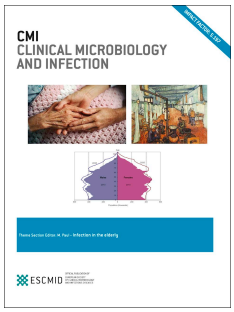
Accepted Manuscript



Outbreak of brainstem encephalitis associated with enterovirus-A71 in Catalonia, Spain (2016): a clinical observational study in a children's reference centre in Catalonia

Dídac Casas-Alba, Mariona F. de Sevilla, Ana Valero-Rello, Claudia Fortuny, Juan-José García-García, Carlos Ortez, Jordi Muchart, Thais Armangué, Iolanda Jordan, Carles Luaces, Irene Barrabeig, Rubén González-Sanz, María Cabrerizo, Carmen Muñoz-Almagro, Cristian Launes

PII: S1198-743X(17)30185-4

DOI: 10.1016/j.cmi.2017.03.016

Reference: CMI 900

To appear in: *Clinical Microbiology and Infection*

Received Date: 19 January 2017

Revised Date: 15 March 2017

Accepted Date: 18 March 2017

Please cite this article as: Casas-Alba D, de Sevilla MF, Valero-Rello A, Fortuny C, García-García J-J, Ortez C, Muchart J, Armangué T, Jordan I, Luaces C, Barrabeig I, González-Sanz R, Cabrerizo M, Muñoz-Almagro C, Launes C, Outbreak of brainstem encephalitis associated with enterovirus-A71 in Catalonia, Spain (2016): a clinical observational study in a children's reference centre in Catalonia, *Clinical Microbiology and Infection* (2017), doi: 10.1016/j.cmi.2017.03.016.

Outbreak of brainstem encephalitis associated with enterovirus-A71 in Catalonia, Spain (2016): a clinical observational study in a children's reference centre in Catalonia.

Dídac Casas-Alba[1], Mariona F de Sevilla[1,2,3], Ana Valero-Rello[2,4], Claudia Fortuny[1,2,3], Juan-José García-García[1,2,3], Carlos Ortez[5], Jordi Muchart[6], Thais Armangué[5], Iolanda Jordan[2,3,7], Carles Luaces[8], Irene Barrabeig[9], Rubén González-Sanz[10], María Cabrerizo[10], Carmen Muñoz-Almagro[2,3,8,11], Cristian Launes[1,2,3].

1) Department of Paediatrics, Hospital Sant Joan de Deu (University of Barcelona), Spain; 2) Paediatric Infectious Diseases Research Group, Institut de Recerca Sant Joan de Déu, Esplugues de Llobregat, Spain; 3) CIBER en Epidemiología y Salud Pública, CIBERESP, Spain; 4) Department of Molecular Microbiology, Hospital Sant Joan de Deu, Spain; 5) Department of Paediatric Neurology, Hospital Sant Joan de Deu (University of Barcelona), Spain; 6) Department of Diagnostic Imaging, Hospital Sant Joan de Deu (University of Barcelona), Spain; 7) Paediatric Intensive Care Unit, Hospital Sant Joan de Deu (University of Barcelona), Spain; 8) Emergency Departament, Hospital Sant Joan de Deu (University of Barcelona), Spain; 9) Epidemiological Surveillance Unit of Health Region, Barcelona-South, Public Health Agency of Catalonia, Hospitalet de Llobregat, Spain; 10) Enterovirus Unit, National Centre for Microbiology, Institute of Public Health "Carlos III", Madrid, Spain; 11) School of Medicine, Universitat Internacional de Catalunya, Barcelona, Spain.

KEY WORDS: Enterovirus; molecular epidemiology; types; EV-A71; brainstem; encephalitis.

RUNNING TITLE: Brainstem encephalitis and EV-A71 in Catalonia, Spain

CORRESPONDING AUTHOR:

Cristian Launes

Paediatric Infectious Diseases Research Group, Paediatrics Department

Institut de Recerca Pediàtrica Hospital Sant Joan de Deu

P. Sant Joan de Déu, no. 2, 08950 Esplugues, Barcelona, Spain

Phone: +34.93.280.55.69, Fax: +34.93.280.36.26; E-mail: claunes@sjdhospitalbarcelona.org

FUNDING: This study was partially supported by a grant from the Spanish National Health Institute [grant number PI15CIII-00020] and "Fundación Godia". Dr Armangue is supported by a grant from Instituto Carlos III (CM14/00081) and Dodot Procter & Gamble research grant sponsored by Asociación Española de Pediatría (AEP) (DN040579, TA).

TRANSPARENCY DECLARATION: The funders have not influenced the design or analysis, nor have they had any role in preparing the manuscript.

CONFLICT OF INTEREST: The authors declare no potential conflicts of interest.

ABSTRACT

Objectives: To describe the characteristics of an outbreak of brainstem encephalitis and encephalomyelitis related to enterovirus (EV) infection in Catalonia (Spain), a setting where these manifestations were uncommon.

Methods: Clinical and microbiological data from patients with neurological symptoms associated with EV detection admitted to a reference paediatric hospital between April and June 2016 were analysed.

Results: Fifty-seven patients were included. Median age was 27.7 months (p25-p75:17.1-37.6). Forty-one (72%) were diagnosed with brainstem encephalitis, 7 (12%) with aseptic meningitis, 6 (11%) with encephalitis, and 3 (5%) with encephalomyelitis (2/3 with cardiopulmonary failure). Fever, lethargy and myoclonic jerks were the most common symptoms. Age <12 months, higher white-blood-cell count, and higher procalcitonin levels were associated with cardiopulmonary failure. Using a PAN-EV real-time PCR, EV was detected in faeces and/or nasopharyngeal aspirate in all the patients, but it was found in cerebrospinal fluid only in patients with aseptic meningitis. EV was genotyped in 47/57 and EV-A71 was identified in 40/47, being the only EV type found in patients with brainstem symptoms. Most of the detected EV-A71 strains were subgenogroup C1. Intravenous immunoglobulins were used in 34 patients. Eight cases (14%) were admitted to the intensive care unit. All the patients but 3, those with encephalomyelitis, showed a good clinical course and had no significant sequelae. No deaths occurred.

Conclusions: The 2016 outbreak of brainstem encephalitis in Catalonia was associated with EV-A71 subgenogroup C1. Despite the clinical manifestations of serious disease, a favourable outcome was observed in the majority of patients.

INTRODUCTION

An outbreak of enterovirus (EV) infection affecting more than 100 children with acute brainstem symptoms was reported in Catalonia (Spain) between April and June 2016. EV detected in the first patients were typed as EV-A71 [1,2].

EV-A71 infections characteristically present as hand-foot-mouth disease (HFMD) or herpangina, but they are also associated with neurological pathologies [3,4], causing epidemics of aseptic meningitis, brainstem encephalitis, encephalomyelitis, and acute flaccid paralysis (AFP) largely restricted to infants [4]. The first association of EV-A71 with an outbreak of neurological disease occurred in California between 1969 and 1972 [5], although a subsequent retrospective study detected EV-A71 in samples from 1963 from the Netherlands [6]. In Europe, there were large outbreaks in Bulgaria and Hungary in the 1970s [7,8]. Since then, big outbreaks of EV-A71-associated illness with severe presentations have only been reported in the Asia-Pacific region, while sporadic cases and small outbreaks of mild to moderate disease have been reported worldwide [9-11]. Recent global concern about EV-A71-related neurological disease has increased due to the 2016 outbreak in Spain [2].

The great genetic diversity within EV, due to their error-prone RNA-dependent RNA polymerase [12] and the *intra*- and *inter*-species recombination events among EVs [13-15], together with the different socioeconomic conditions of each setting, may lead to differences in clinical expression and outcomes from one outbreak to another. Furthermore, facing to treat a completely unknown disease in our setting was even more difficult since various treatment approaches had been used in Southeast Asia [16-20], with little strong clinical evidence of their benefit. The WHO summarised the evidence and issued clinical guidelines for management of HFMD/EV-A71 illness of different severities [21].

The objectives of this study were to describe the clinical characteristics of patients with EV infection associated with acute neurologic manifestations during the outbreak, as well as to

detect variables associated with more severe disease and poorer prognosis. The study also describes the molecular EV typing of these cases.

METHODS

Study design

Epidemiological, microbiological, and clinical data were prospectively collected from all children with enterovirus-related neurological disease who were seen in or transferred for hospitalization to a tertiary paediatric hospital (Hospital Sant Joan de Déu, University of Barcelona) from April 15 to June 30, 2016. This hospital is a 300-bed reference medical centre for high-complexity pathologies that provides health care services to a paediatric population of ≈300,000 subjects. The centre has participated in a Spanish EV molecular surveillance network since 2010.

Inclusion criteria and definitions

Case definitions of the WHO Guide to Clinical Management and Public Health Response for HFMD [21] were used to define the inclusion criteria. Patients with the clinical diagnosis of brainstem encephalitis, encephalomyelitis with/without autonomic dysfunction, encephalitis, and aseptic meningitis were included if EV infection was detected in any sample and no other cause was associated with the clinical symptoms.

The Vietnamese Ministry of Health HFMD Classification and Management Guidelines were used to stratify the severity of patients with brainstem encephalitis or encephalomyelitis [22]. Ataxia was considered a Grade 2b Group 2 criterion [16]. See web-only Supplementary Table S1. The major variables considered in estimating severity were the Vietnamese classification, the presence of cardiopulmonary failure, and the persistence of neurologic symptoms with a modified Rankin Scale ≥ 2 (neurologic symptoms with significant disability) at days 14 and 30 from the onset of disease [23].

Management, including microbiological diagnosis and imaging tests

A protocol for the diagnosis and management of cases was established and it was applied prospectively during the study period.

Baseline assessment at presentation included history and physical examination, blood and CSF analysis, bacterial cultures, and collection of nasopharyngeal swab and stool samples in all the cases. An in-house PAN-Enterovirus real time-PCR [24] was performed in plasma and CSF in all patients. In addition, nasopharyngeal aspirate and stools were collected for EV detection by PAN-Enterovirus real time-PCR in patients with brainstem encephalitis or encephalomyelitis. RT-PCR for herpes simplex virus (HSV) in CSF was tested. The FilmArray Meningitis-Encephalitis (FA-M/E) panel was also tested in the CSF of the first 20 patients with brainstem symptoms of the outbreak [1]. Imaging studies were performed to all children with brainstem encephalitis and encephalomyelitis, except those with mild symptoms or quick recovery. The imaging studies included brain and spine magnetic resonance image (MRI) (diffusion-weighted, fast-spin-echo, T1 and T2-weighted, fluid-attenuation-inversion-recovery, and spoiled-gradient-echo sequences for the brain study, and T1 and T2-weighted for the spine study).

Treatment conformed to several guidelines for diagnosis and treatment of HFMD, recommending supportive care and consideration of IVIG in severe cases [16,21]. The clinical, radiological, and pathological assessments described for the Australian 2013 outbreak [20] were taken into special consideration to prescribe IVIG and/or methylprednisolone. IVIG (1 g/kg/day, once daily, for 2 days) was administrated to patients with lethargy, invalidating or persistent ataxia, progressive worsening, or paresis. Methylprednisolone (30 mg/kg/day, 1 time/day, for 3 days) was given to patients with clinical or MRI signs of myelitis and patients with brainstem MRI lesions and persistent symptoms in whom IVIG had already been administered.

Outpatient follow-up after discharge was performed on all patients at days 14 and 30 from onset of disease.

EV-positive samples were genotyped at the Enterovirus Unit of the National Centre for Microbiology using a RT-nested PCR in the 3'-VP1 region specific for species EV-A, B and C and sequencing according to a previously described procedure [25]. To study the relationships between Spanish EV-A71 strains and those circulating in other countries, a phylogenetic analysis was performed. Multiple sequence alignments were performed by the ClustalW program. Genetic distances were calculated using the maximum composite likelihood (MCL) nucleotide distance model, and statistical significance of phylogenies estimated by bootstrap analysis with 1000 pseudoreplicate datasets. Phylogenetic trees were constructed using the neighbour-joining (NJ) method in the MEGA software 6.0.

Statistical analysis

Descriptive statistics are reported in terms of absolute frequencies and percentages. Data comparisons of categorical variables were performed using Pearson Chi-square test or Fisher exact test when appropriate. Continuous non-normal distributed variables were described as median value and interquartile range (IQR) and compared using Mann-Whitney U test and Kruskal-Wallis analysis. Spearman's rho correlation coefficient was used to analyse the correlation between the time from the onset of neurologic symptoms to the initiation of therapies and the duration of symptoms after the onset of therapies. Statistical analysis was performed with SPSS v22.0 software (Armonk, NY: IBM Corp). A P-value < 0.05 was considered statistically significant. Relative risks and the 95% confidence intervals were calculated with MedCalc® software.

The institutional ethics board approved the study and informed consent was obtained from parents or carers.

RESULTS

Patient demographics

Sixty-three patients were admitted with neurological-symptoms and EV detection during the study period. In 6 of them, the informed consent could not be obtained and they were excluded from the study.

Of the 57 patients, 41 (72%) were classified with brainstem encephalitis following the WHO Classification, 7 cases (12%) were diagnosed with aseptic meningitis, 6 cases (11%) with encephalitis, and 3 cases (5%) with encephalomyelitis, two of them with cardiopulmonary failure.

Overall, the median age at disease onset was 27.7 months (IQR: 17.1-37.6) and 33/57 were males (57.9%). The first case was diagnosed on April 27 and the outbreak lasted 10 weeks. Figure 1.

Non-neurological manifestations

Supplementary Table S2 shows the main clinical symptoms of patients. Fever (axillary temperature ≥ 38ºC at home or in hospital) was the initial manifestation in all patients, with the median peak body temperature being 39ºC (IQR:38.5-39.5). The mean time from the onset of fever to admission was 48 hours (IQR:24-72 hours) and the fever lasted a median of 3 days (IQR:2-4 days). There were no differences in peak body temperature between patients with aseptic meningitis and patients with brainstem encephalitis or encephalomyelitis; however, fever lasted less in patients with aseptic meningitis. Table 1.

Among the muco-cutaneous manifestations, herpangina was observed only in 8 (14%) patients, whilst petechial rash on extremities was the main observed exanthema (11, 19%) followed by HFMD vesicular exanthema (10, 18%).

Neurological manifestations

The mean time from the onset of fever to the onset of neurological symptoms was 24 hours (IQR:0-72 hours). Within the first 24 hours of fever, the most common neurological symptoms were lethargy and/or irritability (17 (30%) patients). Myoclonic jerks, tremor, ataxia and/or cranial nerve involvement appeared subsequently in 44 children, mainly after 24 hours of fever (27 (61%) patients). Nystagmus and/or strabismus were observed in 8 patients (14%). The 3 patients with encephalomyelitis developed paresis with a marked weakness especially in the neck and shoulder region after 24 hours of fever, two of them experiencing bulbar palsy and autonomous nervous system (ANS) dysfunction in the form of cardiopulmonary failure as well. One patient had a typical febrile seizure.

Laboratory and imaging findings

White-blood-cell (WBC) count and procalcitonin were high in patients with severe ANS dysfunction, but normal in all the others. Table 1. No patient had significant alterations in plasma C-reactive protein. CSF white-cell count was significantly higher in patients with aseptic meningitis than in patients with brainstem encephalitis and/or encephalomyelitis. Table 1.

Forty-seven MRIs were performed, among which 25 (53%) were abnormal: 15 had bulbar involvement and 18 had medullar involvement. The most common MRI findings were high intensity lesions on T2-weighted images in the dorsal pons and medulla, midbrain, and dentate nuclei. In cases with medullar involvement, MRI showed bilateral high intensity lesions on T2-weigthed images in the anterior horn cells of the spinal cord and also in the posterior chords in some cases.

Virological findings

The PAN-Enterovirus real time-PCR detected EV genome in the CSF of 6/57 (11%) patients, being all of them patients with the diagnosis of aseptic meningitis. Negative patients in CSF were tested for EV detection in faeces and nasopharyngeal aspirate using the same PAN-

Enterovirus real time-PCR. The rates of detections were 40/46 (87%) in faeces and (35/51, 69%) in nasopharyngeal aspirate. In only 2 of 33 patients was EV detected in plasma.

On the other hand, the FilmArray M/E panel detected EV genome in CSF of 4/20 patients with brainstem symptoms who were negative for EV detection in CSF using the PAN-Enterovirus real time-PCR [1]. HHEV-6 was detected in 3 of 20 and the result was interpreted as latency in CSF [1].

No bacterial culture from CSF or blood was positive. No patient tested positive for HSV infection in CSF.

Detected EV was genotyped in 47 of 57 (82%) patients, being EV-A71 the most frequently identified type (40/47), followed by echovirus-30 (4/47), coxsackievirus(CV)-B1 (2/47), and CV-A10 (1/47). EV-A71 was the only serotype detected in patients with brainstem encephalitis or encephalomyelitis. Table 2 shows EV types and the WHO clinical diagnosis. EV-A71 was detected in the CSF from one brainstem encephalitis case and in another CSF sample, an EV-A was identified, but the specific serotype could not be determined. The rest of EV-A71 were typed from respiratory and/or stool detections.

Phylogenetic analysis carried out with 38 EV-A71 strains obtained in this study and others available in GenBank showed that most of the Spanish ones (35/38) belonged to subgenogroup C1 and only 3/38 to subgenogroup C2. Figure 2. Furthermore, Spanish C1 strains formed a subclade together with the variant Germany strain detected in 2015 [15, 26].

Management

Thirty-three patients (58%) received IVIG, among whom 26 also received steroids (46%). No patient with aseptic meningitis or encephalitis received treatment. The median lag time between the onset of fever and the administration of IVIG was 3 days (IQR:3-5), and from admission to its administration was 1 day (IQR:1-2). The median time from the onset of fever to corticosteroid administration was 4 days (IQR:3.5-5 days) and a median of 2 days (IQR:1-3)

passed from admission to its administration. Steroids were initiated in 6 cases with normal MRI, because of cranial nerve dysfunction (2 cases) and persistent symptoms (mainly, ataxia) despite the administration of IVIG (4 cases). Four patients did not receive steroids because of complete recovery before abnormal MRI results.

Outcomes and variables associated with a more severe disease

The neurological symptoms lasted a median of 5 days (IQR:3.5-8 days), and the median hospital stay was 7 days (IQR:4.5-9 days). Eight cases (14%) were admitted to the intensive care unit (ICU), due to decreased consciousness level for a short-observation period (6 cases) and severe shock (2) requiring mechanical ventilation and inotropes. The median ICU stay was 3.5 days (IQR:1.5-17.5).

Forty-four patients (77%) recovered fully while they were hospitalized and the remaining 13 patients (23%) were discharged with symptoms, ranging from mild ataxia in 10 patients, to paresis in 3. Paresis affected neck musculature chiefly (2 patients had hypotony on horizontal and vertical suspension, oral feeding disability and shoulder weakness, and 1 patient was bending the neck laterally). At day 30, all of them were improving and oral feeding was successfully recovered. Three additional patients presented persistent hyperreflexia in some extremity with no significant disability at day 30. There were no deaths.

With regard to epidemiological variables associated with severe ANS dysfunction, 2/9 patients < 12 month-old underwent cardiopulmonary failure, whereas no older patients had it (p=0.02). Higher white blood cell counts, higher procalcitonin blood levels, lower CSF white-cell counts were more common in patients with cardiopulmonary failure. As for MRI results, all the patients with cardiopulmonary failure had bulbar inflammatory lesions (2/2), but this finding was also found in patients without ANS dysfunction (13/45), so the differences were not significant. Table 1.

Sequelae with significant disability at day 30 were related to the presence of encephalomyelitis independently of cardiopulmonary failure, using both the case definitions of the WHO (relative-risk (RR): 96.2; 95%CI: 6-1557) and stage 4 of the Vietnamese classification (RR: 31; 95%CI: 6-163). Table 3. We did not find any correlation between the prompt initiation of IVIG and the duration of neurological symptoms after the onset of treatment (Spearman rho=-0.24, p=0.20). However, the lag time between the onset of symptoms and the onset of corticosteroids, and the duration of neurological symptoms after the onset of treatment, correlated inversely (Spearman rho=-0.56, p=0.01). No paresis/ANS dysfunction was observed in patients who had been administered corticosteroids and/or IVIG.

DISCUSSION

This is the first report of an outbreak of central nervous system (CNS) disease associated with EV-A71 detection in Spain. In all the patients with brainstem or encephalomyelitis symptoms in whom EV could be typed, EV-A71 was found. The other EV types were in the minority and were only found causing benign entities. The outbreak occurred in spring, showing a similar seasonal pattern to that reported for other EVs in Spain [9,25]. It affected children of around 2 years of age, like other EV-A71 epidemics elsewhere [4,20,27]. Although the EV-A71 outbreak seemed to be largely restricted to Catalonia, some sporadic cases in the rest of Spain occurred (no published data). Furthermore, a smaller concomitant outbreak in France has recently been reported [28]. EV-A71 is classified into 7 genogroups, A–G, on the basis of the diversity of the nucleotide sequences of the VP1 protein capsid. In the last years, increasing epidemic activity of genogroups B3-B5 and C1-C5 has been reported in the Asian-pacific region while genogroup C1 to C5 viruses were also detected in Australia. In Europe, most of the EV-A71 detected belongs to C1 and C2 [12]. The phylogenetic analysis showed that most of the EV-A71 detected in 2016 in Catalonia belonged to subgenogroup C1. Our centre participates in a Spanish EV

surveillance network that reported low-level circulation of EV-A71 in recent years, associated with non-severe neurological symptoms [10,29]. These EV-A71 C1 viruses had not previously been detected in Spain [10] and were closely related to a new cluster of EV-A71 C1 viruses identified in Germany and France in 2015 and 2016, respectively, suggesting that this new variant has spread from the North to the South of Europe [15,26,28]. The Public Health Agency of Catalonia conducted an epidemiological research and they did not find an association between the first cases suggesting a common origin neither a clear connection between cases [30].

Similarly to poliomyelitis, detection of EV-A71 from sterile sites is specific but usually insensitive [21], while detection from rectal and throat swabs is more sensitive but less specific and may include asymptomatic carriage. The clinical similarity between the patients as well as the detection of EV-A71 in all the cases of brainstem encephalitis and encephalomyelitis guided us to the etiologic diagnosis of the outbreak. Wide networks of EV molecular surveillance in European countries may help to identify new virulent variants and to assist in early detection of the etiology and epidemiological connections of similar outbreaks, which in turn may help to guide clinical management and foster optimal diagnostic strategies.

We found a very low rate of patients with EV-A71 and mucocutaneous manifestations, which is concordant with previous literature reporting a wide variability of manifestations depending on the dermatotropism and neurotropism of the circulating strain [8,31]. This fact made a prompt diagnosis harder. Initial manifestations could be indistinguishable among patients with meningitis, encephalitis, brainstem encephalitis, and encephalomyelitis, but patients with the later two progressively developed myoclonic jerks, tremor, ataxia, and, among a minority, cranial nerve involvement. In patients with aseptic meningitis, fever duration was shorter, pleocytosis was higher, and the CSF was more likely to be positive for EV, as in other studies [32]. Consequently, patients with a suspected diagnosis of aseptic meningitis and persisting fever should undergo close clinical observation and may require further investigation.

316 Regarding variables associated with a more severe disease, most of our patients were
317 classified in the Stage 2b Group 2 of the Vietnamese classification at the peak of the disease.
318 During hospitalization, some patients worsened to Stage 2b Group 2, but no patient developed
319 ANS dysfunction (Stages 3 and 4), in contrast to other series [26]. There were no deaths, which
320 seems plausible considering that the case-fatality rate for this disease is low (0.4%) [26].
321 Nevertheless, two cases had signs of severe ANS dysfunction at admission. Both of them were
322 < 12 months old, in line with other series that have inversely correlated the age at onset with
323 the severity [18,33,34]. Leucocytosis has also been described in the most severely affected
324 children, as has a more prolonged fever [19,35]. As with the outbreak in Australia in 2013 [20],
325 this study also supports the usefulness of the 2011 WHO guidelines for establishing the risk of
326 sequelae using the case definitions of each clinical entity. Persistent paresis at day 30 was only
327 observed in the 3 patients with encephalomyelitis.
328 The role of IVIG treatment in reducing acute morbidity and mortality rates is controversial [16-
329 20]. Despite the main indication of IGIV treatment being clinical severity [16,21], we used the
330 MRI to guide the treatment, following some other experiences [20]. The frequency and type of
331 MRI findings in our patients with severe symptoms (Stage 2b Group2) were consistent with
332 previous reports [20,36,37], but we found an unexpected number of patients with non-severe
333 symptoms (Stage 2a) and bulbar involvement on MRI. Furthermore, some patients with
334 abnormal MRI were not treated due to the timing of imaging and recovery. This limits the
335 conclusions that can be drawn about the role of MRI in treatment planning, as some patients
336 may not need to be treated if they continue to improve despite abnormal imaging.
337 Additionally, no conclusions can be drawn concerning the worsening of neurological
338 manifestations without IVIG treatment, as all the patients with severe disease received it.
339 Regarding steroids, they are a common treatment in viral and inflammatory myelitis [38], and
340 their use in EV-A71 disease has been documented [20, 39]. The prompt use of corticosteroids
341 in children with no severe symptoms has been also associated with increased risk of

subsequent severe disease in other observational studies [40,41]. In our series, most of the patients received the treatment after several days of symptoms, and a worsened clinical course was not observed in them. Additional research about the effectiveness and safety of IVIG and steroids is imperative.

One of the main limitations of this study is that the small number of patients with ANS dysfunction limits the extrapolation from the data. It is also an observational study and this fact limits the conclusions about the efficacy of treatments.

To conclude, 57 patients with CNS disease due to EV, sought treatment in our department. Most of them were infants with brainstem encephalitis, a manifestation rarely observed in our setting previously. EV-A71 was detected in all the cases of brainstem encephalitis or encephalomyelitis. Most of the EV-A71 belonged to subgenogroup C1, which was closely related to a new cluster of EV-A71 C1 viruses identified in Germany and France in 2015 and 2016. Treatment with IVIGs and corticosteroids was used according to the severity, but no conclusions about their efficacy can be drawn. Age < 12 months, longer fever, higher WBC-count, and higher procalcitonin levels were related to a more severe disease. All the patients but the 3 who presented with signs of encephalomyelitis before treatment showed a good clinical course and had no significant sequelae at day 30.

ACKNOWLEDGMENTS

Thanks to all the clinical, laboratory and diagnostic imaging personnel who take care of patients and who organized the response and management of the outbreak in our centre.

FUNDING

This study was partially supported by a grant from the Spanish National Health Institute [grant number PI15CIII-00020] and “Fundación Godia”. Dr Armangue is supported by a grant from

Instituto Carlos III (CM14/00081) and a Dodot Procter & Gamble research grant sponsored by Asociación Española de Pediatría (AEP) (DN040579, TA).

The funders have not influenced the design or analysis, nor have they had any role in preparing the manuscript.

CONFLICT OF INTEREST

The authors declare no potential conflicts of interest.

387 **REFERENCES**

388 1. Launes C, Casas-Alba D, Fortuny C, Valero-Rello A, Cabrerizo M, Muñoz-Almagro C. Utility of
389 Filmarray Meningitis/Encephalitis panel during an outbreak of enterovirus brainstem
390 encephalitis in Catalonia (2016). J Clin Microbiol 2016;55:336-8.

391

392 2. European Centre for Disease Prevention and Control. Enterovirus detections associated
393 with severe neurological symptoms in children and adults in European countries. 2016 Aug 08
394 [cited 2017 Jan 5]. http://ecdc.europa.eu/en/publications/Publications/01-08-2016-RRA-
395 Enterovirus%2071-Spain,%20France,%20Netherlands.pdf

396

397 3. Ooi MH, Wong SC, Lewthwaite P, Cardosa MJ, Solomon T. Clinical features, diagnosis, and
398 management of enterovirus 71. Lancet Neurol 2010;9:1097-105.

399

400 4. McMinn PC. An overview of the evolution of enterovirus 71 and its clinical and public health
401 significance. FEMS Microbiol Rev 2002;26: 91-107.

402

403 5. Schmidt NJ, Lennette EH, Ho HH. An apparently new enterovirus isolated from patients with
404 disease of the central nervous system. J Infect Dis 1974;129:304-9.

405

406 6. Van der Sanden S, Koopmans M, Uslu G, Van der Avoort H, on behalf of the Dutch Working
407 Group for Clinical Virology. Epidemiology of enterovirus 71 in The Netherlands, 1963 to 2008. J
408 Clin Microbiol 2009;47:2826–33.

7. Chumakov M, Voroshilova M, Shindarov L, Lavrova I, Gracheva L, Koroleva G, et al. Enterovirus 71 isolated from cases of epidemic poliomyelitis-like disease in Bulgaria. Arch Virol 1979;60:329-40.

8. Nagy G, Takátsy S, Kukán E, Mihály I, Dömök I. Virological diagnosis of enterovirus type 71 infections: experiences gained during an epidemic of acute CNS diseases in Hungary in 1978. Arch Virol 1982;71:217-27.

9. Rodà D, Pérez-Martínez E, Cabrerizo M, Trallero G, Martínez-Planas A, Luaces C, et al. Clinical characteristics and molecular epidemiology of enterovirus infection in infants < 3 months in a referral paediatric hospital of Barcelona. Eur J Pediatr 2015;174:1549-53.

10. Cabrerizo M, Tarragó D, Muñoz-Almagro C, Del Amo E, Domínguez-Gil M, Eiros JM, et al. Molecular epidemiology of enterovirus 71, coxsackievirus A16 and A6 associated with hand, foot and mouth disease in Spain. Clin Microbiol Infect 2014;20:O150-6.

11. Akhmadishina LV, Govorukhina MV, Kovalev EV, Nenadskaya SA, Ivanova OE, Lukashev AN. Enterovirus A71 Meningoencephalitis Outbreak, Rostov-on-Don, Russia, 2013. Emerg Infect Dis 2015;21:1440-3.

12. Tee KK, Lam TT, Chan YF, Bible JM, Kamarulzaman A, Tong CY, et al. Evolutionary genetics of human enterovirus 71: origin, population dynamics, natural selection, and seasonal periodicity of the VP1 gene. J Virol 2010;84:3339–50.

13. Huang SW, Hsu YW, Smith DJ, Kiang D, Tsai HP, Lin KH, et al. Reemergence of enterovirus 71 in 2008 in Taiwan: dynamics of genetic and antigenic evolution from 1998 to 2008. J Clin Microbiol 2009;47:3653–62.

14. Muslin C, Joffret ML, Pelletier I, Blondel B, Delpeyroux F. Evolution and emergence of enteroviruses through intra- and inter-species recombination: Plasticity and phenotypic impact of modular genetic exchanges in the 5' untranslated region. PLoS Pathog 2015;11:e1005266.

15. Karrasch M, Fischer E, Scholten M, Sauerbrei A, Henke A, Renz DM, et al. A severe pediatric infection with a novel enterovirus A71 strain, Thuringia, Germany. J Clin Virol 2016;84:90-5.

16. Chea S, Cheng YB, Chokephaibulkit K, Chotpitayasunondh T, Rogier van Doorn H, Hafy Z, et al. Workshop on use of intravenous immunoglobulin in hand, foot and mouth disease in Southeast Asia. Emerg Infect Dis 2015;21.

17. Wang SM, Lei HY, Huang MC, Su LY, Lin HC, Yu CK, et al. Modulation of cytokine production by intravenous immunoglobulin in patients with enterovirus 71-associated brainstem encephalitis. J Clin Virol 2006;37:47-52.

18. Chang LY, Hsia SH, Wu CT, Huang YC, Lin KL, Fang TY, et al. Outcome of enterovirus 71 infections with or without stage-based management: 1998 to 2002. Pediatr Infect Dis J 2004;23:327-32.

19. Ooi M, Wong S, Mohan A, Podin Y, Perera D, Clear D, et al. Identification and validation of clinical predictors for the risk of neurological involvement in children with hand, foot, and mouth disease in Sarawak. BMC Infect Dis 2009;9:3.

20. Teoh HL, Mohammad SS, Britton PN, Kandula T, Lorentzos MS, Booy R, et al. Clinical characteristics and functional motor outcomes of enterovirus 71 neurological disease in children. JAMA Neurol 2016;73:300-7.

21. Cardosa J, Farrar J, Yeng C. A Guide to Clinical Management and Public Health Response for Hand, Foot and Mouth Disease (HFMD). Geneva, Switzerland: World Health Organization Western Pacific Region. 2011 [cited 2016 Oct 15]. http://www.wpro.who.int/publications/docs/GuidancefortheclinicalmanagementofHFMD.pdf

471 22. Qui PT, Khanh TH, Trieu HT, Giang PT, Bich NN, Thoa le PK, et al. Intravenous magnesium
472 sulfate for the management of severe hand, foot, and mouth disease with autonomic nervous
473 system dysregulation in Vietnamese children: study protocol for a randomized controlled trial.
474 Trials. 2016;17:98.

475

476 23. van Swieten JC, Koudstaal PJ, Visser MC, Schouten HJ, van Gijn J. Interobserver agreement
477 for the assessment of handicap in stroke patients. Stroke 1988;19:604-7.

478

479 24. Selva L, Martinez-Planas A, García-García JJ, Casadevall R, Luaces C, Muñoz-Almagro C.
480 Comparison of an in-house real-time RT-PCR assay with a commercial assay for detection of
481 enterovirus RNA in clinical samples. Eur J Clin Microbiol Infect Dis 2012;31:715-9.

482

483 25. Cabrerizo M, Echevarría JE, González I, de Miguel T, Trallero G. Molecular epidemiological
484 study of HEV-B enteroviruses involved in the increase in meningitis cases occurred in Spain
485 during 2006. J Med Virol 2008;80:1018-24.

486

487 26. Böttcher S, Obermeier PE, Neubauer K, Diedrich S, Laboratory Network for Enterovirus
488 Diagnostics . Recombinant Enterovirus A71 Subgenogroup C1 Strains, Germany, 2015. Emerg
489 Infect Dis. 2016;22:1843-6.

490

491 27. Khanh TH, Sabanathan S, Thanh TT, Thoa le PK, Thuong TC, Hang Vt, et al. Enterovirus 71-
492 associated hand, foot, and mouth disease, Southern Vietnam, 2011. Emerg Infect Dis
493 2012;18:2002-5.

494

495 28. Antona D, Kossorotoff M, Schuffenecker I, Mirand A, Leruez-Ville M, Bassi C, et al. Severe
496 paediatric conditions linked with EV-A71 and EV-D68, France, May to October 2016. Euro
497 Surveill 2016;21. pii: 30402. doi: 10.2807/1560-7917.ES.2016.21.46.30402.

498

499 29. Cabrerizo M, Díaz-Cerio M, Muñoz-Almagro C, Rabella N, Tarragó D, Romero MP, et al.
500 Molecular epidemiology of enterovirus and parechovirus infections according to patient age
501 over a 4-year period in Spain. J Med Virol 2017;89:435-42.

502

503

504 30. Subdirecció General de Vigilància i Resposta a Emergències de Salut Pública (Agència de
505 Salut Pública de Catalunya). Informe investigació epidemiológica quadres neurològics aguts per
506 enterovirus en población pediátrica. 201 [cited 2017 Feb 28].
507 http://canalsalut.gencat.cat/web/.content/contingut_responsiu/salutAZ/E/enterovirus/neurol
508 ogics_enterovirus.pdf

509

510

511 31. Ho M, Chen ER, Hsu KH, Twu SJ, Chen KT, Tsai SF, et al. An epidemic of enterovirus 71
512 infection in Taiwan. Taiwan Enterovirus Epidemic Working Group. N Engl J Med 1999;341:929–
513 35.

514

515 32. Volle R, Bailly JL, Mirand A, Pereira B, Marque-Juillet S, Chambon M. Variations in
516 cerebrospinal fluid viral loads among enterovirus genotypes in patients hospitalized with
517 laboratory-confirmed meningitis due to enterovirus. J Infect Dis 2014;210:576-84.

518

519 33. Chang L-Y, Huang L-M, Gau SS-F, Wu YY, Hsia SH, Fan TY, et al. Neurodevelopment and
520 cognition in children after enterovirus 71 infection. N Engl J Med 2007;356:1226–34.

521

522 34. Xing W, Liao Q, Viboud C, Zhang J, Sun J, Wu JT, et al. Hand, foot, and mouth disease in
523 China, 2008-12: an epidemiological study. Lancet Infect Dis 2014;14:308-18.

524

525 35. Chang L, Lin T, Hsu K, Huang YC, Lin KL, Hsueh C, et al. Clinical features and risk factors of
526 pulmonary oedema after enterovirus-71-related hand, foot, and mouth disease. Lancet
527 1999;354: 1682-6.

528

529 36. Shen WC, Chiu HH, Chow KC, Tsai CH. MR imaging findings of enteroviral
530 encephalomyelitis: an outbreak in Taiwan. AJNR Am J Neuroradiol 1999;20:1889-95.

531

532 37. Chen CY, Chang YC, Huang CC, Lui CC, Lee KW, Huang SC. Acute flaccid paralysis in infants
533 and young children with enterovirus 71 infection: MR imaging findings and clinical correlates.
534 AJNR Am J Neuroradiol 2001; 22:200-5.

535

536 38. Defresne P, Meyer L, Tardieu M, Scalais E, Nuttin C, De Bont B, et al. Efficacy of high dose
537 steroid therapy in children with severe acute transversemyelitis. J Neurol Neurosurg Psychiatry
538 2001;71:272-4.

539

540 39. Nolan MA, Craig ME, Lahra MM, Rawlinson WD, Prager PC, Williams GD, et al. Survival
541 after pulmonary edema due to enterovirus 71 encephalitis. Neurology 2003;60:1651-6.

542

543 40. He Y, Yang J, Zeng G, Shen T, Fontaine RE, Zhang L, et al. Risk factors for critical disease and
544 death from hand, foot and mouth disease. Pediatr Infect Dis J. 2014;33:966-70.

545

546 41. Mou J, Dawes M, Li Y, He Y, Ma H, Xie X, Griffiths S, Cheng J. Severe hand, foot and mouth
547 disease in Shenzhen, South China: what matters most? Epidemiol Infect 2014;142:776-88.

548 **Table 1.** Epidemiological, clinical, laboratory, and imaging variables associated with more severe disease in children with EV-related neurologic disease (n=57).

	no.	**Age** (months)[a]	**Sex** (males)	**Peak fever** (ºC)[a]	**Duration of fever**[a] (days)	**White blood cell count**[a] (cells*10^3/µl)	**CSF white cell count**[a] (cells/mm3)	**Procalcitonin** (ng/m)[a]	**Bulbar involvement on MRI** (Yes/no. patients who underwent MRI)
WHO clinical classification [21]									
CNS involvement stage without ANS dysfunction	55	27.8 (18.4-37.7)	31/55	39.0 (38.5-39.5)	3 (2-4)	12.4 (11-15)	182 (105-442)	0.1(0.07-0.15)	13/45
Cardiopulmonary failure stage	2	8.2 (7.7-8.6) p=0.07	2/2 p=0.55	38.3 (38.1-38.5) p=0.14	4 (3-5) p=0.43	19.4 (17.4-21.4) **p=0.03**	55 (10-100) p=0.09	1 (0.40-1.60) **p=0.02**	2/2 p=0.10
Vietnam HFMD classification [22]									
Aseptic meningitis	7	12.3 (0.7-37.7)	4/7	38.3 (38.0-39.2)	1 (0.5-3)	12.4 (10.1-15.4)	560 (200-690)	0.09 (0.06-0.11)	-
2a	9	18.4 (5.1-48.9)	6/9	39.0 (38.3-39.8)	3 (1-4)	11.0 (9.9-12.7)	88 (52-180)	0.10 (0.10-0.25)	1/5
2bG1	9	27.9 (22.6-36.9)	5/9	39.0 (38.0-39.8)	3 (0.5-4.5)	11.9 (11.5-13.3)	198 (135-370)	0.09 (0.09-0.20)	1/9
2bG2	30	28.1 (20.6-42.0)	16/30	39.0 (38.7-39.6)	3.5 (3-4)	12.8 (11.7-15.3)	180 (112-385)	0.08 (0.05-0.14)	11/29
4	2	8.2 (7.9-8.6) p=0.09	2/2 p=0.73	38.3 (38.1-38.5) p=0.25	4 (3-5) **p=0.02**	19.4 (17.4-21.4) p=0.05	55 (10-100) **p=0.02**	1.00 (0.40-1.60) p=0.10	2/2 p=0.08
NRL exploration with persistent paresis at day 30									
Yes	3	8.6 (8.2-16.7)	2/3	38.5 (38.3-39.1)	4 (3.5-4.5)	17.4 (15.0-19.4)	50 (30-75)	0.09 (0.07-0.14)	3/3
No	54	27.8 (18.2-37.8) p=0.12	31/54 p=1.00	39.0 (38.5-39.5) p=0.59	3 (2-4) p=0.27	12.4 (11.1-15.1) p=0.07	185 (112-445) **p=0.02**	1.00 (0.40-1.60) **p=0.01**	12/42 **p=0.01**

549

550 CSF: cerebrospinal fluid; MRI: magnetic resonance imaging; WHO: World Health Organization; CNS: central nervous system; ANS: autonomic nervous system; HFMD: hand-foot-mouth disease;
551 NRL: neurologic.

552 a) Median (interquartile range)

553 **Table 2.** Clinical characteristics and enterovirus (EV) positive rates according to EV

554 genotypes.

	Patients in whom EV could be genotyped				**Patients in whom EV could not be genotyped** n = 10
	EV-A71 n=40	**Other EV types** n=7	**p-value**[a]	**Total** n=47	
WHO clinical classification [21]					
• Aseptic meningitis	2 (28.6%)	5 (71.4%)		7	0
• Encephalitis	4 (66.6%)	2 (33.3%)		6	0
• Brainstem encephalitis	31 (100%)	0 (0%)	< 0.01	31	10[b]
• Encephalomyelitis	1 (100%)	0 (0%)		1	0
• Encephalomyelitis with ANS dysfunction.	2 (100%)	0		2	0
Positive samples in cerebrospinal-fluid	4/37[c]	5/7	< 0.01	9/44	2/8
Positive samples in respiratory specimens	27/40	2/4	0.59	29/44	6/7
Positive samples in faeces	29/35	3/3	1	32/38	8/8

555 WHO: World Health Organization; ANS: autonomous nervous system.

556 a) Comparing proportions between groups EV-A71 and other EV types

557 b) In one case, EV from species A was identified but it could not be typed

558 c) The 4 detections were made using the FilmArray meningitis/encephalitis panel [1]

559 **Table 3.** Outcomes according to the WHO clinical classification [21] and the

560 Vietnamese HFMD classification [22].

	Length of hospital stay (days)	IVIG treatment (Yes)	NRL symptoms (mRS ≥2) at day 14	NRL symptoms (mRS ≥2) at day 30
WHO clinical classification				
Meningitis	3 (2-5)	0/7	0/7	0/7
Encephalitis	4.5 (3-6.2)	1/6	0/6	0/6
Brainstem encephalitis	8 (6-9)	30/41	9/41	0/41[a]
Encephalomyelitis	10 (7.5-11.0)	1/1	1/1	1/1
Encephalomyelitis and cardiopulmonary failure	51 (38-64)	2/2	2/2 **p=0.01**	2/2 **p<0.01**
Vietnam HFMD classification				
Aseptic meningitis	3 (2-5)	0/7	0/7	0/7
2a	4 (3-6.5)	2/9	0/9	0/9
2bG1	9 (5.5-10.5)	6/9	2/9	0/9
2bG2	8 (6-10)	24/30	8/30	1/30[a]
4	51 (38-64)	2/2	2/2 **p=0.01**	2/2 **p<0.01**

561 IVIG: Intravenous immunoglobulin; NRL: neurologic; mRS: modified Rankin Scale; WHO: World Health Organization;

562 HFMD: hand-foot-mouth disease.

563 a) Three other patients had discrete hyperreflexia in an extremity without significant disability.