MAJOR ARTICLE

Variations in Cerebrospinal Fluid Viral Loads Among Enterovirus Genotypes in Patients Hospitalized With Laboratory-Confirmed Meningitis Due to Enterovirus

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Background. Acute enterovirus (EV) meningitis is a major cause of hospitalization among adults and children. It is caused by multiple EV genotypes assigned to 4 species (EV-A, EV-B, EV-C, and EV-D).

Methods. We determined viral loads in the cerebrospinal fluid (CSF) of 156 patients of all ages with EV meningitis during a 5-year observational prospective study. The virus strains were genotyped, and their time origin was determined with Bayesian phylogenetic methods.

Results. The CSF viral loads ranged between 3.4 and 7.5 $\log_{10}$ copies/mL (median, 4.9 $\log_{10}$ copies/mL). They were higher in neonates than in infants and children ($P = .02$) but were comparable in adults. Viral loads were associated with EV genotypes ($P < .001$). The EV strains were identified in 152 of 156 patients and assigned to 23 genotypes within the EV-A and EV-B species. The most frequent genotypes, echoviruses 6 and 30, were associated with different viral loads ($P < .001$). The highest viral loads were in meningitis cases caused by coxsackievirus A9, B4, and B5 genotypes. Most patients infected by a same genotype were infected by a major virus variant of recent emergence.

Conclusions. The variations in CSF viral loads in patients at the onset of EV meningitis are related to genotypic differences in the virus strains involved.

Keywords. Enterovirus meningitis; pleocytosis; adults; children; infants; neonates; Enterovirus genotypes; CSF viral load; meningitis pathophysiology.

Human enterovirus (EV) diseases can be caused by 100 genotypes that are classified according to genetic relationships into 4 species designated, EV-A, EV-B, EV-C, and EV-D [1]. A number of EV genotypes infect the central nervous system and are responsible for clinical manifestations including meningitis, encephalitis, and, more rarely, paralytic myelitis, cerebellar ataxia, and Guillain–Barré syndrome [2–5]. The 61 genotypes within the EV-B species are the most common cause of acute meningitis, a self-limiting inflammation of the meninges characterized by favorable outcome and a major reason for admission to hospital of children and young adults [6–9]. EV meningitis occurs as outbreaks of variable size, from local community outbreaks to large epidemics [10, 11].

Diagnosis of EV infection in patients with suspected meningitis relies on examining cerebrospinal fluid (CSF) with rapid nucleic acid amplification tests (reverse-transcription polymerase chain reaction [RT-PCR]), a procedure that avoids unnecessary investigations and antimicrobial administration [7, 8]. The early differential identification of EV genotypes involved in neurological syndromes is important for outbreak and patient management, in particular when enterovirus 71

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(EV71) and poliovirus genotypes are involved [12, 13]. Epidemiological surveys suggest that neurotropism and pathogenic features vary among EV genotypes [4]. Data regarding the CSF viral loads in patients with neurological conditions are scant but would prove helpful in elucidating further the history of EV infections. The quantitative genome detection of herpesviruses in patients with neurological diseases showed a clear relationship between high viral load in CSF and the severity and outcome of disease [14, 15]. A few methods have been developed to determine EV RNA levels in clinical samples [16, 17], but none have been used prospectively in large observational studies. The investigation of systemic coxsackievirus B3 (CVB3) infections in 10 neonates suggested a relationship between higher viral load in blood and younger age and greater disease severity [18]. We recently described a method based on TaqMan real-time RT-PCR coupled with an internal control RNA to quantify the EV genomes in CSF [19]. As a first step toward the investigation of pathogenic features of neurotropic EVs, we determined the concentration of EV RNA in CSF specimens from patients with acute meningitis and analyzed possible relationships between CSF viral loads and biological, clinical, and virological characteristics.

METHODS

Patients and Clinical Samples

This study was approved by the local committee for the protection of human subjects (Comité d'Ethique des Centres d'Investigation Clinique de l'Inter-Région Rhône-Alpes-Auvergne, France) with a waiver of informed consent (institutional review board number 5044).

A total of 156 patients with laboratory-confirmed EV meningitis were included in the study. They were hospitalized between 1 January 2008 and 31 December 2012 at the University Hospital of Clermont-Ferrand (n = 133) and between June and August 2011 in the Pediatric Unit of the Hospital of Versailles (n = 23). Patients were distributed as follows: 19 (12%) were neonates (median age, 21 days; range, 9–28 days), 27 (17%) were infants (median age, 40 days; range, 30–458 days), 57 (37%) were children (median age, 6 years; range, 2–15 years), and 53 (34%) were adults (median age, 31 years; range, 17–57 years). All neonates were full-term, and no patient was immunocompromised. In all patients, bacteriological investigations and results of tests for molecular detection of herpes simplex virus were negative.

EV molecular diagnosis was based on analysis of CSF specimens obtained at patient admission, using either the NucliSENS EasyQ^{R} Enterovirus kit manufactured by bioMérieux (Clermont-Ferrand) or Cepheid's Xpert EV assay on a SmartCycler (Versailles). The leftover part of each EV-positive CSF samples or RNA extract was stored at −80°C before analysis within 8 days to determine the viral load and genotype (see below).

Data were gathered from medical charts and hospital computer records, using a standardized questionnaire, which included patients' clinical history, date and time of admission, and CSF characteristics. Symptom duration was defined as the interval between the onset of symptoms and lumbar puncture. The onset of symptoms was estimated to have occurred at 8 AM, 2 PM, 8 PM, and 2 AM when it was recorded in the morning, the afternoon, the evening, and at night, respectively. Pleocytosis was defined as a CSF white blood cell (WBC) count of >19 cells/mm^3 for patients aged ≤28 days and as ≥10 cells/mm^3 for older patients [20]. CSF protein concentration was classified as normal if it was ≤0.9 g/L for newborns <30 days old and ≤0.45 g/L for older patients.

Viral Load Determination

Viral RNA was extracted from 200 µL of CSF by a NucliSens EasyMAG extractor (bioMérieux) and eluted in 25 µL of elution buffer. Internal control was added before the extraction step, and quantification of the EV genome was performed with our in-house real-time RT-PCR assay, developed on the Rotor-Gene 6000 (Qiagen) [19]. The lowest concentration of viral RNA quantified with ≥90% probability is 15 copies/µL (95% confidence interval [CI], 10–20 copies/µL), equivalent to 1875 copies/mL (3.3 $\log_{10}$ copies/mL). The absence of PCR inhibitors in all CSF specimens tested was indicated by the effective amplification of internal control.

Enterovirus Genotyping and Phylogenetic Analysis

Enterovirus strains were genotyped by nucleotide sequencing of viral genes encoding the VP4/VP2 and VP1 capsid proteins with our previously described method [21]. Viral RNA extracted from clinical specimens (throat, plasma, or CSF) was used for complementary DNA synthesis, followed by gene amplification. The nucleotide sequences of PCR products were determined, and the VP1 sequences were deposited in GenBank under the accession numbers HG793656–785. The sequences were used to identify the EV strain in each patient as described earlier [21]. We generated a data set by aligning the VP1 sequences determined in 130 patients to the reference sequences of 17 EV-B genotypes. The time origin of virus lineages was analyzed in a Bayesian statistical framework with a Markov Chain Monte Carlo (MCMC) method implemented in the BEAST program, version 1.7.5 [22, 23] using previously described approaches [24]. The general-time-reversible model with an invariant class of nucleotide substitution and a gamma distribution of substitution rates was used to estimate nucleotide substitutions. The Bayesian skyline model [24] was used as tree prior under a relaxed molecular clock model as described earlier [25]. MCMC analyses were run for 60 million generations, sampling a tree every 3000 steps. A maximum clade credibility (MCC) tree was produced with the TreeAnnotator and FigTree programs (available at: http://beast.bio.ed.ac.uk/TreeAnnotator and http://tree.bio.ed.ac.uk/software/figtree, respectively). Statistical support for the tree nodes was assessed by their posterior

probability. Time to the most recent common ancestor (tMRCA) at each node in the phylogeny was calculated from the median height parameter in the MCC tree. Statistical uncertainty in the tMRCA calculations was estimated as a 95% highest posterior density (HPD) interval.

Statistical Analysis

Statistical analysis was performed using Stata software, version 12 (StataCorp, College Station, TX). The tests were 2-sided, with a type I error set at an α level of 0.05. The characteristics of patients were presented as the mean ± standard deviation or the median and interquartile range (IQR) for each age group, for continuous data, and as the number of patients and associated percentages, for categorical parameters. Viral loads, expressed in genome copies per milliliter, were log-transformed to base 10 for these analyses. Comparisons between groups were analyzed with the χ^2 or Fisher exact test, for categorical variables, followed by the Marascuilo procedure, and with analysis of variance or the Kruskal–Wallis test, for quantitative variables (normality verified by the Shapiro-Wilk test and evaluated for homoscedasticity by the Bartlett test), followed by an appropriate post hoc multiple comparisons test (ie, the Tukey–Kramer or Dunn test). Relations between quantitative parameters were explored by correlation coefficients (ie, Pearson or Spearman coefficients). A linear regression model (with EV load as the dependent variable) was considered in multivariate situation by backward and forward stepwise for the factors considered significant in univariate analysis (entry in the model for $P < .1$) and according to relevant biological and clinical parameters, such as red blood cell (RBC) count, protein level in the CSF, and duration of symptoms (adjustment factors). The interactions between factors were tested. Results were expressed as regression coefficients and 95% CIs. To investigate possible bias caused by a traumatic tap during lumbar puncture, we also calculated the WBC counts and protein concentrations by subtracting 1 WBC for every 500–1000 RBCs in the CSF and 0.01 g/L protein for every 1000 RBCs/mm^3 [26]. The results obtained with the 2 methods were comparable.

RESULTS

Relationship Between CSF Viral Loads and Clinical and Biological Characteristics

The viral loads determined in 156 sequential patients with EV meningitis ranged between 3.4 and 7.5 with a median of 4.9 $\log_{10}$ copies/mL of CSF (IQR, 4.3–5.4 $\log_{10}$ copies/mL). The median CSF viral loads were significantly different between age groups ($P = .02$; Figure 1*A*). Viral loads in neonates (5.4 $\log_{10}$ copies/mL) were higher than those in infants (4.4 $\log_{10}$ copies/mL; $P = .007$) and children (4.7 $\log_{10}$ copies/mL; $P = .01$) but were not different from viral loads in adults (5.0 $\log_{10}$ copies/mL). There was no relation between CSF viral loads and fever, headache, and stiff neck, but the EV RNA levels were elevated in 13 patients (mainly adults) with vertigo and paresthesia (5.1 vs 4.8 $\log_{10}$ copies/mL; $P = .03$; Supplementary Table 1).

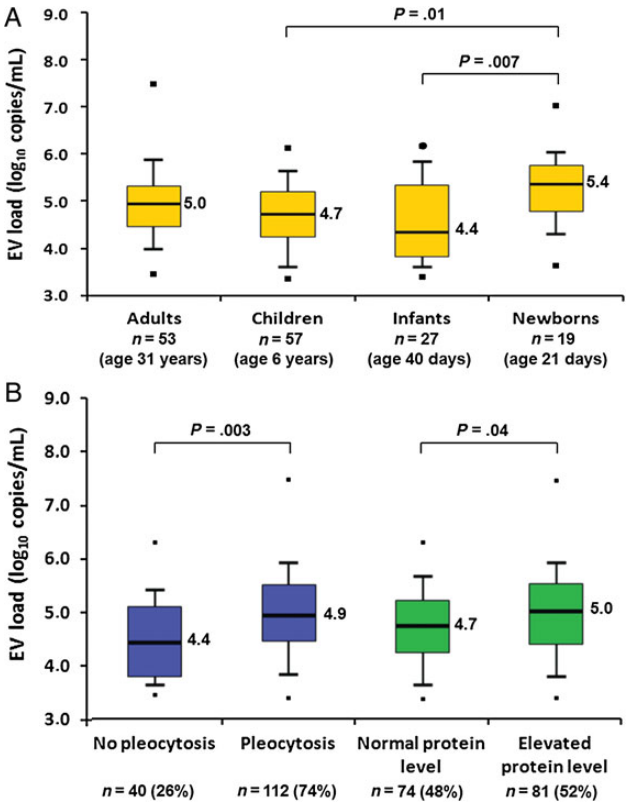

Figure 1. Enterovirus (EV) cerebrospinal fluid (CSF) RNA levels in the study population (n = 156) of patients with laboratory-confirmed meningitis. The box plots represent CSF viral loads stratified by age group (*A*) and pleocytosis or protein levels in the CSF specimens (*B*). Results are reported as medians (horizontal lines in boxes; values are indicated in the figure), interquartile ranges (top and bottom edges of boxes), and the first and ninth decile (small horizontal lines extended below and above the boxes). Points represent the lowest and highest values. Only statistically significant differences are displayed.

A relationship was observed between viral loads and the number of leukocytes and the concentration of proteins in CSF (Figure 1*B*). Pleocytosis (median WBC count, 100 cells/mm^3; range, 10–1600 cells/mm^3) was present in 112 of 152 patients (74%). WBC count differed between age groups ($P < .01$). The median values in adults, children, infants, and neonates were 82, 97, 4, and 6 cells/mm^3, respectively. WBC count was not performed in 4 patients (3 neonates and 1 infant) because of the presence of a coagulum. The median WBC counts in CSF with pleocytosis did not differ between age groups (100 cells/mm^3; $P = .58$). Pleocytosis was not observed in 11 of 16 newborns (69%), 16 of 26 infants (62%), 4 of 57 children (7%), and 9 of 53 adults (17%). The viral loads were higher in the presence of pleocytosis (median, 4.9 $\log_{10}$ copies/mL) than in

the absence of pleocytosis (4.4 $\log_{10}$ copies/mL; $P = .003$) (Figure 1*B*). This difference was observed in adults (5.0 vs 4.2 $\log_{10}$ copies/mL; $P = .003$) and newborns (5.8 vs 4.9 $\log_{10}$ copies/mL; $P = .04$), but not in children and infants, owing to the effects of sampling size. Among the 40 patients with pleocytosis who were admitted within the first 24 hours of illness, 25 (62%) had a CSF neutrophil percentage of >55% and a median CSF viral load of 5.05 $\log_{10}$ copies/mL. The other 15 patients had a viral load of 4.40 $\log_{10}$ copies/mL ($P = .07$). Among the 69 patients admitted later, 39 (57%) had lymphocyte percentage of 55% and a viral load of 4.91 $\log_{10}$ copies/mL. The other 30 patients had a viral load of 4.95 $\log_{10}$ copies/mL ($P = .68$).

CSF protein concentration was high in 81 of 155 patients (52%) and was higher in adults (0.59 g/L) than in children (0.36 g/L; $P < .001$) and infants (0.51 g/L; $P = .03$). The viral load was higher in patients with elevated protein concentrations (5.0 $\log_{10}$ copies/mL) than in those with normal protein level (4.7 $\log_{10}$ copies/mL; $P = .04$; Figure 1*B*). The protein levels were higher when pleocytosis was present ($P = .04$), particularly among adults.

Relationship of Viral Load, Age, and Pleocytosis With Symptom Duration

There was a difference in symptom duration before lumbar puncture between the age groups (Figure 2): it was shorter in neonates (0.8 days) and infants (0.7 days) than in children (1.2 days; $P \leq .008$ for both comparisons, compared with neonates and infants) and adults (1.3 days; $P < .001$, compared with neonates and infants). Although there was no relationship between symptom duration and viral load (R = .12; $P = .14$), the patients who had CSF pleocytosis also had a longer symptom duration (1.2 vs 0.8 days; $P = .001$).

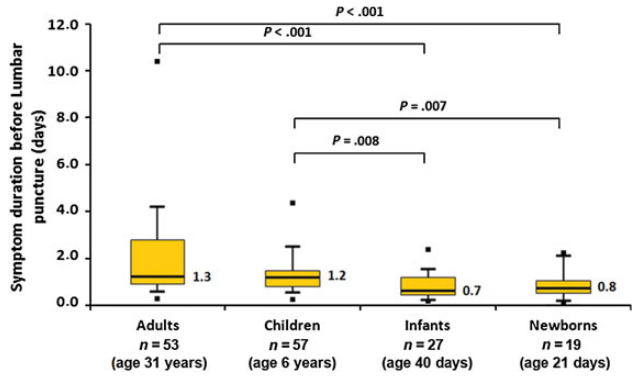

Figure 2. Number of days between the onset of symptoms and the lumbar puncture after patient's admission to the hospital. The symptom duration is represented as box plots and compared between age groups. Results are reported as medians (horizontal lines in boxes; values are indicated in the figure), interquartile ranges (top and bottom edges of boxes), and the first and ninth decile (small horizontal lines extended below and above the boxes). Points represent the lowest and highest values. Only statistically significant differences are displayed.

A multivariate analysis that adjusted for RBC count, protein level in the CSF, and duration of symptoms confirmed that patient age and CSF pleocytosis were related to CSF viral loads, but symptoms of vertigo and paresthesia were not (Table 1).

Relationships Between CSF Viral Loads and EV Genotypes

EV strains were genotyped with viral sequences VP4/VP2 (16 patients) or VP1 (136 patients) (Supplementary Figure 1). In 4 patients, it was not possible to identify EV strains because of low CSF viral loads. The 7 most frequent EV genotypes belonged to the EV-B species: echovirus (E) genotypes 30 (37%), 6 (13%), 11 (8%), and 18 (7%) and CV genotypes A9 (6%), B5 (4%), and B4 (3%). An array of 12 other EV-B and 4 EV-A genotypes was found in 19% of patients. The EV genotypes were distributed differently among the age groups (Figure 3*A*–*D*). Meningitis in young adults was caused by E30 (57% of cases), E6 (9%), CVA9 (9%), and E18 (7%). E6 and E30 genotypes were also the main causes of meningitis in children (25 and 39%, respectively), followed by E11 and CVB5 genotypes (5% each).

Table 1. Multivariate Analysis of Variables Associated With Enterovirus Load in the Cerebrospinal Fluid (CSF) of Patients With Meningitis

Characteristic	Regression Coefficient (95% CI)	*P*
Patient group		
Children	Reference	
Adults	−0.046 (−.39 to .30)	NS
Infants	−0.027 (−.5 to .45)	NS
Newborns	0.77 (.24–1.3)	.005
CSF data		
Samples with pleocytosis	Reference	
Samples without pleocytosis	−0.48 (−.86 to −.1)	.015
Clinical signs		
Absence of vertigo and paresthesia[a]	Reference	
Presence of vertigo and paresthesia	0.44 (−.16 to .89)	.058
Enterovirus genotypes		
E6	Reference	
E30	0.54 (.16–.91)	.005
CVA9	0.96 (.39–1.54)	.001
CVB4	1.16 (.45–1.88)	.002
CVB5	0.44 (−.22 to 1.09)	NS
E11	0.23 (−.36 to .82)	NS
E18	0.47 (−.1 to 1.04)	NS

Results of multivariate regression analysis that adjusted for red blood cells count, protein levels in the CSF, and duration of symptom (sampling time). *P* values of <.05 were considered statistically significant.

Abbreviations: CI, confidence interval; CV, coxsackievirus; E, echovirus; NS, not significant.

[a] Among clinical symptoms of meningitis, only vertigo and paresthesia were considered significant in the univariate analysis and included in the multivariate analysis.

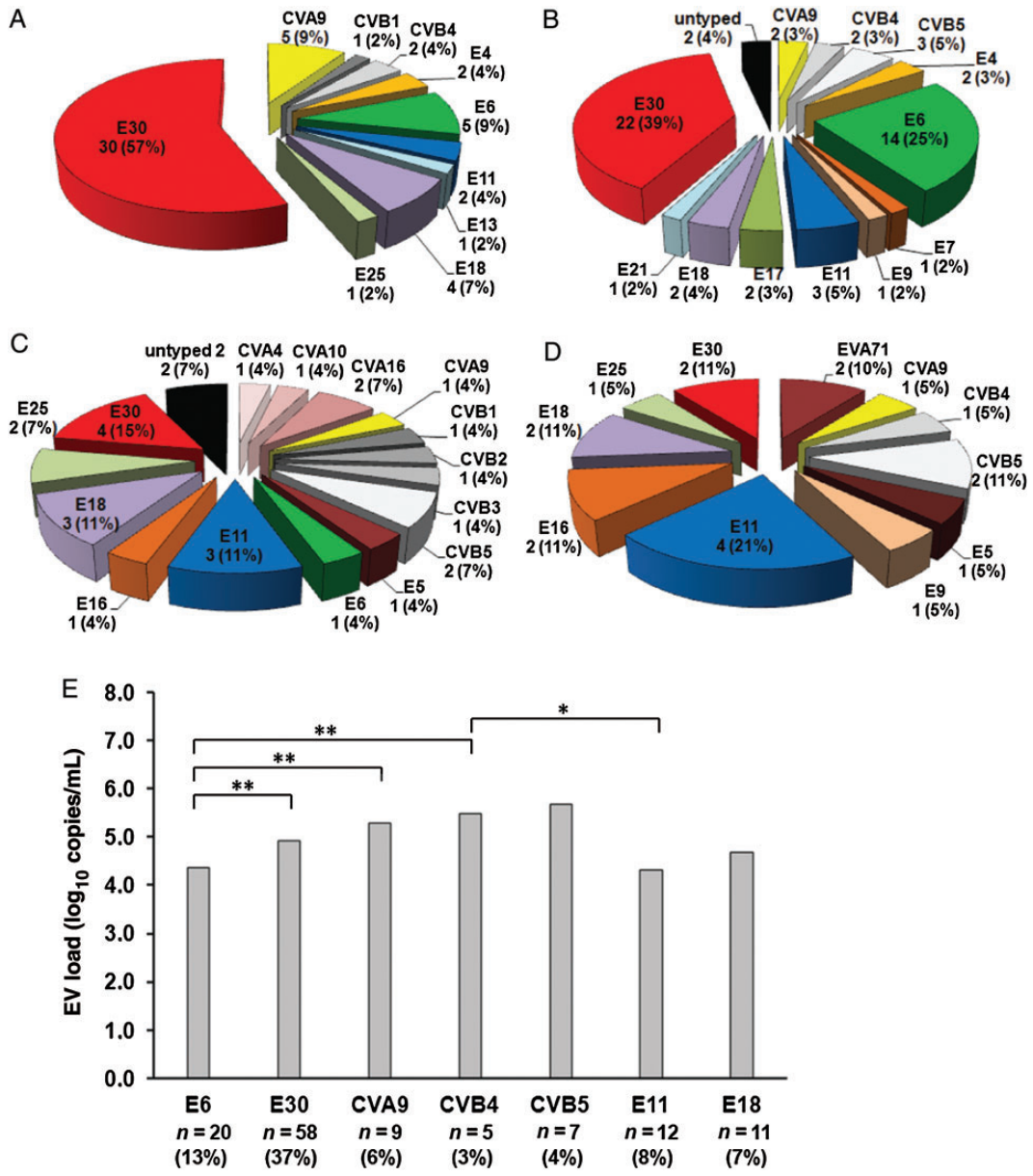

Figure 3. Comparison of cerebrospinal fluid (CSF) viral loads, by enterovirus (EV) genotype. Pie chart distribution of the EV genotypes according to the age of patients, as follows: adults (*A*), children (*B*), infants (*C*), and neonates (*D*). Data represent the number of patients (percentage), by EV genotype. *E*, Comparison of viral loads, stratified by EV genotypes. Results are reported as medians. All statistical comparisons were performed using the Kruskal–Wallis test, followed by the Dunn posttest. **P* < .05 and ***P* < 0.01. Abbreviations: CV, coxsackievirus; E, echovirus.

Infants and neonates were infected by a large array of EVs. E30 predominated in infants (15%), followed by E11 and E18 (11% each). In neonates, E11 (21%) was predominant, followed by E18 and CVB5 (11% each). Notably, EV strains assigned to different CV genotypes of the EV-A species (CVA4, A10, and A16, and EVA71) were also found in infants and neonates.

We observed relationships between CSF viral loads and EV genotypes ($P < .001$). Among the 7 most frequent EV genotypes (Figure 3*E*), the CSF viral loads in E6-infected patients (median, 4.4 $\log_{10}$ copies/mL) were lower than viral loads in patients infected with E30, CVA9, and CVB4 (4.9, 5.2, and 5.5 $\log_{10}$ copies/mL, respectively; $P < .01$, by the Kruskal–Wallis test followed by the Dunn posttest). Similarly, the CSF viral loads in E11-infected patients (4.3 $\log_{10}$) were lower than those in CVB4-infected patients ($P < .05$, by the Kruskal–Wallis test followed by the Dunn post test). Multivariate analysis confirmed that EV genotypes were associated with CSF viral loads (Table 1). E11 and CVB5 mainly infected younger infants (Table 2); E6, E18, and CVB4 were found in children; and CVA9 and E30 were found in adults ($P = .003$). The CSF protein levels differed

Table 2. Variables Associated With Enterovirus Serotypes in Cerebrospinal Fluid Specimens From Patients With Meningitis

Characteristic	Most Frequent Enterovirus Genotype							
	E6 (n = 20)	E30 (n = 58)	CVA9 (n = 9)	CVB4 (n = 5)	CVB5 (n = 7)	E11 (n = 12)	E18 (n = 11)	*P*
Age, y (d)	6	18	31	12	0.2 (69)	0.1 (45)	5	.003
Proteins concentration, g/L	0.4	0.5	0.6	0.8	0.5	0.6	0.5	.013
Leukocyte count, cells/mm^3	57	66	90	165	783	9	4	.002
Symptom duration, d	1.1	1.2	2.2	1.4	1.2	0.9	1.2	NS

Data are median values. *P* values of <.05 were considered statistically significant.

Abbreviations: CV, coxsackievirus; E, echovirus; NS, not significant.

between genotypes ($P = .013$): E6-infected patients had lower levels than E11- and CVB4-infected patients, and protein levels were lower in E30-infected patients than in those infected with E11 (Table 2). Differences were also determined between CSF WBC counts and genotypes ($P = .002$): patients infected with CVB5 had a greater number of WBCs than patients infected with the other EV genotypes.

Phylogenetic Clustering and Time Origin of EV-B Strains

The phylogenetic clustering and time origin of lineages corresponding to the 130 virus strains assigned to the EV-B species were estimated with their VP1 sequences (Figure 4*A*). The Bayesian phylogenetic tree clearly indicated that all patients with E30 meningitis were infected by the same variant. This virus emerged about 6 years before its first detection within the study population (Figure 4*B*). Similarly, most patients with E6 meningitis were infected by a main virus variant, which emerged in 2008, 3 years before the 2011 outbreak. Two patients with higher viral loads had E6 meningitis caused by genetically distinct virus strains (Figure 4*B*). The E11 and CVA9 phylogenetic patterns were similar to the E6 pattern: all but 1 or 2 patients were infected by a main virus variant, which arose 2 years before the initial detection in the study population (Figure 4*B*). In patients with E18 meningitis, 2 variants that emerged in 2002 and 2009 were found with similar frequencies (Figure 4*B*).

DISCUSSION

This prospective study explored the genotype-specific CSF viral loads in 156 patients of all ages hospitalized with EV meningitis. Our study is the first to provide consistent and evidence-based data suggesting that the EV RNA levels in the CSF of patients with acute meningitis are related to the genotype of the virus strain involved.

Although the exact incidence of EV meningitis is unknown, it is highest among children and young adults [27–29], 2 groups that represented 37% and 34%, respectively, of our study population. In addition, the interval between the onset of symptoms and lumbar puncture was similar in the 2 groups, and the median CSF viral load was moderate (4.74 and 4.96 $\log_{10}$ copies/mL in children and adults, respectively). A study of a single meningitis epidemic in 2000 caused by EV genotypes E6, E13, and E30 reported a median CSF viral load of 4.5 $\log_{10}$ copies/mL in 61 young patients, mainly children [16]. A decisive finding of our investigation was that >64% of children and adults were infected with the same 2 EV genotypes, E6 and E30, but in different proportions: E6 caused meningitis in 9% of adults and 25% of children, whereas E30 meningitis was seen in 57% of adults and 39% of children. As we also found that CSF viral loads were higher in patients with E30 meningitis than in those with E6 meningitis, we concluded that the amount of virus in the CSF early after the onset of symptoms was mainly determined by viral genotype in the 2 patient groups. Phylogenetic investigations revealed that the E6 and E30 meningitis cases were caused by virus variants that emerged a few years before their involvement in the seasonal epidemics in France in 2009 (E30) and 2011 (E6; Supplementary Figure 2). From the above phylogenetic data, we concluded that the EV RNA levels in the study population may be related to genotypic features, as shown for E30. In contrast, the E6 data suggested that subgenotypic features may be involved in the RNA levels because the 2011 variant was clearly distinct from the E6 viruses identified in earlier seasons [30, 31].

We found that the absence of CSF pleocytosis, as reported in previous studies, was associated with a shorter duration of symptoms in most of the youngest patients [32, 33]. These results indicate that, as suggested by Sato et al [34], in patients admitted when the disease was at an initial stage, the circulating blood leukocytes had not yet infiltrated the CSF compartment. The neonates had higher CSF viral loads than the infants and children but not the adults. In other respects (eg, symptom duration before lumbar puncture and diversity of EV genotypes involved), infections in neonates and infants had similar characteristics. Kawashima et al analyzed the CSF in 21 patients aged 13 days to 11 months and found high viral loads in 4 of 11 neonates and 2 of 10 infants without, however, identifying the virus strains involved [17]. In 24 cases of E9 infections, including neonates and infants, Dalwai et al determined mean

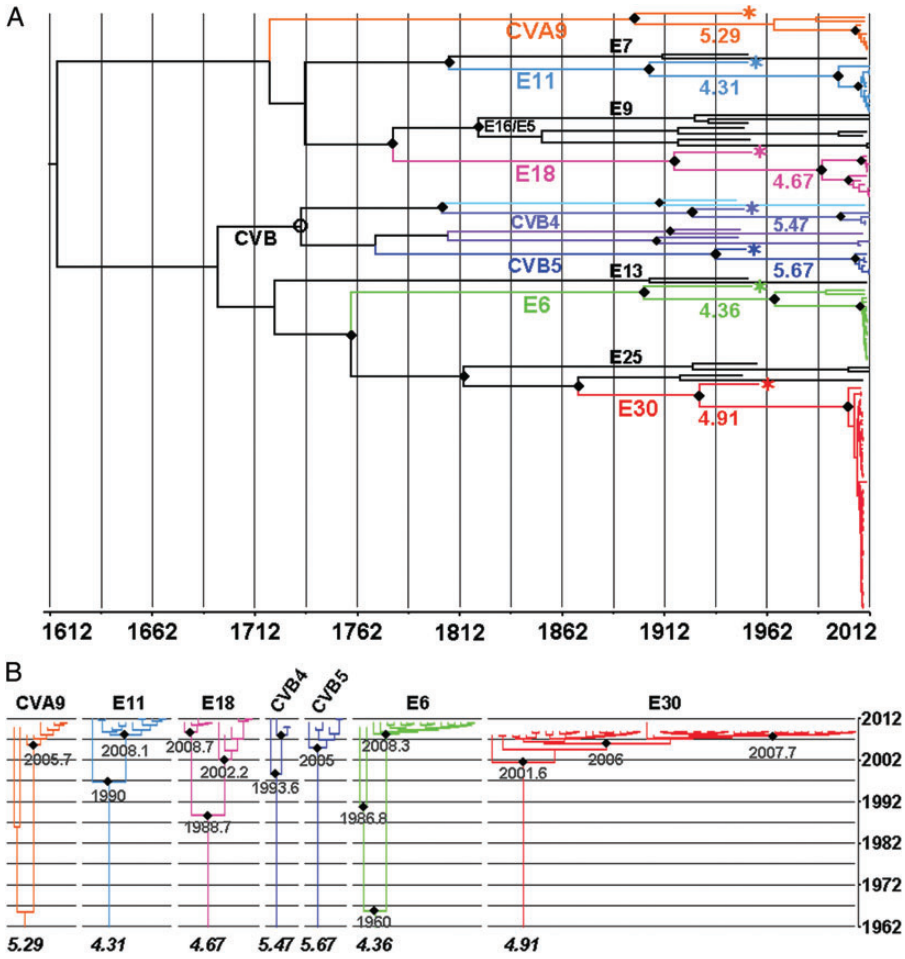

Figure 4. *A*, Bayesian phylogenetic tree (chronogram) of viral strains identified in 130 patients with meningitis caused by 17 enterovirus (EV) types assigned to the EV-B species. *B*, Expanded views of the phylogenetic tree, which show the relationships between sequences of coxsackievirus A9 (CVA9), echovirus 11 (E11), E18, CVB4, CVB5, E6, and E30 over the most recent period. Sequences are explicitly dated in calendar years. The chronogram was reconstructed with the viral $1D^{VP1}$ gene sequences determined in patients and the reference sequences of the 17 EV types. Each tip represents a virus strain in 1 patient. The main EV genotypes encountered in the study population are shown with different colors. Branches labeled with asterisks indicate sequences used as references. For greater clarity, the branches of the other genotypes were shown with black branches, and their reference sequences were not indicated. Statistical consistency of nodes estimated as posterior probability (pp) was indicated by full diamonds (pp > .95) and open circles (pp > .90). In panel *A*, the values below branches are the median cerebrospinal fluid (CSF) viral loads (in $\log_{10}$ copies of viral RNA/milliliter). In panel *B*, the time origin (ie, time to the most recent common ancestor) is indicated below each node.

viral loads of 4.0 and 4.6 $\log_{10}$ copies/mL in patients with mild and severe encephalitis, respectively [35]. However, we suggest that the increased viral loads in severe encephalitis cases may have resulted from a higher proportion of neonates in this group. Otherwise, the CSF viral loads in the above studies are consistent with our findings. The neonatal period is marked by a high susceptibility to pathogens, including EVs [36]. The increased susceptibility observed early in childhood was reflected in our study by the large array of 19 EVs with which the neonates and infants were infected. However, since neither genotype was predominant and the 2 patient populations were relatively small, we did not attempt to analyze the distribution of genotypes. Overall, we cannot exclude the possibility that factors related to younger age were involved in the higher CSF viral loads found in the neonates of our study population, as there is increasing evidence that the homeostasis of innate and adaptive immunity has specific characteristics in the neonatal period [37, 38].

Two other findings were strongly suggestive of a link between EV genotypes and the CSF viral loads in patients with meningitis. First, we found that the highest viral loads were in patients with meningitis caused by CVB4 and CVB5, whereas the lowest were seen in cases of meningitis caused by E6 and E11. We also found that the patients with E11 meningitis were infected by a same variant, which emerged in 2008, 2 years before its first detection in patients, which is consistent with the phylogenetic

data of E6. The second finding is related to meningitis caused by 5 CVB genotypes that form a subgroup within human EVs because they share a common tissue tropism owing to their use of the same cell receptors [39]. In our study population, 16 patients were infected with CVB genotypes, and their CSF was characterized by a higher WBC count than in meningitis cases caused by the other EVs. As CVB genotypes are lymphotropic [40] and lymphocytosis was predominant in these patients, the high CSF viral loads in CVB meningitis might have resulted from infected lymphocytes.

Two salient points emerged from our study. First, we observed that the CSF viral loads were correlated with pleocytosis and protein levels. We showed that there was a link between the absence of pleocytosis and the time at which lumbar puncture was performed, whereas the absence of leukocytes in the CSF of patients with EV meningitis is generally thought to be related to younger age. Second, we found that the highest viral loads were in meningitis caused by EV genotypes such as E30 and CVB, which otherwise had had a high prevalence in hospitalized patients over the previous 10 years. In contrast, 2 variants of E6 and E11 were related to the lowest viral loads and were caused by virus variants that emerged only recently. Overall, we conclude that the differences in CSF viral loads in patients with EV meningitis were related to either the genotype or subgenotype of the involved virus strain. This study raises the important issue of the precise origin in blood of viral RNA detected in the CSF during the early stage of EV meningitis.

Supplementary Data

Supplementary materials are available at *The Journal of Infectious Diseases* online (http://jid.oxfordjournals.org/). Supplementary materials consist of data provided by the author that are published to benefit the reader. The posted materials are not copyedited. The contents of all supplementary data are the sole responsibility of the authors. Questions or messages regarding errors should be addressed to the author.

Notes

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Disclaimer. The authors alone are responsible for the content and writing of the paper.

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Potential conflicts of interest. All authors: No reported conflicts.

All authors have submitted the ICMJE Form for Disclosure of Potential Conflicts of Interest. Conflicts that the editors consider relevant to the content of the manuscript have been disclosed.

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