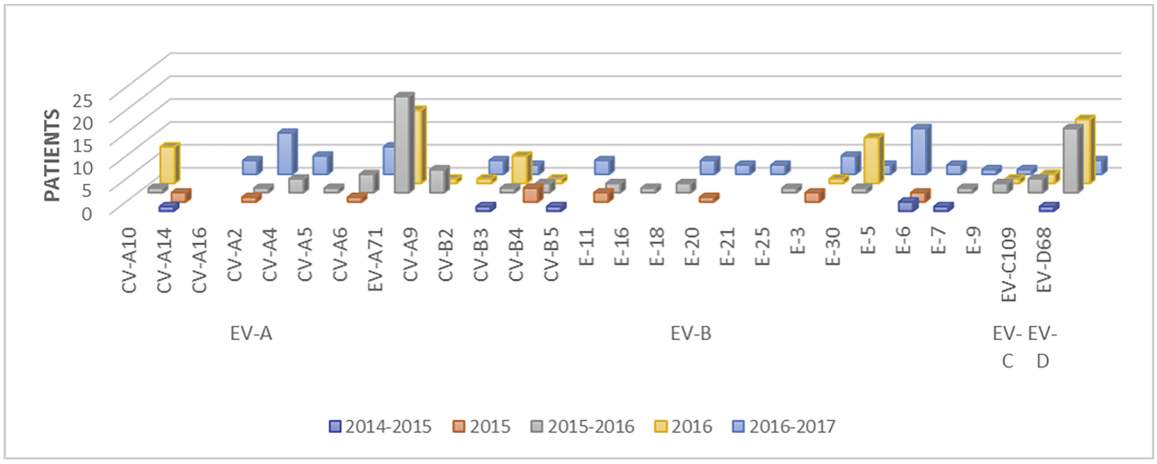

Fig. 3. Distribution of enterovirus (EV) types through the three consecutive seasons and inter-season periods. CV refers to coxsackievirus and E to echovirus.

attended at the emergency department or admitted to the hospital. Demographic features (sex and age) and clinical data were retrospectively collected from EV laboratory-confirmed cases.

The inclusion criteria, according to the hospital diagnostic pipeline, included all paediatric patients with respiratory infection or neurologic complication by EV infection (mainly during the 2016 outbreak). Upper RTI (URTI) included those respiratory infections from the nose to the larynx, excluding HFMD and herpangina. Lower RTI (LRTI) included recurrent wheezing, asthma, bronchiolitis, and pneumonia; LRTI severity was studied in patients requiring admission due to the RTI, to ensure that the length of hospital stay or the respiratory support were only related to the disease. Aseptic meningitis was defined as an inflammatory process of the leptomeninges causing signs and symptoms indicative of meningeal irritation, with a cerebrospinal fluid (CSF) study characterised by pleocytosis, normal or increased protein levels, and the absence of microorganisms on Gram stain and on routine culture. Rhombencephalitis was referred to inflammatory diseases affecting the hindbrain (brainstem and cerebellum), causing dysfunction of these structures, with normal or elevated leukocyte count in CSF. Typical magnetic resonance (MR) findings were considered in the diagnostic confirmation of rhombencephalitis, with the isolation of EV in respiratory samples. According to the World Health Organisation (WHO), AFP is defined as a sudden onset of paralysis/weakness in one or more limbs of a child < 15 years old with the absence or decreased myotatic reflexes.

3.2. Detection of EVs from respiratory specimens

Detection of EV was performed by specific real-time multiplex RT-PCR assays (Anyplex II RV16 assay, from October 2014 to November 2016, or Allplex Respiratory Panel Assay, from December 2016 to May 2017, Seegene, Korea). For samples received between October 2014 and November 2016, an additional real-time RT-PCR (Seegene, Korea) was performed to improve the detection of all EVs due to the inaccurate detection of some EVs, as previously described [11]. Prior to PCR-based assays, total nucleic acids were extracted using NucliSense easyMAG (bioMérieux, France) according to the manufacturer's instructions.

3.3. Phylogenetic analysis and characterisation of EVs

VP1 amplification and sequencing were performed based on the protocol recommended by the WHO [12], with minor modifications. PCR products purification was performed using Exo-SAP-IT (USB, Affymetrix Inc., USA) for single PCR products (350bp), or in case of unspecific PCR products in the 2% agarose gel electrophoresis, using Genelute Gel Extraction Kit (Sigma-Aldrich, USA). Purified PCR products were sequenced using the ABI Prism Big Dye Terminator cycle sequencing kit v3.1 (Thermo Fisher Scientific, USA) on the ABI PRISM 3130XL Genetic Analyser (Thermo Fisher Scientific, USA). Nucleotide sequences were edited and assembled using MEGA v5.2 [13] and preliminarily analysed by the Enterovirus Genotyping web-based Tool [14]. A multiple alignment of sequences together with reference sequences to EV types belonging to the four species (EV-A, -B, -C, and -D) downloaded from NCBI GenBank (Supplementary Table 1) was performed using the MUSCLE software in MEGA v5.2 [13]. Prior to phylogenetic analysis, the molecular evolutionary models of nucleotide substitutions were fitted to the multiple sequence alignments using evolutionary analysis, conducted in MEGA v5.2 [13]. The phylogenetic trees were constructed using a neighbor-joining distance method as implemented in MEGA v5.2 with the evolutionary model with the lowest Bayesian information criterion score [13]. The topological accuracy of the internal branch was evaluated by the bootstrap method (1000 replicates).

3.4. Statistical analysis

Statistical analysis was performed using SPSS v22 (SPSS Inc., USA). Continuous variables were described by the median and the interquartile range (IQR) and categorical variables by frequencies and proportions. Mann-Whitney and Chi-squared tests were calculated to assess associations between categorical variables. P values < 0.05 were considered to be statistically significant.

4. Results

During the study, 10,260 samples from 5703 patients were received and processed for laboratory confirmation. A total of 397 (4%) samples from 376 (7%) patients were EV laboratory-confirmed (Fig. 1). Moreover, other respiratory viruses were also detected, such as influenza viruses (FLUV) (21%), RVs (16%), respiratory syncytial virus (RSV) (9%), adenovirus (4%), coronaviruses (3%), human metapneumovirus (HMPV) (2%), bocavirus (1%), and parainfluenza viruses (1%).

4.1. Molecular characterisation

Partial VP1 coding-region was successfully sequenced from 273/397 (69%) samples from 260/376 (70%) patients. Phylogenetic analysis of partial VP1 sequences revealed that 210/260 (81%) strains fell within EV-A (82, 40%), EV-B (90, 42%), EV-C (5, 2%) and EV-D (33, 15%), in addition to 50/260 (19%) RVs (RV-A: 30, 60%; RV-B: 2, 4%; RV-C: 14, 28%; and undetermined RV: 4, 6%). Most EV detections (179/210; 86%) were in children < 5 years old and up to 27 different EV types

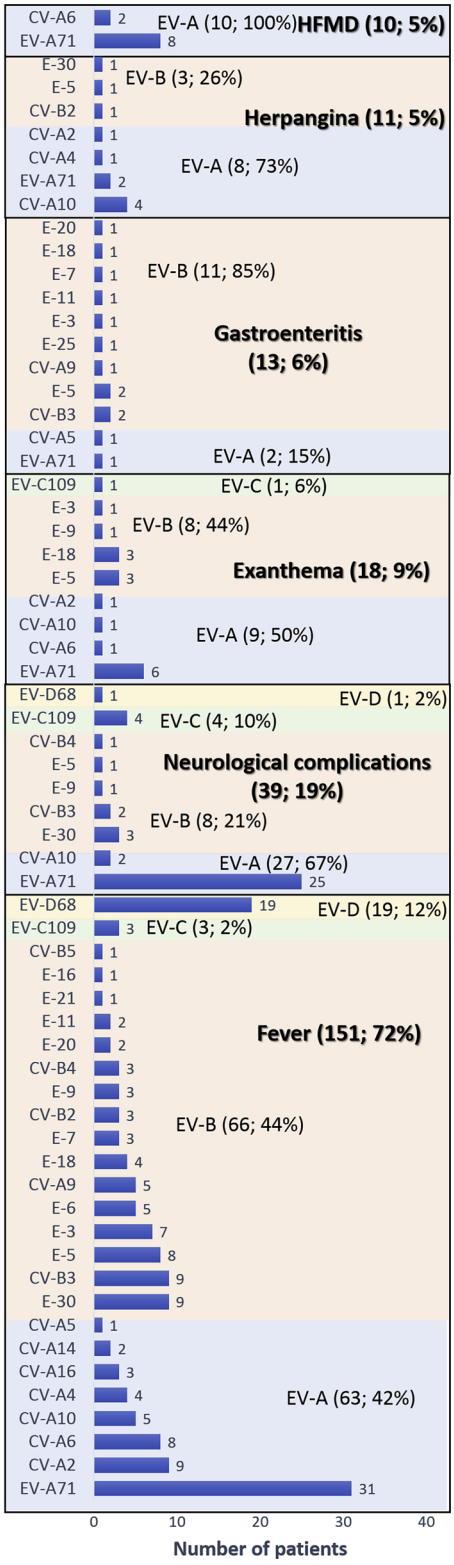

Fig. 4. Description of the clinical outcomes found among enterovirus-confirmed patients by specie and type. The outcomes (number; percentage) are represented by a black square and enterovirus (EV) species (number; percentage) are distinguished by colours: EV-A (blue); EV-B (orange); EV-C (green); EV-D (yellow). CV refers to coxsackievirus and E to echovirus. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

were distinguished. The EV seasonality was variable along the study, showing detection peaks during spring and autumn (Fig. 2). While some types of EV were sporadically detected, like echovirus (E)-25 and coxsackievirus (CV)-A5, other types were most frequently observed, particularly EV-A71 (35 belonging to subgenogroup C1, and 2 to C2) (Supplementary Fig. 1) and CV-A6 (11/210; 5%) in EV-A; E-30 (13/210; 6%) and CV-B3 (10/210; 5%) in EV-B and EV-D68 (29 belonging to clade B3, and 3 to B2) (Supplementary Fig. 2) in EV-D (Fig. 3).

4.2. Clinical manifestations

Regarding the clinical syndromes (Fig. 4), 151 (72%) patients had initially fever, followed by RTI (122; 58%) with a cough, dyspnoea or wheezing. A total of 39/210 (19%) neurological affections, such as meningitis, rhombencephalitis or AFP were observed. Occasionally, patients had exanthema (18/210; 9%), gastroenteritis (13/210; 6%), herpangina (11/210; 5%), and HFMD (10/210; 5%).

4.2.1. Respiratory symptoms

A total of 74/122 (61%) patients had LRTI and 48/122 (39%), URTI. EV-D68 (29; 24%) was the main virus related to RTI and children < 2 years old were the most affected population ($p = 0.915$) (Tables 1 and 2). In the LRTI group, 59/74 (80%) patients were admitted to the hospital due to the RTI (42% due to EV-D68) and the median length of stay was 3 days (IQR 2–5). Most patients (46/59; 78%) required respiratory support (51% conventional oxygen; 17% high flow nasal cannula; and 8% mechanical ventilation). Moreover, 5 (8%) patients required intensive care unit (ICU) admission (3 EV-D68, 1 CV-B4, and 1 not typed), in which the median ICU stay was 6 days (IQR 4–8.5), longer in the EV-D68 group (median 7 days; IQR 4–10) compared to CV-B4 (median 4 days; IQR 2–7) ($p = 0.35$).

Hospitalisations associated with EV-D68-related LRTI (25/28; 89%) were moderate and 22/28 (79%) were children < 5 years old. A total of 14 (50%) had recurrent wheezing or asthma and 14 (50%) had fever. The median length of stay was 3 days (IQR 2–5), in which 19/28 (68%) required respiratory support (58% conventional oxygen; 26% high flow nasal cannula; and only one required intubation and invasive mechanical ventilation) and 2/28 (7%) required non-invasive mechanical ventilation.

4.2.2. Neurological complications

Most of the studied cases (39; 19%) were from the 2016 spring outbreak occurred in Catalonia, of which 34 (87%) corresponded to rhombencephalitis, 4 (10%) to aseptic meningitis, and 1 (3%) to AFP. A total of 34/39 (87%) were children < 5 years old (Table 3). Aseptic meningitis was related to E-30, E-5, E-9, and EV-C109. The main symptoms were fever, headache, and positive meningeal signs, with pleocytosis in the CFS. EV laboratory-confirmation in the CSF was positive in all patients but one. Otherwise, rhombencephalitis was the principal neurological complication, in which most cases (25/34; 74%) were detected during the outbreak associated with EV-A71. A total of 20/25 (80%) patients had symptoms with MR findings suggesting rhombencephalitis and the remaining (5/25; 20%) had symptoms but MR was normal. Before the neurologic complications, 5/25 (20%) of cases began with EV-A71-related HFMD, 3/25 (12%) with herpangina (2 EV-A71 and 1 CV-A10) and 6/25 (24%) with exanthema (5 EV-A71 and 1 EV-C109). Only one patient developed permanent neurologic disabilities due to cardiorespiratory failure and hypoxic-ischemic encephalopathy, in addition to one AFP case, but associated with EV-D68 infection [15].

Table 1
Different enterovirus infections in the upper and lower respiratory tract. In upper respiratory tract infections, all cases different from herpangina or hand-foot-mouth disease (HFMD) were included. Percentages are calculated in columns.

EV* type	LRTI* N (%)	URTI* N (%)	Total N (%)
EV-D68	**28 (39)**	1 (2)	**29 (24)**
EV-A71	**4 (5)**	**5 (10)**	**9 (7)**
CV*-A6	**6 (8)**	2 (4)	**8 (7)**
CV-A2	2 (3)	**5 (10)**	7 (6)
CV-A4	**4 (5)**	3 (7)	7 (6)
E*-3	2 (3)	**4 (8)**	6 (5)
CV-A9	3 (4)	3 (7)	6 (5)
E-30	1 (1)	**4 (8)**	5 (4)
E-5	2 (3)	3 (7)	5 (4)
E-6	0 (0)	**4 (8)**	4 (3)
CV-A10	2 (3)	2 (4)	4 (3)
CV-B5	3 (4)	0 (0)	3 (2)
CV-B3	2 (3)	1 (2)	3 (2)
CV-B4	0 (0)	3 (7)	3 (2)
E-18	2 (3)	1 (2)	3 (2)
CV-B2	2 (3)	1 (2)	3 (2)
CV-A16	2 (3)	1 (2)	3 (2)
CV-A14	1 (1)	1 (2)	2 (2)
E-20	2 (3)	0 (0)	2 (2)
E-11	1 (1)	1 (2)	2 (2)
E-7	1 (1)	0 (0)	1 (1)
EV-B	1 (1)	0 (0)	1 (1)
EV-D	1 (1)	0 (0)	1 (1)
CV-A5	1 (1)	0 (0)	1 (1)
E-9	0 (0)	1 (2)	1 (1)
E-16	1 (1)	0 (0)	1 (1)
E-25	0 (0)	1 (2)	1 (1)
E-21	0 (0)	1 (2)	1 (1)
Total	**74 (61)**	**48 (39)**	**122**

*EV: Enterovirus.
*LRTI and URTI: Lower and Upper respiratory tract infections.
*CV: Coxsackievirus.
*E: Echovirus.

Table 2
Respiratory symptoms distribution by age groups. Percentages are calculated in columns.

Age groups	LRTI* N (%)	URTI* N (%)	TOTAL N (%)
< 2 years	35 (47)	25 (52)	**60 (49)**
2-4 years	29 (39)	18 (38)	**47 (39)**
5-14 years	7 (10)	5 (10)	**12 (10)**
15-64 years	3 (4)	0 (0)	**3 (2)**
TOTAL	**74 (61)**	**48 (39)**	**122**

* LRTI and URTI: Low and Upper respiratory tract infections; p = 0.915.

Table 3
Neurological complications distribution by age groups. Percentages are calculated in columns.

Age groups	AFP* N (%)	Meningitis N (%)	Rhombencephalitis N (%)	TOTAL N (%)
< 2 years	0 (0)	0 (0)	18 (53)	**18 (46)**
2-4 years	1 (100)	2 (50)	13 (38)	**16 (41)**
5-14 years	0 (0)	2 (50)	3 (9)	**5 (13)**
TOTAL	**1 (3)**	**4 (10)**	**34 (87)**	**39**

* Acute flaccid paralysis.

5. Discussion

The present study describes the epidemiology, the genetic diversity and the clinical features related to respiratory EV laboratory-confirmed infections in paediatric patients attended at a tertiary university hospital in Spain. In comparison with other respiratory viruses, the prevalence of EV was relatively low (7%) during the study period. However, regarding the disease presentation and compared to RVs, which were more prevalent but also more related to mild respiratory disease (unpublished data), clinical complications due to EV infection are reason enough to consider EVs as subjects under surveillance.

In temperate countries, EV circulation showed a clear pattern of seasonality with a major prevalence during late summer and early autumn, even though a second peak during spring it was also detected, as usually reported in Catalonia [7]. Moreover, the age is a determinant for the susceptibility, as the younger the age, the higher the susceptibility. Children < 5 years old were the most susceptible population to EV infection, as also described before [4,16].

The phylogenetic analysis of partial VP1 sequences revealed that most EVs belonged to EV-A and EV-B, while a minor percentage belonged to EV-C and EV-D. EV-A71 and EV-D68 were the most prevalent EV, particularly EV-A71 genogroup C (C1 and C2) and EV-D68 genogroup B (B2 and B3), which are the predominant genogroups circulating around Europe and worldwide [8]. EV-A71 and EV-D68 should be considered subjects under surveillance, not only because of the 2014 EV-D68-related outbreak and its relatedness to AFP, but also for the 2016 rhombencephalitis outbreaks associated with EV-A71 infection in several European countries [7,17].

An outbreak of EV-D68 was reported in North-America in 2014, characterised by severe respiratory illness and, occasionally accompanied by neurological complications [18]. Nonetheless, in the European region, a study reported a very low rate (2%), mainly associated with mild disease, with few severe cases [8]. The EV-D68 circulation was also low (32/5703; 0.6%) in the present study, although it showed a higher detection rate during 2016, like other Spanish reports [1,19] as well as during the current year 2018 in our hospital and recently reported data (October 2018) from the European Non-Polio Enterovirus Network (unpublished data). Regarding the clinical manifestations, most of EV-D68 detected were associated with LRTI, highlighting its possible association with severe respiratory illnesses [20]. Similarly to other European cases [21,22], only one case, a 2-year-old girl, developed AFP in February 2016 [15]. EV-D68 usually causes more severe disease in patients with asthma or reactive airway, as previously reported [23,24], but herein only one case was reported. Therefore, due to the relatedness of EV-D68 infection to severity, non-polio EV surveillance has been enhanced, and since EV-D68 has a predominant respiratory tropism, respiratory specimens should be studied to monitor EV-D68 circulation [25].

Otherwise, the second most predominant EV was EV-A71, mostly detected in specimens of cases involved in the 2016 rhombencephalitis outbreak occurred in Catalonia and other Spanish regions [1,7,10]. Neurologic manifestations of EV-A71 mainly occurred in children < 5 years old and rhombencephalitis was the most common neurologic presentation, but the EV-A71 detection at hospital level might be underestimated because of the severity of the disease. A question which really remained unanswered was whether EV-A71 simultaneously circulated in the community during the outbreak, but related to mild diseases such as HFMD or herpangina, and hence, attended at the Primary Care Centres. Likewise, CV-A6 is well-known to cause HFMD [26], and it could have circulated in the community during the study period. However, CV-A6 was not frequently detected in our series (11/210), maybe due to the mildness of the disease that does not require to go to the hospital. A recent French study conducted an ambulatory clinic-based surveillance to report the underestimation of EV detected only in hospitals [27].

It is well-known that one EV type can be related to several clinical manifestations, and inversely. CV-A10, E-5, E-30, CV-B3, and EV-C109 were also associated with neurological affections, as previously described [28–31], in addition to milder diseases. Instead, E-6 was only related to respiratory symptoms or fever, in discordance with previous studies [32]. Despite their low prevalence, all EVs are considered to be

neurotropic [33] and therefore, they should be subject under surveillance.

A relevant percentage of specimens confirmed as EV using the multiplex PCR-based assay (19%), were finally characterised like RVs. The high genetic similarity between RVs and EVs is an important factor that affects the specificity of diagnostic molecular methods, which are specially designed to distinguish between both viruses [11].

In summary, the present study reports the virological and clinical surveillance of EV in a tertiary hospital in Spain from 2014 to 2017. It reports recent and valuable information regarding the EV prevalence, seasonality, genetic diversity, and associated clinical features. In addition, this study remarks the necessity to perform EV surveillance in primary care settings to predict the early circulation in the community of all EVs, but those related to severe diseases such as EV-D68 or EV-A71.

Author contribution section

C.A., J.V. and A.A. conceived the presented idea and wrote the manuscript. C.A. developed the theory, performed the molecular characterisation by phylogenetic analysis and interpretation of the results. F.F., S.R., MC.M., and P.A. contributed in the sample preparation and performed the diagnosis. J.V. and M.B. collected all the clinical data and described the major clinical findings of the manuscript. T.P. and C.R. helped in supervising the project. All authors discussed the results and contributed to the final manuscript.

Conflict of interest

The authors declare no conflicts of interest.

Ethical approval

Institutional Review Board approval (PR(AG)173/2017) was obtained from the Vall d'Hebron University Hospital (HUVH) Clinical Research Ethics Committee.

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Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.jcv.2018.11.004.

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