

## Enteroviruses in Spain: virological and epidemiological studies over 10 years (1988–97)

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### SUMMARY

A total of 15662 clinical samples were analysed for enterovirus (EV) isolation in cell cultures during a 10-year period (1988–97). Furthermore, 210 isolates of EV obtained in primary laboratories within Spain from patients with meningitis were characterized. The total number of EV typed was 758, including 727 non-polio EV and 31 Sabin-like (SL) polioviruses. Twenty-eight EV serotypes were represented. Echoviruses comprised 90% (653/727) of fully typed non-polio EV. The four most prevalent serotypes were echovirus 30, echovirus 9, echovirus 6 and echovirus 4. Echovirus 30 was the main serotype associated with meningitis. Echovirus 9 was the aetiological agent in 20 outbreaks of meningitis while the occurrence of echovirus 6 was localized in 1 year (1997). Coxsackieviruses A and B occurred in 3 and 7% of the non-polio EV respectively. Coxsackievirus B5 presented the relative greater abundance. This paper examines the epidemiology of EV in Spain to serotype level over a 10-year period with special attention to non-polio EV associated with meningitis.

### INTRODUCTION

The enteroviruses (EV) comprise a large genus, established in 1957, within the picornaviridae family. Human EV were sub-classified into poliovirus (PV, serotypes 1–3), coxsackievirus groups A and B (CAV, serotypes 1–22 and 24; CBV, serotypes 1–6) and echoviruses (EV, serotypes 1–7, 9, 11–27, and 29–33) and the newer enterovirus serotypes 68–71 [1–3].

Sixty-six immunologically distinct serotypes are known to cause infections in humans. Examples of the acute diseases in which the aetiological role of EV is well documented are poliomyelitis, Bornholm disease, acute meningitis, encephalitis, acute myocarditis, diabetes mellitus type 1, orchitis, and upper res-

piratory tract infections [4, 5]. However, most infections are mild or asymptomatic, especially in children. Severity depends on age and constitution of the host, but also on virulence of the circulating virus [6]. Sometimes EV may also result in serious or even fatal diseases such as myocarditis, hepatitis, aseptic meningitis or meningo-encephalitis.

Polioviruses (PV) are associated with central nervous system (CNS) infection and may be implicated in the pathogenesis of some chronic neurological diseases such as post-poliomyelitis syndrome [7]. At present, while eradicated from the Americas and much of the developed world, poliomyelitis continues to be a target priority of the WHO in developing countries. Control of PV infection in much of the world has focused attention on the non-polio EV (NPEV) [8]. Aseptic meningitis is by far the most common and clinically vexing infections where EV are involved. At

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least 85% of the cases of aseptic meningitis for which an aetiology can be determined, particularly among children and infants are due to EV [9, 10].

EV have a worldwide distribution. Within a given geographical locality, some serotypes may be endemic, with little or only gradual change from year to year. In contrast, other serotypes may be introduced periodically, causing epidemics, with few isolations reported in intervening years [11].

Epidemiological surveillance plays a crucial role in understanding the changing patterns of EV infection and disease association. Such knowledge may help in the control of infectious diseases [12–15]. Although complete identification of EV does not contribute significantly to patient management, it is essential for epidemiological purposes, establishing the dominant virus each year or in each outbreak [13]. The ability to detect rapidly and distinguish enteroviral-associated illness from that due to bacteria and other viruses is an essential goal for prognostic, therapeutic, and epidemiological purposes [16].

In Spain, after the introduction of mumps vaccine, the leading viral cause of aseptic meningitis was EV [17]. Since oral poliomyelitis vaccination was introduced in Spain in 1963 [18], surveillance has assisted public health officials in recognizing outbreaks of enteroviral disease. Moreover, in the last 8 years an increase of outbreaks of aseptic meningitis associated with NPEV has been observed [19].

This paper analyses the occurrence of different EV during a 10-year period (1988–97), using the data in The National Centre for Microbiology, CNM (Institute de Salud Carlos III, Majadahonda, Madrid). Furthermore, special attention is also focused on the outbreaks of aseptic meningitis during these years. Because our laboratory is the only one in Spain which serotypes enterovirus isolates, we receive samples from patients from different regions of Spain and also virus isolates (obtained by other primary laboratories) for identification and typing. Therefore, we are able to ascertain the frequency and aetiology of enteroviral diseases and the distribution patterns of enteroviruses.

## MATERIALS AND METHODS

### Patients and samples

A total of 15662 clinical samples over a 10-year period (1988–97) from hospitalized patients of different regions of Spain suffering diseases related to EV, mostly aseptic meningitis, were studied. In this period,

we obtained 999 enterovirus isolates and, in addition, 210 EV isolates from patients with aseptic meningitis were sent to our laboratory by seven primary laboratories. The source of EV isolates was cerebrospinal fluid (CSF) (617), stool (504) and throat swabs (88). More than 50% of the total number of virus isolates were associated with 60 different outbreaks of aseptic meningitis. Other isolates were from clinical samples from cases of sporadic aseptic meningitis and samples from patients (children under 1 year) with minor illness such as fever and upper respiratory infections.

### Virus isolation

All samples were inoculated onto cell cultures for the isolation of EV in four continuous cell lines [Buffalo green monkey kidney (BGM), human rhabdomyosarcoma (RD), human lung carcinoma (A-549), and human embryonic fibroblast cells (HEF)], selected for their support of EV replication [20]. For the PV investigation stools samples were also studied as is recommended by WHO [21].

Cell cultures demonstrating cytopathic effect (CPE) were serotyped by the standard method of neutralization, using pools of Lim Benyeesh–Melnick antisera [22]. In the last 3 years alternative pools have been used (RIVM pools, National Institute of Public Health and the Environment, The Netherlands).

Polioviruses were characterized by a neutralization procedure using monoclonal antibodies to differentiate Sabin-derived vaccine strains from wild-type strains. These monoclonal antibodies were kindly supplied by Dr P. Minor (NIBSC, National Biological Standards Board, Hertfordshire, UK). In the last 5 years, the intratypic differentiation of polioviruses was also carried out by restriction fragment length polymorphism (RFLP) after RT nested-PCR [23].

## RESULTS

Of the 1209 EV isolates, 758 (598 from our laboratory and 160 from other primary laboratories) were fully typed: 727 were NPEV and 31 were Sabin-like polioviruses (not associated with poliomyelitis cases) with the following distribution: 20 PV1, 9 PV2 and 2 PV3 (Table 1). Of the fully typed EV, 465 were associated with aseptic meningitis outbreaks, 252 from cases of sporadic aseptic meningitis and 41 with minor illness. The remaining 451 EV isolates were

Table 1. *Distribution of enterovirus serotypes in Spain (1988–97)*

Year ... Isolates* ...	1988	1989	1990	1991	1992	1993	1994	1995	1996	1997	Total
EV-2	0	0	0	0	1	0	0	0	0	0	1
EV-4	0	0	0	48	9	1	1	0	0	0	59
EV-5	0	0	0	0	0	4	0	7	8	0	19
EV-6	12	5	0	2	0	8	2	1	3	102	135
EV-7	2	1	0	0	3	1	1	14	3	3	28
EV-9	0	2	1	0	57	76	1	4	7	0	148
EV-11	0	0	0	0	0	3	1	1	15	1	21
EV-14	0	0	0	0	1	1	0	0	0	0	2
EV-15	0	0	0	0	1	0	0	0	0	0	1
EV-16	0	0	0	0	0	0	0	1	0	0	1
EV-17	0	0	2	1	7	5	0	1	0	0	16
EV-18	0	0	0	0	0	0	0	0	5	5	10
EV-20	0	0	0	0	0	0	2	0	0	0	2
EV-21	0	0	0	0	1	0	2	0	0	0	3
EV-22	0	0	0	0	0	0	0	0	0	1	1
EV-22/23	0	1	0	0	0	0	0	0	0	0	1
EV-24	0	0	1	0	0	0	0	0	0	0	1
EV-25	1	0	0	0	1	2	1	0	0	0	5
EV-27	0	0	0	0	1	0	0	0	0	0	1
EV-30	0	0	0	1	52	1	11	12	88	26	191
EV-31	0	0	0	2	3	2	0	0	0	0	7
Total echoviruses	15	9	4	54	137	104	22	41	129	138	653
CAV-9	0	4	0	0	2	0	0	0	2	6	14
CAV-16	0	4	3	0	0	0	0	0	0	0	7
CBV-1	0	0	1	0	0	0	0	0	4	0	5
CBV-2	0	0	0	0	0	0	1	0	0	0	1
CBV-4	0	0	0	1	6	0	3	0	4	2	16
CBV-5	0	0	0	0	9	2	0	0	3	12	26
CBV-6	0	3	0	0	0	1	1	0	0	0	5
Total NPEVs	15	20	8	55	154	107	27	41	142	158	727
Polio 1†	2	2	0	0	1	3	1	5	3	3	20
Polio 2†	0	0	0	0	3	0	1	2	2	1	9
Polio 3†	0	0	0	0	0	1	0	0	0	1	2
Total Polioviruses	2	2	0	0	4	4	2	7	5	5	31
NPEVs isolates‡	49	46	12	95	40	43	24	48	53	41	451
Total enteroviruses	66	68	20	150	198	154	53	96	200	204	1209

\* EV, echovirus; CAV, Coxsackie A virus; CBV, Coxsackie B virus; NPEVs, non-polio enteroviruses.

† All poliovirus typed were Sabin-like (not associated with poliomyelitis-cases).

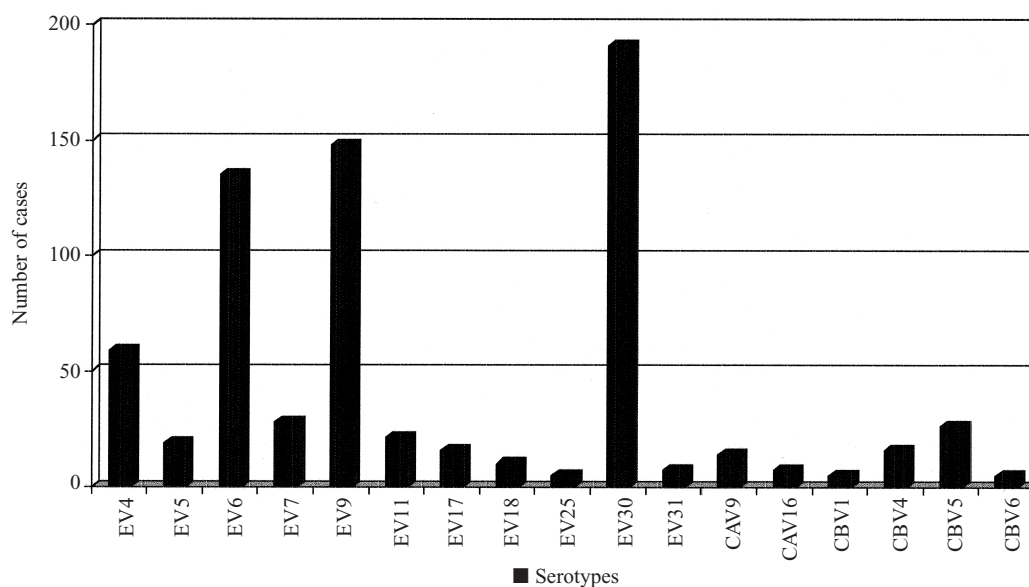
‡ These isolates were not fully typed (see explanation in the text).

NPEV but were not fully typed (152 came from patients with aseptic meningitis outbreaks in which other samples had been fully typed and 299 were isolated from stools from patients with sporadic aseptic meningitis and minor illness cases).

Table 1 shows the distribution of EV serotypes over a 10-year period. The EV incidence was highest in 1992, 1993, 1996 and 1997 with a total of 154, 107, 142 and 158 NPEV identified respectively. There was a substantial variation in the annual number of viruses recovered during the time of the study, with a marked

difference both in the serotypes detected and in the occurrence of the peaks.

Among the 727 NPEV identified, 90% were echoviruses (653 isolates). Echovirus 30 was the most prevalent echovirus serotype identified (29%, 191/653) especially in 1996 with a total of 88 isolates (68%, 88/129). The second most prevalent serotype was EV-9 with a total of 148 isolates, which was 23% (148/653) of the total echovirus isolates. This serotype circulated mainly in 1993 (73%, 76/104) and in 1992 (42%, 57/137), sharing the main circulation with EV-



**Fig. 1.** Number of total isolates of the most common serotypes of NPEV in Spain during the period 1988–97. EV, Echovirus; CAV, Coxsackie A virus; CBV, Coxsackie B virus.

**Table 2.** Number of outbreaks of aseptic meningitis and their associated enterovirus serotypes in Spain (1988–97)

Serotypes*	1988	1989	1990	1991	1992	1993	1994	1995	1996	1997	Total
EV-4	0	0	0	7	0	0	0	0	0	0	7
EV-6	2	0	0	0	0	0	0	0	0	4	6
EV-7	0	0	0	0	0	0	0	1	0	0	1
EV-9	0	0	0	0	10	9	0	1	0	0	20
EV-30	0	0	0	0	4	0	2	1	7	2	16
EV-31	0	0	0	0	0	1	0	0	0	0	1
CAV-9	0	1	0	0	0	0	0	0	0	0	1
CBV-6	0	1	0	0	0	0	1	0	0	0	2
Mix	0	1	0	0	1	0	1	0	0	1	4
Non-identified	0	1	0	0	1	0	0	0	0	0	2
Total	2	4	0	7	16	10	4	3	7	7	60

\* EV, echovirus; CAV, Coxsackie A virus; CBV, Coxsackie B virus; Mix, two or three enterovirus serotypes isolated; Non-identified, outbreaks in which the serotypes of the non-polio EV could not be identified.

30 (38%, 52/137). Echovirus 6 was the third most prevalent serotype identified with 135 isolates (21%, 135/635), especially in 1997 (74%, 102/138). Echovirus 4 was highly prevalent in 1991 with 89% (48/54). For the years 1991, 1993, 1996 and 1997 only one serotype, EV-4, EV-9, EV-30 and EV-6, respectively was isolated (Table 1).

Coxsackie B viruses were isolated from 53 cases (7%, 53/727). CB-5 was the commonest, (the third most prevalent serotype of all enteroviruses typed in 1997; 12 cases), followed by CBV-4 (16 cases). Coxsackie A viruses were identified in 21 cases (3%, 21/727) and the most prevalent was CAV-9 identified in 14 cases (Table 1). Figure 1 shows the most

common types of NPEV isolated during the 10-year period.

#### Annual distribution of enteroviruses associated with outbreaks of aseptic meningitis

Of the total EV typed, 465 corresponded to 58 different aseptic meningitis outbreaks. In two other outbreaks, the serotype of NPEV could not be identified. The number of aseptic meningitis outbreaks and their associated enterovirus are shown in Table 2. Figure 2 shows the four main EV serotypes associated with the number of outbreaks in each year of the study. The years with the highest number of entero-

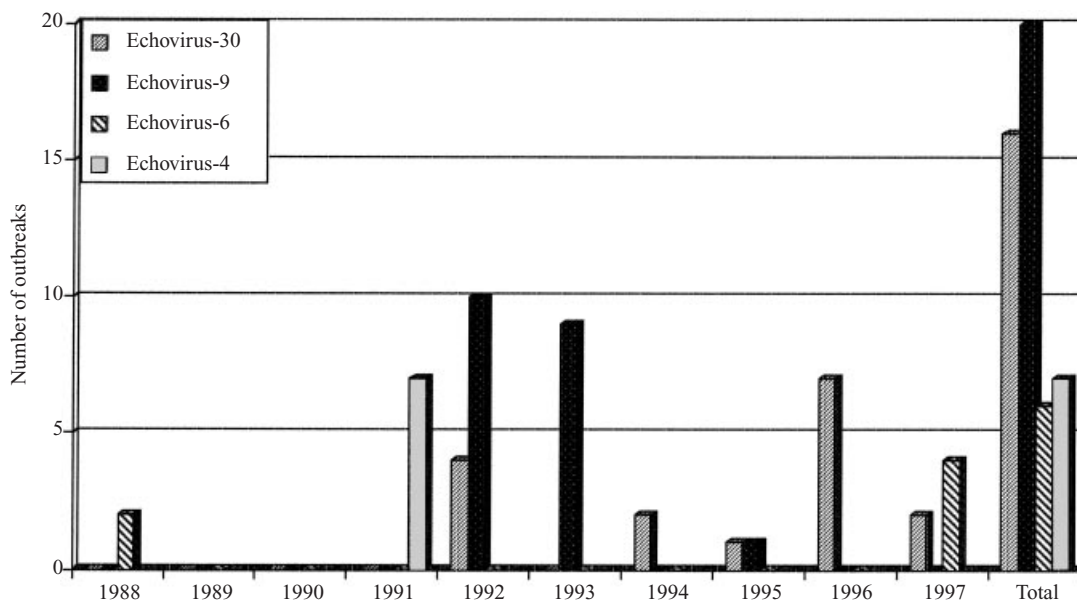


Fig. 2. The four main enterovirus serotypes associated with aseptic meningitis outbreaks during the period 1988–97.

virus isolates (1991, 1992, 1993, 1996 and 1997, Table 1) also show the highest number of aseptic meningitis outbreaks (between 7 and 16, Table 2). However, the correlation between the number of outbreaks and the total number of EV isolates was not absolute. For instance, in 1992 and 1997 the number of EV detected was similar, even though the number of corresponding outbreaks and the EV involved were different. The 16 outbreaks in 1992 were due to EV-9 and EV-30 while in the 7 outbreaks of 1997, EV-6 and EV-30 were involved. Aseptic meningitis outbreaks occurred every year with the exception of 1990, the year with the fewest EV isolated. The main serotypes associated with aseptic meningitis outbreaks were EV-9 (33%, 20/60), EV-30 (27%, 16/60), EV-4 (12%, 7/60) and EV-6 (10%, 6/60) (Fig. 2).

Table 3 shows the 10-year distribution of aseptic meningitis outbreaks corresponding to 11 out of the 17 Autonomous Communities of Spain. The geographical distribution of the main EV serotypes associated with outbreaks is shown in Figure 3. In the North, the most prevalent serotype causing outbreaks was EV-30 in Comunidad Gallega (CG) and País Vasco (PV). In the Mediterranean coast, with a total of 20 outbreaks, the Comunidad Valenciana (CV), in the middle of this coast, had the most outbreaks (12); 6 of them were associated with EV-9. In Castilla-León (C-L), 9 outbreaks were distributed in 4 provinces with EV-30 and EV-9 being the most prevalent serotypes. In Aragón (A), Northeast, 3 outbreaks were detected in which the EV-30 and EV-4 were

prevalent. In the Centre of Spain three Autonomous Communities presented a total of 11 outbreaks, EV-9 was identified in 7 of them. Finally, Andalucía (A), in the South presented a total of 8 outbreaks, and the main serotypes found were EV-4, EV-30 and EV-9.

### Major outbreaks

Figure 4 shows the occurrence of the most prevalent serotypes of echoviruses circulating during the period 1988–97. Five major waves of epidemic activity due to EV-30, EV-9, EV-6 and EV-4 were found. An analysis of the temporal presentation of the outbreaks and sporadic cases and the geographical distribution of outbreaks (Table 3) suggests the following pattern of spread. In the spring of 1992, EV-30 started to circulate in the North and Northwest of Spain (León, May 1992 and La Coruña, June 1992). The virus persisted in this area until the end of the year (La Coruña, November 1992), but it did not seem to spread outside of this region. The virus appeared 4 years later, 1996, again in the North and Northwest (Santiago de Compostela, País Vasco, Burgos) and at this time spread Northeast (Zaragoza) and East (Murcia).

In late spring and summer of 1992, EV-9 started to circulate in the central (Madrid, June 1992) and Southern (Sevilla, July 1992) regions of the country, spreading widely to the North and West (Palencia, October 1992) and the Mediterranean coast (Tarragona, October 1992 and Murcia, Alicante, Castellón,

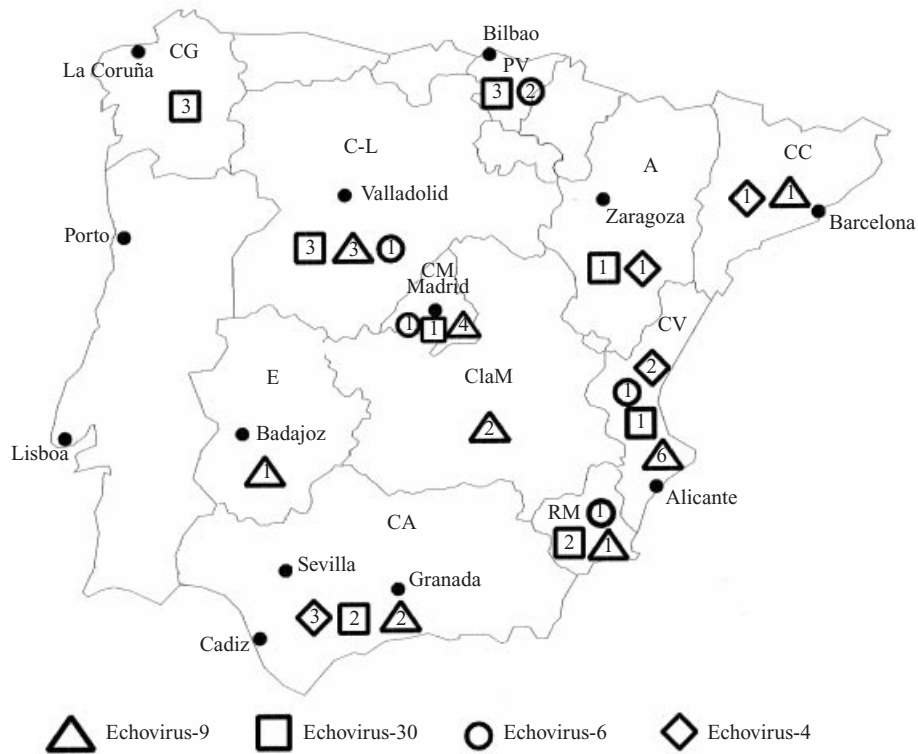
Table 3. *Distribution of aseptic meningitis outbreaks in the Autonomous Communities of Spain (1988–97)\**

AC†	City	1988	1989	1990	1991	1992	1993	1994	1995	1996	1997	Total
(CG)	Orense										1 Mix	1
(CG)	Coruña					2EV30		1CBV6				3
(CG)	Santiago									1EV30		1
(PV)	Alava									1EV30		1
(PV)	Bilbao									2EV30	2EV6	4
(C-L)	León		1Mix			1EV30						2
(C-L)	Palencia					1EV30, 1EV9						2
(C-L)	Valladolid		1CAV9					1EV9				2
(C-L)	Burgos							1EV9		1EV30	1EV6	3
(CM)	Madrid		1CBV6			1EV9	3EV9	1EV30				6
(CM)	Alcalá										1EV6	1
(C la M)	Guadalajara						1EV9					1
(C la M)	Ciudad Real					1Non-i	1EV9					2
(A)	Teruel		1Non-i		1EV4							2
(A)	Zaragoza									1EV30		1
(E)	Badajoz						1EV9					1
(CV)	Castellón					1EV9	1EV31					2
(CV)	Valencia				1EV4	1EV9						2
(CV)	Alicante	1EV6			1EV4	3EV9			1EV7,1EV9		1EV30	8
(RM)	Murcia	1EV6				1EV9		1Mix		1EV30	1EV30	5
(CC)	Gerona				1EV4							1
(CC)	Tarragona					1EV9						1
(CA)	Granada				1EV4							1
(CA)	Cádiz				1EV4	Mix						2
(CA)	Málaga				1EV4		1EV9		1EV30			3
(CA)	Sevilla					1EV9						1
(CA)	Córdoba							1EV30				1
Total		2	4	0	7	16	10	4	3	7	7	60

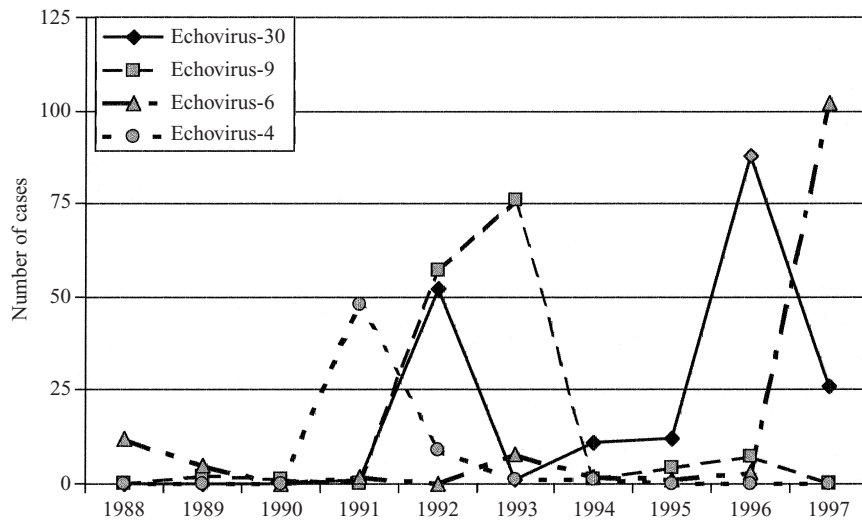
\* EV, Echovirus; CAV, coxsackie A virus; CBV, coxsackie B virus; Non-i, Non-identified.

† AC, Autonomous Communities; CG, Comunidad Gallega; PV, País Vasco; C-L, Castilla y León; CM, Comunidad de Madrid; C la M, Castilla la Mancha; A, Aragón; E, Extremadura; CV, Comunidad Valenciana; RM, Región de Murcia; CC, Comunidad Catalana; CA, Comunidad Andaluza.





**Fig. 3.** The geographical distribution in Spain of the four main enterovirus serotypes associated with aseptic meningitis outbreaks. CG, Comunidad Gallega; PV, País Vasco; C-L, Castilla y León; CM, Comunidad de Madrid; C la M, Castilla la Mancha; A, Aragón, E, Extremadura; CV, Comunidad Valenciana; RM, Región de Murcia; CC, Comunidad Catalana; CA, Comunidad Andaluza.



**Fig. 4.** Prevalence of four main enterovirus serotypes during the period 1988–97.

Valencia, November–December 1992). In the first half of 1993, EV-9 continued circulating in the centre of Spain (Ciudad Real, April 1993, Madrid, Guadalajara, May 1993, Valladolid and Burgos, June 1993), but also in the Mediterranean coast (Málaga, January 1993). The enteroviral activity seemed to return to background levels at the early summer of 1993 because

no further specimens from outbreaks of aseptic meningitis were received for studying at the CNM from June 1993 to June 1994.

Echovirus 6 started to circulate in May of 1988 in the Mediterranean coast (Murcia) and spread to Alicante in December. The circulation of this virus was at a low level during the rest of the years in the

period of study and no outbreaks due to EV-6 were noted in Spain until 1997. In this year 102 isolates were typed as EV-6 resulting in the most numerous serotype identified in any 1 year of the 10-year study. In 1997, EV-6 started to circulate in the north of the country at the beginning of the year and was recovered from CSF samples until the end of summer (Bilbao, February–October). Afterwards it was isolated from samples from patients in the Northwest (Burgos, April–June) and spread to the Centre in the summer months (Alcalá de Henares, June–September).

Echovirus 4 started to circulate among the population of the Northeast of the country in early 1991 (Teruel, February–March 1991), then it spread over the Mediterranean coast (Alicante, Murcia, Granada, June–July 1991) to reach Southern provinces of Andalucía (Málaga and Cádiz, September 1991) later in the year.

## DISCUSSION

This study details the pattern of spread of different EV serotypes in Spain over a 10-year period. Most EV infections are subclinical or cause only minor non-specific symptoms, while our data are mostly based on isolates from hospitalized patients with severe disease. However, the distribution of EV infections in hospitalized patients should broadly reflect the spread of EV across Spain. Our laboratory policy consisted of identifying viral isolates from CSFs and throat swabs. CSF is readily available from patients with neurological diseases and virus isolation from this source has a high significance. However, examination of this type of sample may limit the range of viruses recovered [13]. Faeces is the best specimen for EV detection, although it is known that presence of EV in this sample does not provide evidence of aetiology since viral shedding may occur in the absence of symptoms [4]. Enteroviruses are worldwide in their distribution. In temperate climates they cause epidemics during the summer and autumn months, while in tropical areas infections occur with high incidence throughout the year [24]. In Spain EV infections have also been reported in winter [25].

Isolation of EV may be relatively simple but their further identification can be very demanding. Typing depends on the capacity of the virus to grow in cell cultures, and to be neutralized with specific antiserum. Some isolates remained unidentified because they represented an untypable enterovirus or a mixture of various EV [3, 13]. In both cases the combinations of

specific pools prepared by the collaborator laboratories of the WHO, were not able to neutralize them. Further identification could only be possible by the use of monotypic antiserum which is rarely available in diagnostic and reference laboratories. Other reasons for failure are the microbial contamination of clinical samples, specially throat swabs, or the low number of infective viral particles in specimens precluding the propagation in cell cultures [7, 26]. We have found, in agreement to other authors, that echoviruses are more frequently isolated than CAV and CBV from symptomatic humans [13, 27, 28]. Among the CAV and CBV, the highest number of cases was due to CBV-5. CBV-3 was not isolated during the 10-year study period, probably because our study was focused on aseptic meningitis outbreaks and CBV-3 is not considered as an epidemic type [29]. As in other countries, a small number of serotypes were identified as the cause of aseptic meningitis in Spain, EV-30, EV-9, EV-6 and EV-4 being the most common serotypes [9, 30, 31]. Echovirus 30 was the virus mostly implicated in the aseptic meningitis syndrome (16 outbreaks and numerous sporadic cases), being in 1996 the sole serotype associated with every outbreak. It has been found as the predominant serotype in other Mediterranean countries (e.g. France; 1974–85 [30] and also in Japan; 1981–91) [31]. However, EV-9 was associated with 20 aseptic meningitis outbreaks, in 2 years (1992–3). The analysis of these data suggests an important relation between EV-9 and epidemic aseptic meningitis [1], showing a slight difference with EV-30. On the other hand, EV-4 was associated with all seven outbreaks in 1991 and EV-6 caused sudden and explosive epidemics in 1997. It is of interest that the geographical distribution of EV-4 was limited to the Mediterranean coast and to the South of Spain. The other three echoviruses were widely distributed throughout Spain independently of the number of outbreaks associated with them. However, it must be taken into account that our results are mostly related to aseptic meningitis, therefore, these results cannot be compared with studies in which the relationship between EV and different diseases has been established [27, 28].

Since 1996, an intensive surveillance of acute flaccid paralysis is being achieved in order to obtain in our country the WHO certificate for the Eradication of Poliomyelitis. In this context, our laboratory has been chosen as the reference laboratory for a Spanish laboratory network, with the purpose of showing the absence of wild PVs in our country. The ability to



differentiate between wild and vaccine-derived PVs and the knowledge of the circulating NPEV will play a crucial role in the control of enteroviral infectious diseases and epidemiological surveillance in the future.

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