Molecular epidemiology of enterovirus 71, coxsackievirus A16 and A6 associated with hand, foot and mouth disease in Spain

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Abstract

Hand, foot and mouth disease (HFMD) is a childhood illness frequently caused by genotypes belonging to the enterovirus A species, including coxsackievirus (CV)-A16 and enterovirus (EV)-71. Between 2010 and 2012, several outbreaks and sporadic cases of HFMD occurred in different regions of Spain. The objective of the present study was to describe the enterovirus epidemiology associated with HFMD in the country. A total of 80 patients with HFMD or atypical rash were included. Detection and typing of the enteroviruses were performed directly in clinical samples using molecular methods. Enteroviruses were detected in 53 of the patients (66%). CV-A6 was the most frequent genotype, followed by CV-A16 and EV-71, but other minority types were also identified. Interestingly, during almost all of 2010, CV-A16 was the only causative agent of HFMD but by the end of the year and during 2011, CV-A6 became predominant, while CV-A16 was not detected. In 2012, however, both CV-A6 and CV-A16 circulated. EV-71 was associated with HFMD symptoms only in three cases during 2012. All Spanish CV-A6 sequences segregated into one major genetic cluster together with other European and Asian strains isolated between 2008 and 2011, most forming a particular clade. Spanish EV-71 strains belonged to subgenogroup C2, as did most of the European sequences circulated. In conclusion, the recent increase of HFMD cases in Spain and other European countries has been due to a larger incidence of circulating species A enteroviruses, mainly CV-A6 and CV-A16, and the emergence of new genetic variants of these viruses.

Keywords: Coxsackievirus, genotyping, HFMD, molecular epidemiology, phylogenetic analyses
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Introduction

Hand, foot and mouth disease (HFMD) is a childhood illness characterized by fever and vesicular lesions on the palms of the hands, soles of the feet, oral mucosa and tongue. The major causative agents of HFMD are enteroviruses (EVs), in particular the serotypes belonging to the A species (EV-A) [1]. Two types, Coxsackievirus A16 (CV-A16) and enterovirus 71 (EV-71), are involved in most cases of HFMD [2,3], but other EV-A types and some echoviruses have also been associated with this pathology [4,5].

Although HFMD is classically a mild disease, large outbreaks in the Asia-Pacific region have been described since 1997, with a high incidence of fatal cardiopulmonary and neurological complications, particularly when the causative virus is EV-71 [6–9]. Currently, HFMD is considered endemic and is an important public health issue in much of the region. In Europe and North America, it is not a notifiable disease but epidemiological studies of outbreaks caused by different EV-A types (CV-A16, CV-A10 and CV-A6) have been

described in recent years [10–14]. EV-71 has also been detected in several European countries and the USA and, although rare, infections with fatal outcome have been reported [15–18].

Onychomadesis or nail shedding is a disorder in children newly recognized as a complication in the course of HFMD. Onychomadesis often occurs weeks after initial symptom onset and was first reported in 2000 in five North American children [19]. Subsequent studies established a clear link for these diseases [20,21]. Between the summer of 2008 and early 2009, HFMD outbreaks, followed by onychomadesis and associated with different EV types, were first described in various Spanish regions [22–25].

During the last 25 years in Spain, an EV surveillance system has provided considerable data on the types circulating in association with different pathologies, mainly with aseptic meningitis [26,27]. Since 2010, however, the Spanish National Centre for Microbiology (CNM) has increasingly received more samples from local outbreaks and sporadic cases of HFMD that occurred in different regions, both for virological diagnosis and/or type identification. The aim of the current study was to investigate the involvement of the different EV types associated with the HFMD cases in Spain from 2010 to 2012.

Materials and Methods

Patients and clinical samples

From January 2010 to December 2012, the CNM in Madrid received 80 clinical samples from patients with signs and symptoms of HFMD ($N = 60$) or with fever and atypical rash ($N = 20$) from 16 different hospitals in 12 regions of the country. In 2010 a total of 11 cases were received, 24 were received in 2011, and in 2012 that number was increased to 45 cases. Of the 80 cases included in the study, 54 were sporadic and 26 were from six local outbreaks: in Mallorca, June 2010; in Vigo, September 2011; in Madrid, November 2011 and March 2012; in Gran Canaria (Canary Islands), May 2012; and in Caceres, November 2012.

Each specimen was sent with a standardized form with recorded information on patient demographics (age, sex), clinical diagnosis (HFMD, onychomadesis after HFMD, rash/exanthema or febrile syndrome) and date of sample collection. The mean age of the patients was 13.7 years (ranging from 11 days to 34 years). Clinical specimens were 28 throat swabs (35%), 21 (26%) stools, 17 (21%) vesicular swabs and 14 (18%) sera. Onychomadesis occurred in 16 (27%) sporadic cases after HFMD. In them, clinical samples were collected during onychomadesis, at 25.6 days (range 12–42 days) after the acute HFMD phase.

Enterovirus detection and genotyping

EVs had been detected directly in clinical samples by RT-PCR of the 5′-untranslated region (UTR) of the viral genome as described elsewhere [26] and carried out in the hospital of origin or in the Viral Detection Unit of the CNM.

EV typing was performed in the Enterovirus Unit of the CNM. Samples positive for the 5′-UTR PCR were subjected to a species EV-A, B and C specific RT-nested PCR in the 3′-VP1 region as previously reported [28]. Amplified DNA was directly sequenced using a BigDye Terminator kit (Applied Biosystems, Foster City, CA, USA) and both inner PCR primers.

Phylogenetic analyses

Type identification was determined by phylogenetic comparison of EV-A sequences obtained with the prototype strains. To study the relationships between Spanish strains and those circulating in other countries, additional phylogenetic analyses were performed with the nucleotide sequences of Spanish strains assigned to types CV-A6 and EV-71 and the respective homologous sequences available from GenBank. Multiple sequence alignments were performed by the ClustalW program. Genetic distances were calculated using the maximum composite likelihood nucleotide distance model, and statistical significance of phylogenies estimated by bootstrap analysis with 1000 pseudoreplicate datasets. Phylogenetic trees were constructed using the neighbour-joining method in the MEGA software 4.0. The sequences obtained in this study have been deposited in GenBank under accession numbers KC688834-KC688865.

Results

Clinical and epidemiological data of patients with EV and non-EV infections

Of the total of 80 clinical samples studied, 53 were positive for EVs (66%), 43 from patients with HFMD diagnosis and 10 from patients who presented with fever and atypical rash. There were no statistical differences in mean age, male/female rate and the distinct syndromes presented between patients with EV infection and those negative for EVs (data not shown).

Most of the EV-infected patients (45/53, 85%) were children ($p < 0.001$), aged between 11 days and 7 years (mean age, 1.7 years), but also young people (8/53, 15%; mean age, 31.4 years) were included. The majority of patients were male (30/53), but this was not statistically significant (p 0.2438).

Shedding of nails occurred after initial HFMD infection in 12 (28%) of the 43 EV-positive patients. Clinical samples were

taken during onychomadesis symptoms at a mean of 27.1 days following HFMD.

According to the clinical syndromes presented, those with onychomadesis after HFMD were younger (1.7 ± 0.9 years) than those with HFMD alone or atypical rash (8.2 ± 2.3 years, 5.4 ± 10.4 years, respectively), but the differences are not statistically significant.

Regarding the seasonal distribution, HFMD cases mainly occurred in two annual periods, between March and June and between September and December, matching the epidemic peaks of overall EV circulation in the country (Fig. 1).

Prevalent enterovirus genotypes

Fifty (94%) of the 53 EVs detected in HFMD/atypical rash cases were genotyped. Most EVs (96%) were assigned to a type within the EV-A species. CV-A6 was predominant, accounting for 60% of the cases. This was followed by CV-A16, but other minority A types were also detected. Two cases (4%) were positive for species B EVs (E-18 and CV-B4).

Through the national EV surveillance system, the CNM receives a mean of 350 EVs/year for genotyping that are associated with neurological diseases, mainly meningitis and febrile syndromes. Most are characterized as echovirus or coxsackievirus B within EV-B species. During the present study period, however, EV-A types were also identified in a significant percentage of patients presenting febrile syndromes only (6%). It was therefore considered of interest to include these 12 fever cases in the current molecular epidemiological study. Hence, in total, CV-A6 was identified in 34 (55%) of the 62 typed cases, CV-A16 in 11 (18%), EV-71 in 8 (13%), CV-A4 and CV-A8 in two (3% each) and CV-A2, CV-A5 and CV-A14 in one (2% each).

Data on the main genotypes detected according to the clinical pathologies of patients are included in Table 1. There are no statistical differences in type variability between the different syndromes. However, CV-A infections were predominantly responsible for HFMD cases ($p < 0.05$) while EV-71 was more frequent in infant febrile syndromes ($p < 0.001$). Patients infected by EV-71 were significantly younger than those infected by another EV-A ($p < 0.01$). Finally, the onychomadesis symptoms after HFMD were not associated with a specific EV type ($p < 0.05$).

During 2010 (Fig. 2), CV-A16 was the most frequently detected type, followed by CV-A6. In 2011, CV-A6 was the predominant type while CV-A16 was not detected. Other CV-A types were also identified in the same year. Finally, during 2012, CV-A16 circulated again, together with CV-A6 and other CV-As. EV-71 was detected during the 3 years, but mainly during 2012 (6/8, 75%).

Distribution of the three predominant genotypes (CV-A6, CV-A16 and EV-71) throughout the 3-year study period is shown in Fig. 1. During almost all of 2010, CV-A16 was the only type identified in HFMD cases but in October, this was replaced by the infrequent CV-A6, which then circulated until the end of 2011. However, in 2012, a co-circulation of CV-A16 and CV-A6 was observed. With respect to EV-71, this was associated with HFMD or rash only in three cases from 2012. The other five EV-71s were identified in infant febrile syndromes.

Phylogenetic analyses

The reconstructed tree in the 3′-half VP1 region showed that all EV-A sequences clustered with their respective prototype strain with bootstrap values higher than 95% (data not shown). Phylogenetic analysis carried out with several Spanish CV-A6 strains ($n = 24$) and others available in GenBank showed that the Spanish ones segregated into one major genetic cluster together with strains detected between 2008 and 2011 in

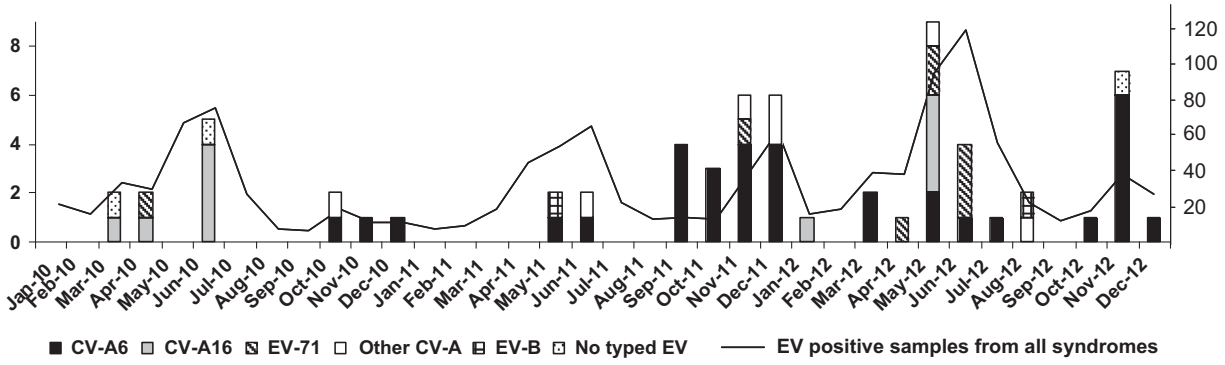

FIG. 1. Distribution of the number of EV-positive samples from all syndromes analysed in the Enterovirus Unit of the CNM between January 2010 and December 2012 (right-axis) and prevalent types from HFMD/rash/fever cases studied (left-axis) by month. CV-A, coxsackievirus A; EV, enterovirus; EV-B, enterovirus B species.

TABLE 1. Demographic and clinical data associated with the different EV genotypes

	Enterovirus genotypes				
	CV-A6 (*n* = 34)	**CV-A16 (*n* = 11)**	**EV-71 (*n* = 8)**	**Other EV (*n* = 9)**	**p value***
Mean age, years	7.9 ± 2.1	4.1 ± 2.9	0.4 ± 0.2	1.1 ± 0.3	**0.0030**
Male/female ratio	19/15	6/5	7/1	5/4	0.2105
HFMD symptoms	21 (62)	5 (45)	1 (12)	2 (22)	**0.0343**
HFMD symptoms + onychomadesis	4 (12)	1 (9)	1 (12)	5 (55)	**0.0159**
Rash + fever	7 (20)	2 (18)	1 (12)	0	0.9810
Infant febrile syndromes	2 (6)	3 (27)	5 (64)	2 (22)	**0.0008**

Data are mean ± standard deviation for age and *N* (%) of patients for clinical symptoms.
*Significant variations between four groups were evaluated using the Student *t*-test for age and chi-squared test for clinical symptoms. The p value below 0.05 was considered to be significant and appears in bold.

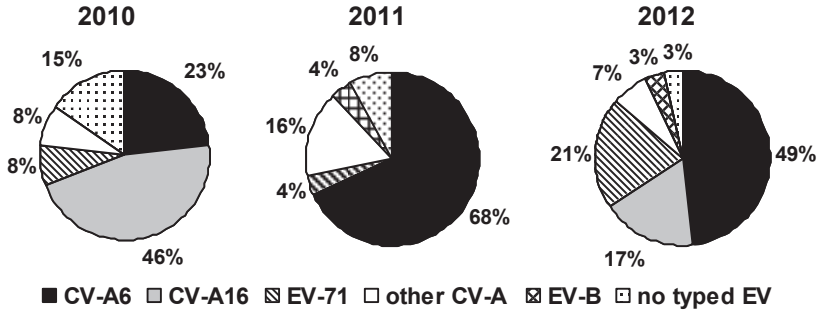

FIG. 2. Distribution of EV types associated with HFMD alone, HFMD plus onychomadesis, atypical rash and febrile syndromes by year. CV-A, coxsackievirus A; EV, enterovirus; EV-B, enterovirus B species.

Europe (Finland, France and Spain (Valencia)) and Asia (Japan, China and India). Furthermore, all Spanish strains except for three (from 2012) formed a particular sub-cluster (Fig. 3a). In the EV-71 tree (Fig. 3b), the eight Spanish strains detected between 2010 and 2012 belong to subgenogroup C2.

Discussion

During the last 12 years, large HFMD outbreaks associated with EV-71 infections and fatal cases in children under 5 years occurred in the Asia-Pacific region, representing a significant public health threat [6–9]. In several European countries, including Spain, the number of HFMD cases reported has increased considerably since 2008 [10,12,14,22–25]. In Europe HFMD is currently not a reportable disease, unlike in many Asian countries, so epidemiological data are still scarce. Nevertheless, the data indicate a recent emergence of circulating EV-A types in regions outside of the Asia-Pacific region, as in Europe or North America [10–14,22].

In the present study, 80 cases with HFMD symptoms or atypical rash, collected between 2010 and 2012 from different Spanish cities, were analysed. The number of cases received in 2012 was four-fold higher than in 2010. EV infections were confirmed in 66% of the cases. Detection failure may be due to several reasons. In patients with a rash, other microorganisms or non-infectious agents could be the cause of the disease. On the other hand, the most appropriate samples for viral diagnosis of HFMD are vesicular swabs, but, unfortunately, this type of specimen was available only in 17 (21%) of the cases. Finally, the viral load in some samples could be below the detection limit of the technique if they were taken several days after the acute phase of the disease. However, the percentage of EV detection described in the current study is comparable to that reported previously in Finland, France or North America, which ranged from 64 to 74% [10,13,14].

The most frequently detected genotype was CV-A6, followed by CV-A16 and EV-71. Other EV-A types were also identified in minor proportions. CV-A10, however, was not found, although this genotype, together with CV-A6, was one of the main causative agents of the outbreaks described during 2008–2010 in Finland, France and Spain [10,14,22].

Interestingly, however, although CV-A6 was previously detected during the outbreak that occurred in Valencia in 2008 [22], this infrequently detected type was first identified in our laboratory in October 2010. It then replaced CV-A16, the unique HFMD-associated genotype detected until then [27]. During 2011, CV-A6 was the predominant type causing HFMD, while CV-A16 was undetectable. In 2012, however,

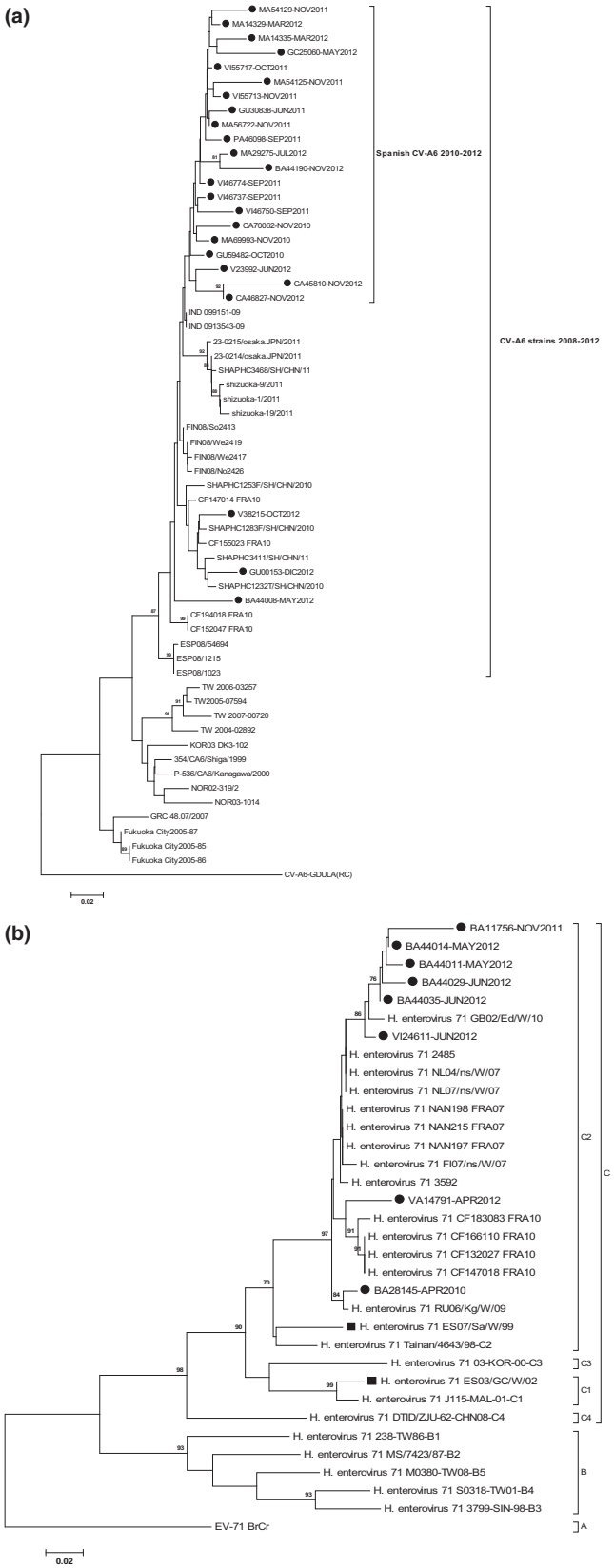

FIG. 3. Phylogenetic analysis of Spanish (a) CV-A6 and (b) EV-71 sequences and others available in GenBank in the 3′-VP1 region of the genome. Spanish strains are indicated by black circles. Trees were constructed using the neighbour-joining method based on the maximum composite likelihood distances model. Bootstrap resampling was used to demonstrate the robustness of grouping; values >80% are shown. In the EV-71 tree, two old Spanish sequences from 1999 and 2002, respectively, were also included (black squares). The EV-71 classification into genogroups A, B and C and subgenogroups C1–C4 is indicated.

CV-A16 re-emerged and both EVs co-circulated during the first 5 months of the year. Again, during the autumn of 2012 CV-A6 predominated (Fig. 1).

Regarding EV-71, this type was associated with HFMD for the first time in 2012. In Spain, the incidence of EV-71 had been very low and always caused neurological manifestations [27]. Indeed, only 37% of the EV-71 detected in the present study was associated with rash or HFMD symptoms. Other infants with EV-71 infections had a clinical presentation only of fever. Therefore in Spain, although the EV-71 circulation seems to have increased in recent years, its infection is still associated more with febrile syndromes in young children than with HFMD. Phylogenetic analysis showed that all Spanish EV-71s belonged to subgenogroup C2, like most of the strains circulating in Europe in recent years, and had been assigned to subgenogroups C1 and C2 within genogroup C [15–18]. However, the C4 subgenogroup, which was associated with large outbreaks in China [9], seems not to have spread in Spain and other European countries.

With respect to clinical outcome, CV-A types can cause HFMD or atypical rash both in children and young people, although it is more common in children ($p < 0.01$). However, onychomadesis after HFMD was restricted to children between 1 and 4 years. Comparison with the clinical presentations in previous HFMD outbreaks indicates that encephalitis and convulsions were reported in five cases from Finland in 2008 [10] and in one from Japan [29], while none of the patients from our series or from others presented with severe neurological manifestations [11–14,22–25]. With respect to the emergence after HFMD of onychomadesis, nail shedding was reported in HFMD cases from Finland, Spain, Taiwan, North America and Japan [10,13,21–25,29], but not in those from France or Croatia [12,14]. Several genotypes (CV-A6, CV-A10, CV-A16 and CV-B1) were identified there. In the Spanish study of the 2008 outbreak [22], the authors suggested an association between the emergence of this complication after HFMD and detection of a mixed infection (the EV type that causes HFMD plus CV-B1). In the current study, onychomadesis was observed in 12 of the EV-positive patients. In 92% of them, EV was typed, despite the samples having been taken 12–42 days after HFMD. No link between onychomadesis and a specific genotype was found because seven different types were detected. Furthermore, emergence of onychomadesis was significantly more frequent in cases infected by EV types other than CV-A6, CV-A16 or EV-71.

Our data agree with previous reports suggesting that co-circulation of several CV-A types in Europe and the emergence of genetic variants spreading into new geographical areas caused the HFMD outbreaks since 2008 in several countries. With respect to CV-A6, HFMD outbreaks caused by this type were described in China, Taiwan and Japan in 2005–2011 [8,21,29]. An increase in its prevalence has also been reported in several European countries since 2008 [10,14]. Recently HFMD outbreaks caused by CV-A6 were described in the USA [11,13]. Phylogenetic relationships between available CV-A6 sequences in the 3′-VP1 region showed that the predominant lineage circulation in Asia and Europe after 2008 is a genetic variant distinct from that previously detected in Asia. Hence the increase in circulation of several EV-As and the emergence of new CV-A types, mainly CV-A6, has contributed to the increased incidence of HFMD cases in Spain in recent years. Indeed, a large HFMD outbreak caused by CV-A6 that occurred in a northern city during 2011 was very recently reported [30]. Regarding EV-71, in Spain as in other European countries [15,17,18,27], C2 strains are circulating with a low incidence and only a few cases are associated with HFMD.

In conclusion, the overall epidemiology of HFMD caused by EV-A in Europe is similar to that observed in the Asian region, but the frequency of EV-71 infections is much lower. The reason for this difference is still unclear. Even so, there is a need for reinforced surveillance of HFMD to differentiate benign EV-A genotypes from EV-71 and for better management of future outbreaks.

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Transparency Declaration

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Author Contributions

Study concept and design: M Cabrerizo, D Tarragó, C Muñoz-Almagro, JE Echevarría and G Trallero. Contribution of samples and clinical data: C Muñoz-Almagro, JM Eiros, I López-Miragaya, C Pérez and J Reina. Performing the experiments: M Cabrerizo, D Tarragó, E del Amo, M Domínguez-Gil, I López-Miragaya, A Otero and I González. Analysis of the data: M Cabrerizo, D Tarragó, C Muñoz-Almagro, JM Eiros, I López-Miragaya, C Pérez, J Reina, JE Echevarría and G Trallero. Preparation of manuscript: M Cabrerizo. Drafting and revising the article: D Tarragó, C Muñoz-Almagro, JM Eiros, I López-Miragaya, C Pérez, J Reina, JE Echevarría and G Trallero.

References

1. Knowles NJ, Hovi T, Hyypiä T *et al.* Picornaviridae. In: King AMQ, Adams MJ, Carstens EB, Lefkowitz EJ, eds, *Virus Taxonomy: Classification and nomenclature of viruses: ninth report of the international committee on taxonomy of viruses*. San Diego, USA: Elsevier Academic press, 2012; 855–880.
2. Hosoya M, Kawasaki Y, Sato M *et al.* Genetic diversity of coxsackievirus A16 associated with hand, foot, and mouth disease epidemics in Japan from 1983 to 2003. *J Clin Microbiol* 2007; 45: 112–120.
3. Wong SS, Yip CC, Lau SK, Yuen KY. Human enterovirus 71 and hand, foot and mouth disease. *Epidemiol Infect* 2010; 138: 1071–1089.
4. Itagaki A, Ishihara J, Mochida K *et al.* A clustering outbreak of hand, foot, and mouth disease caused by Coxsackie virus A10. *Microbiol Immunol* 1983; 27: 929–935.
5. Lindenbaum JE, Van Dyck PC, Allen RG. Hand, foot and mouth disease associated with coxsackievirus group B. *Scand J Infect Dis* 1975; 7: 161–163.
6. Podin Y, Gias EL, Ong F *et al.* Sentinel surveillance for human enterovirus 71 in Sarawak, Malaysia: lessons from the first 7 years. *BMC Public Health* 2006; 6: 180.
7. Puenpa J, Theamboonlers A, Korkong S *et al.* Molecular characterization and complete genome analysis of human enterovirus 71 and coxsackievirus A16 from children with hand, foot and mouth disease in Thailand during 2008-2011. *Arch Virol* 2011; 156: 2007–2013.
8. Wu Y, Yeo A, Phoon MC *et al.* The largest outbreak of hand; foot and mouth disease in Singapore in 2008: the role of enterovirus 71 and coxsackievirus A strains. *Int J Infect Dis* 2010; 14: e1076–e1081.
9. Zhang Y, Tan X, Cui A *et al.* Complete genome analysis of the C4 subgenotype strains of enterovirus 71: predominant recombination C4 viruses persistently circulating in China for 14 years. *PLoS ONE* 2013; 8: e56341.
10. Blomqvista S, Klemolaa P, Kaijalainena S *et al.* Co-circulation of coxsackieviruses A6 and A10 in hand, foot and mouth disease outbreak in Finland. *J Clin Virol* 2010; 48: 49–54.
11. Flett K, Youngster I, Huang J *et al.* Hand, foot, and mouth disease caused by Coxsackievirus A6. *Emerg Infect Dis* 2012; 18: 1702–1704.
12. Ljubin-Sternak S, Slavic-Vrzic V, Vilibić-Čavlek T, Aleraj B, Gjenero-Margan I. Outbreak of hand, foot and mouth disease caused by Coxsackie A16 virus in a childcare centre in Croatia, February to March 2011. *Euro Surveill* 2011; 16: pii=19875.
13. McIntyre MG, Stevens KM, Davidson S *et al.* Severe hand, foot, and mouth disease associated with Coxsackievirus A6 — Alabama, Connecticut, California, and Nevada, November 2011–February 2012. *MMWR Morb Mortal Wkly Rep* 2012; 61: 213–214.
14. Mirand A, Henquell C, Archimbaud C *et al.* Outbreak of hand, foot and mouth disease/herpangina associated with coxsackievirus A6 and A10 infections in 2010, France: a large citywide, prospective observational study. *Clin Microbiol Infect* 2012; 18: E110–E118.
15. Bible JM, Iturriza-Gomara M, Megson B *et al.* Molecular epidemiology of human enterovirus 71 in the United Kingdom from 1998 to 2006. *J Clin Microbiol* 2008; 46: 3192–3200.
16. Perez-Velez CM, Anderson MS, Robinson CC *et al.* Outbreak of neurologic enterovirus type 71 disease: a diagnostic challenge. *Clin Infect Dis* 2007; 45: 950–957.
17. Schuffenecker I, Mirand A, Antona D *et al.* Epidemiology of human enterovirus 71 infections in France, 2000–2009. *J Clin Virol* 2011; 50: 50–56.
18. Vallet S, Legrand-Quillien MC, Dailland T *et al.* Fatal case of enterovirus 71 infection, France, 2007. *Emerg Infect Dis* 2009; 15: 1837–1840.
19. Clementz GC, Mancini AJ. Nail matrix arrest following hand-foot--mouth disease: a report of five children. *Pediatr Dermatol* 2000; 17: 7–11.
20. Bernier V, Labrèze C, Bury F, Taïeb A. Nail matrix arrest in the course of hand, foot and mouth disease. *Eur J Pediatr* 2001; 160: 649–651.
21. Wei SH, Huang YP, Liu MC *et al.* An outbreak of coxsackievirus A6 hand, foot, and mouth disease associated with onychomadesis in Taiwan, 2010. *BMC Infect Dis* 2011; 11: 346.
22. Bracho MA, González-Candelas F, Valero A, Córdoba J, Salazar A. Enterovirus co-infections and onychomadesis after hand, foot, and mouth disease, Spain, 2008. *Emerg Infect Dis* 2011; 17: 2223–2231.
23. Cabrerizo M, de Miguel T, Armada A *et al.* Onychomadesis after a hand, foot, and mouth disease outbreak in Spain, 2009. *Epidemiol Infect* 2010; 138: 1775–1778.
24. Guimbao J, Rodrigo P, Alberto MJ, Omeñaca M. Onychomadesis outbreak linked to hand, foot, and mouth disease, Spain, July 2008. *Euro Surveill* 2010; 15: pii=19663.
25. Redondo-Granado MJ, Torres-Hinojal MC, Izquierdo-Lopez B. Post viral onychomadesis outbreak in Valladolid. *An Pediatr(Barc)* 2009; 71: 436–439.
26. Cabrerizo M, Echevarria JE, González I, de Miguel T, Trallero G. Molecular epidemiological study of HEV-B enteroviruses involved in the increase in meningitis cases occurred in Spain during 2006. *J Med Virol* 2008; 80: 1018–1024.
27. Trallero G, Avellon A, Otero A *et al.* Enteroviruses in Spain over the decade 1998–2007: virological and epidemiological studies. *J Clin Virol* 2010; 47: 170–176.
28. Casas I, Tenorio A, Echevarria JM, Klapper PE, Cleator GM. Detection of enteroviral RNA and specific DNA of herpesviruses by multiplex genome amplification. *J Virol Methods* 1997; 66: 39–50.
29. Fujimoto T, Iizuka S, Enomoto M *et al.* Hand, foot, and mouth disease caused by coxsackievirus A6, Japan, 2011. *Emerg Infect Dis* 2012; 18: 337–339.
30. Montes M, Artieda J, Piñeiro LD *et al.* Hand, foot, and mouth disease outbreak and coxsackievirus A6, Northern Spain, 2011. *Emerg Infect Dis* 2013; 19. doi: 10.3201/eid1904.121589