Journal of Clinical Virology 89 (2017) 22–29

Contents lists available at ScienceDirect

Journal of Clinical Virology

journal homepage: www.elsevier.com/locate/jcv

Comparative analysis of viral shedding in pediatric and adult subjects with central nervous system-associated enterovirus infections from 2013 to 2015 in Switzerland

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ARTICLE INFO

Article history:
Received 21 October 2016
Received in revised form 25 January 2017
Accepted 28 January 2017

Keywords:
Enterovirus
CNS infection
Viral shedding
Epidemiology

ABSTRACT

Background: Several enterovirus (EV) genotypes can result in aseptic meningitis, but their routes of access to the central nervous system remain to be elucidated and may differ between the pediatric and adult populations.
Objective: To assess the pattern of viral shedding in pediatric and adult subjects with acute EV meningitis and to generate EV surveillance data for Switzerland.
Study design: All pediatric and adult subjects admitted to the University Hospitals of Geneva with a diagnosis of EV meningitis between 2013 and 2015 were enrolled. A quantitative EV real-time reverse transcriptase (rRT)-PCR was performed on the cerebrospinal fluid (CSF), blood, stool, urine and respiratory specimens to assess viral shedding and provide a comparative analysis of pediatric and adult populations. EV genotyping was systematically performed.
Results: EV positivity rates differed significantly between pediatric and adult subjects; 62.5% of pediatric cases (no adult case) were EV-positive in stool and blood for subjects for whom these samples were all collected. Similarly, the EV viral load in blood was significantly higher in pediatric subjects. Blood C-reactive protein levels were lower and the number of leucocytes/mm^3 in the CSF were higher in non-viremic than in viremic pediatric subjects, respectively. A greater diversity of EV genotypes was observed in pediatric cases, with a predominance of echovirus 30 in children ≥3 years old and adults.
Conclusion: In contrast to adults, EV-disseminated infections are predominant in pediatric subjects and show different patterns of EV viral shedding. This observation may be useful for clinicians and contribute to modify current practices of patient care.

1. Background

Enterovirus (EV) is the major cause of aseptic meningitis [1,2] with an incidence peak documented during the warm season in the northern hemisphere [3–7]. In most cases, EV meningitis is self-limited with a low rate of complications or sequelae. Neonates represent the most susceptible population in terms of morbidity and mortality rates [2], and a rapid and accurate diagnosis is of the utmost importance in terms of clinical management to reduce the length of hospital stay, costs, and antibiotic use [8–11]. At present, EV meningitis is mainly diagnosed based on the analysis of cerebrospinal fluid (CSF) with EV-specific real-time reverse transcription (rRT)-PCR assays. The viral load observed in the CSF is relatively low and several studies have demonstrated that some cases of EV-associated meningitis could be missed if only CSF is

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http://dx.doi.org/10.1016/j.jcv.2017.01.008

investigated and they recommend to screen blood specimens in combination [12–14].

The central nervous system (CNS) invasion remains an unclear process. The two most likely possibilities are that it occurs either as a consequence of systemic infection by crossing the blood–brain barrier or via retrograde axonal transport [2]. The fecal–oral route is the most common mode of EV transmission and the primary sites of replication are the gastrointestinal or respiratory tracts. It can be postulated that a significant viral replication in the primary site can then be followed by viremia, thus leading potentially to CNS or other organ infections. This hypothesis is essentially based on animal studies [15–18], and it has not been investigated systematically for EV infection in humans.

Many EV genotypes circulate worldwide and are responsible for EV-associated meningitis, with a high prevalence of echovirus 30 (E30) identified in recent outbreaks observed in several European countries [5,19–23].

2. Objectives

To assess the pattern of viral shedding in the CSF, blood, stool, nasopharyngeal and urine specimens in a prospective manner in human cases. The respective quantitative viral load in the different putative sites of replication or viral shedding in pediatric and adult subjects with acute EV meningitis was investigated over a three-year period, enabling also to investigate potential intra-host EV variability. We provide also an evaluation of the etiology of EV meningitis in the western part of Switzerland during the same period.

3. Study design

3.1. Patients

The study was conducted from January 2013 through December 2015. All pediatric (<16 years old; $n = 37$) and adult (≥16 years-old; $n = 23$) patients admitted to the University Hospitals of Geneva (Geneva, Switzerland) with a diagnosis of EV meningitis were considered for study inclusion. Five additional pediatric patients from the University Hospital Center in Lausanne (Switzerland) were also enrolled in 2015. Only patients with a clinical diagnosis of EV-associated meningitis or meningo-encephalitis confirmed by a positive EV-specific rRT-PCR in CSF by the routine laboratory were included (negative direct CSF examination and/or culture, negative by r(RT)-PCR for any other virus requested by the physicians such as herpes simplex virus, varicella zoster virus and/or human parechovirus). After obtaining signed informed consent, the following specimens were collected specifically for the study, if not normally collected for clinical care: upper respiratory specimens (nasopharyngeal swabs or aspirate), stool or anal swab, and urine. Blood specimens were not specifically collected for the sole purpose of this study and only leftover plasma or serum specimens collected for medical reasons were used. Patients had the possibility to refuse the specific collection of respiratory, anal and/or urine specimens.

3.2. Quantitative rRT-PCR

For each collected specimen, the viral genome was extracted individually from 200 μL using the NucliSENS easyMAG (bioMérieux, Geneva, Switzerland) nucleic acid kit, with an elution volume of 25 μL. The quantitative EV-specific rRT-PCR (Entero/Ge/08) that detects all known EV genotypes was performed as previously described [24,25]. As an internal control, 10 μL of standardized Canine Distemper Virus was added to each sample before extraction as previously described [26].

3.3. EV typing

Extracted RNA from the CSF were reverse transcribed with random hexamers (Roche, Indianapolis, IN, USA) using the reverse transcriptase SuperScript II (Invitrogen, Carlsbad, CA, USA). The VP1 (primers AN88 and AN89 [27]) and VP4/VP2 (primers F848 and adapted R1126 [28]) viral capsid regions were then amplified by PCR and amplicons were purified with the MSB Spin PCRapace kit (Stratec, Birkenfeld, Germany) before sequencing on a ABI Prism 3500XL DNA Sequencer (Applied Biosystems, Rotkreuz, Switzerland). Sequences were analyzed with the Geneious Pro 6.1.8 analysis software (Biomatters Ltd, Auckland, New Zealand). EV typing analysis was performed from blood or stool specimens when the viral load in the CSF or CSF and blood were too low to obtain VP1 or VP4/VP2 amplicons.

3.4. Phylogenetic analysis

Sequence alignment was constructed using the MAFFT v7.017 multiple sequence alignment program [29]. Maximum-likelihood trees for the VP1 and VP4/VP2 capsid regions were built using the PhyML method [30] with one thousand bootstrap replicates.

3.5. Statistical analysis

Differences between groups were tested using the Mann–Whitney U tests for continuous variables and the χ^2 test or Fisher's exact test for categorical variables using GraphPad Prism version 6.00 (GraphPad Software, La Jolla, CA, USA). A two-sided p value of <0.05 was considered significant.

4. Results

4.1. Demographic analysis

During the three-year study period, a total of 60 EV-associated meningitis cases (37 pediatric and 23 adults) confirmed by rRT-PCR were detected at the University Hospitals of Geneva. Overall, the age distribution ranged from 5 days to 40 years old (median age, 4 months; Supplementary Fig. 1a) with a male-to-female ratio of 1.1:1. When the pediatric and adult populations were analyzed individually, the median age was 1 month and 29 years old, respectively, with a male-to-female ratio of 1.6:1 and 0.6:1, respectively. The monthly distribution showed that positive cases were more prevalent during the warm season (April–September) (Supplementary Fig. 1b).

4.2. Viral shedding in pediatric versus adult subjects

Of a total of 42 pediatric patients (37 from Geneva; 5 from Lausanne), stool, blood, upper respiratory and urine specimens were obtained for 32, 37, 25 and 24 cases, respectively (Table 1). Among the 23 adult patients (all from Geneva), stool, blood, upper respiratory, and urine specimens were obtained for 12, 21, 15 and 12 cases, respectively (Table 1).

Urine and respiratory specimens showed a weak positive rate that did not significantly differ between the pediatric and adult populations (Table 1). By contrast, the detection rate in stool and blood specimens was significantly different between the two populations. Positive rates in pediatric cases reached up to 91% and 68%, respectively, whereas they were only 25% and 29%, respectively, in adults. CSF, blood, and stool specimens were obtained for 32 pediatric cases and 12 adults. Out of 32 pediatric patients (62.5%), 20 were positive in stool and blood in addition to the CSF (Fig. 1), 9 were positive only in the CSF and stool samples, and

Table 1
Comparison of EV detection rate in pediatric and adult subjects.

	Stool		Blood (plasma/serum)		Respiratory (nasal swab/aspirate)		Urine	
	Pediatric	Adults	Pediatric	Adults	Pediatric	Adults	Pediatric	Adults
Total no. of specimens	**32**	**12**	**37**	**21**	**25**	**15**	**24**	**12**
No. of positive specimens	**29**	**3**	**25**	**6**	**10**	**2**	**5**	**2**
(%)	(91)	(25)	(68)	(29)	(40)	(13)	(21)	(17)
***p* Value**	***p* < 0.0001**		***p* = 0.0097**		**n.s**		**n.s**	

n.s: not significant.

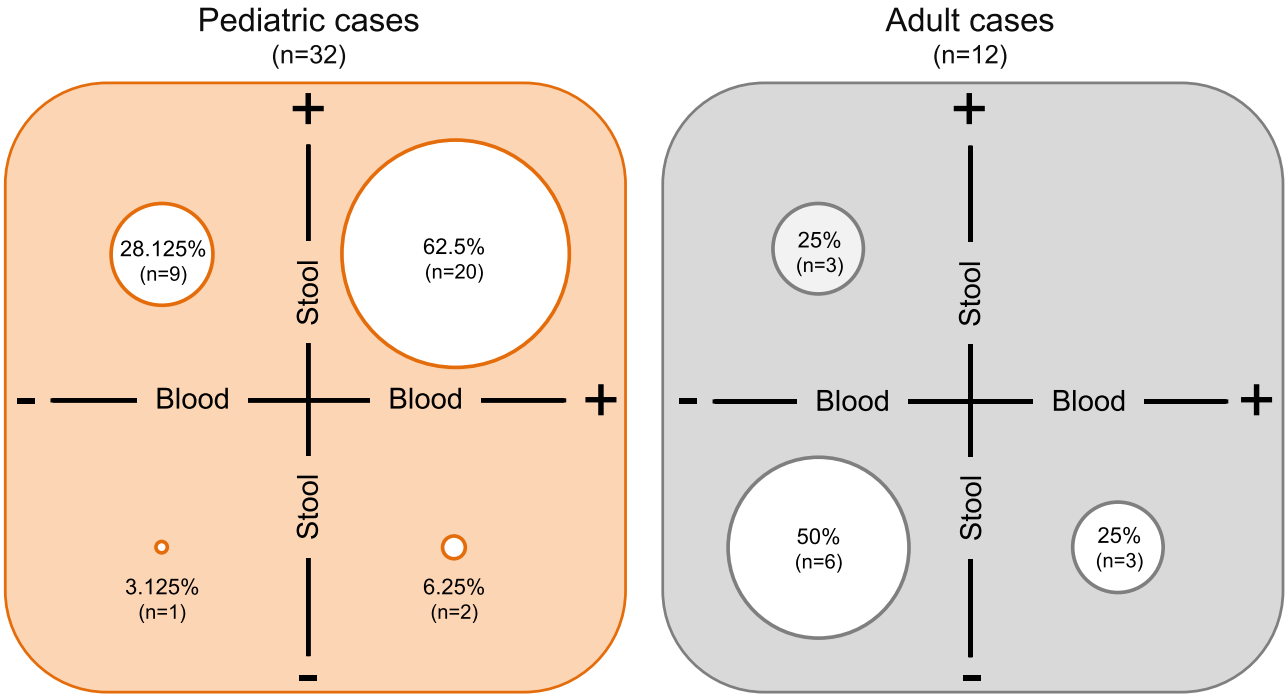

Fig. 1. Comparison of enterovirus detection rates in pediatric and adult subjects for whom cerebrospinal fluid, blood and stool samples were all collected. n: total number; +: EV-positive; –: EV-negative.

2 cases were only positive in the CSF and blood. For the two latter cases, stool could be collected only 4 and 6 days after lumbar puncture. Only one case was positive in the CSF only (3.125%), thus revealing that EV detection was positive in stool and/or blood in addition to the CSF specimen in 31 of 32 (96.9%) patients. Of note, the VP1 and/or VP4/VP2 sequences were obtained for 18 of the 20 pediatric patients who were EV-positive in all three specimens. For each individual patient, the same EV genotype was present in all three specimens and no intra-host variability was observed (data not shown).

An analysis of viral shedding showed a completely different pattern in adult subjects. No case was positive in all the three types of specimens and most adult subjects were only positive in the CSF (50%; Fig. 1).

Viral load measures were assessed for all EV-positive blood, stool, respiratory and urine specimens, and all initial CSF specimens ($n = 59$; 37/42 and 22/23 pediatric and adult subjects, respectively) with a sufficient leftover volume. The viral loads in positive CSF, stool, urine and respiratory specimens were not significantly different between the pediatric and adult populations (Fig. 2a). By contrast, the results obtained with blood specimens showed that the viral load was significantly higher in pediatric cases (range, 1.81×10^2–5.05×10^7 copies/mL; median, 1.89×10^5 copies/mL) compared to adults (range, 2.06×10^3 to 9.8×10^4 copies/mL; median, 7.74×10^3 copies/mL) (Fig. 2a).

We assessed whether a bias could be introduced due to time intervals between the time of lumbar puncture and the collection of other samples collected for the purpose of this study (i.e. stool, respiratory and urine specimens; blood specimens were mostly collected in parallel to CSF for clinical purposes). When the pediatric and adult populations were analyzed separately, a comparative analysis between EV-positive and -negative samples (e.g. pediatric positive versus negative cases in blood) showed equivalent intervals between positive and negative blood, stool and urine specimens (Fig. 2b), irrespective of the population studied, except for respiratory specimens ($p = 0.0254$) in the pediatric population (no difference in adults). A further comparative analysis between the two populations showed that the intervals did not differ between both groups for the stool, urine and respiratory specimens (e.g. all pediatric collected stool versus all adult collected stool), whereas they were significantly different ($p = 0.0056$) for blood specimens (pediatric: median, 0 days; range, –1 to +4 days; adults: median, 0 days; range, 0 to +2 days) (data not shown).

Similarly to non-viremic and viremic adult patients ($p = 0.1979$), the duration of symptoms before lumbar punctures was not significantly different between non-viremic and viremic pediatric subjects ($p = 0.8654$). Furthermore, the higher viral load observed in pediatric cases compared to adult did not result from different durations of symptoms before lumbar punctures between both populations ($p = 0.6736$).

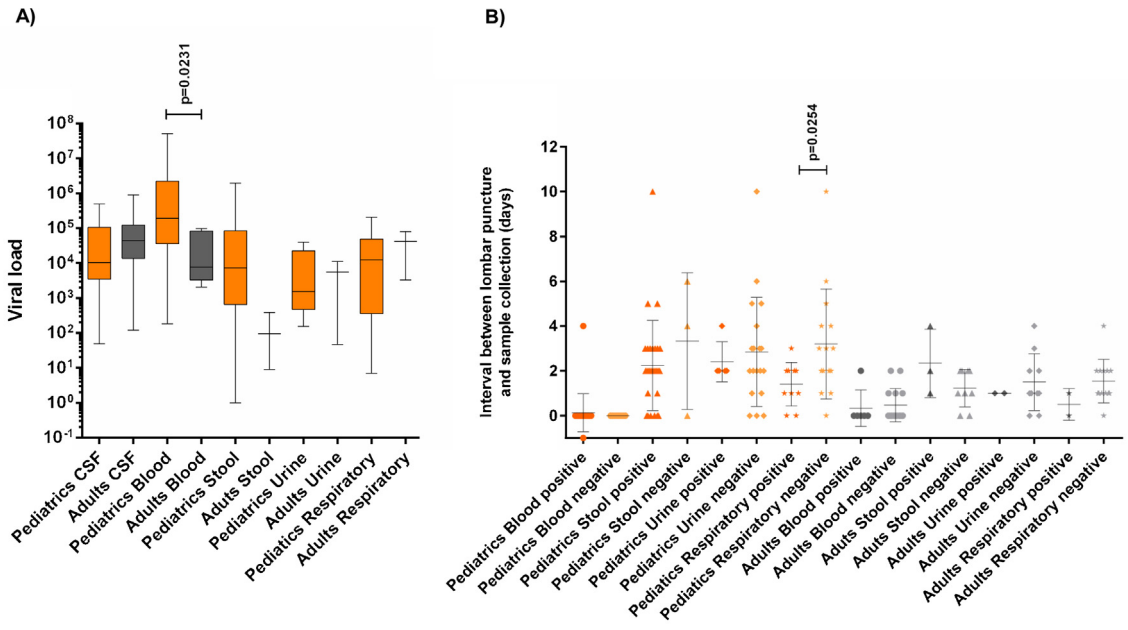

Fig. 2. Analysis of enterovirus shedding. Pediatric subjects analyzed are from the University Hospitals of Geneva and the University Hospital Center in Lausanne; adults are all from the University Hospitals of Geneva. Viral loads (panel a) are given for cerebrospinal fluid (CSF), blood, stool, urine and respiratory specimens. Pediatric and adult viral loads are represented by orange and grey plots, respectively. The CSF, blood and urine viral units are expressed in log10 genome copies/mL, whereas the stool and respiratory viral units are expressed in log10 RNA copies/PCR reaction. The intervals between lumbar puncture and the collection of the different type of samples are represented by orange and grey plots for pediatric and adult cases, respectively (panel b). P values are displayed only when considered statistically significant. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

4.3. Clinical characteristics

Clinical characteristics are presented in Table 2. Headache and fever were the most frequent clinical manifestations reported in children ≥3 years old (100%) and adults (100% and 78.3%, respectively). Fever (100%), irritability (61.3%) and feeding difficulty (48.4%) were the most common clinical features documented in infants and neonates. A higher maximal body temperature was observed in pediatrics subjects compared to adults (Table 2). In contrast to infants and neonates, vomiting was frequently observed in children ≥3 years old (50%) and adults (47.8%). Importantly, no difference in term of clinical severity was observed between the pediatric and the adult populations.

While C-reactive protein (CRP) levels were not significantly different between non-viremic and viremic adult patients ($n = 15$ and 5, respectively), they were significantly lower ($p = 0.0203$) in non-viremic compared to viremic pediatric subjects ($n = 9$ and 21, respectively; Fig. 3a). By contrast, a CSF cell count comparative analysis showed that whereas the number of leucocytes/mm^3 was not significantly different between non-viremic and viremic adult patients ($n = 15$ and 6, respectively), it was significantly higher ($p = 0.0002$) in non-viremic than in viremic pediatric subjects ($n = 10$ and 24, respectively; Fig. 3b). Following the latter finding, EV viral loads in CSF were compared between these two pediatric populations and were not statistically significantly different ($p = 0.4224$; median for viremic and non-viremic pediatric subjects, 1.54×10^4 and 7.85×10^3 EV copies/mL, respectively; data not shown).

4.4. Distribution of EV genotypes

The EV genotype was determined for 62 of 65 cases. All but one genotype (EVA71; EV-A species) belonged to the EV-B species (Fig. 4a, Supplementary Figs. 2 and 3). Overall, the most frequent genotype was E30, representing up to 16.7% and 69.6% of pediatric and adult cases, respectively (Fig. 4b). In 2013 and 2015, E30 represented the most frequent genotype in the adult population (12 of 15 (80%) and 4 of 7 (57.1%) cases, respectively), and the most (2 of 5 cases, 40%) and second most (5 of 25 cases, 20%) frequent genotype in the pediatric population in Geneva, respectively. Of note, E30 was not observed in 2014 in both populations. Furthermore, the phylogenetic analyses showed the presence of two temporal E30 lineages in 2013 and 2015 (Supplementary Figs. 2 and 3).

The second most frequent genotype was coxsackievirus (CV) B5 (19% and 13% of pediatric and adult cases, respectively). Although there is no evidence for the presence of EV genotype restricted to one population, the analysis showed a greater diversity of EV genotypes detected in the pediatric populations (13 genotypes) compared to those present in the adult populations (5 genotypes) over the three-year study period. A predominance of E30 was observed in children ≥3 years old and adults. Indeed, EV genotype was obtained for 6 of 7 pediatric subjects ≥3 years old; five were E30 positive. In contrast, E30 was detected in only two pediatrics subjects <3 years old.

Although E30-associated viremia showed a weak positive rate in the adult subjects (6 of 15 E30 cases), all EV-positive blood specimens in this population were associated to this specific genotype. This observation contrasted with the high positive rate in blood samples that was not correlated to specific EV genotypes in the pediatric population.

5. Discussion

In this prospective study, EV shedding was assessed for EV-associated meningitis subjects and compared between pediatric and adult populations over a three-year period. In parallel, a demographic and epidemiologic surveillance was performed.

In agreement with previous studies [3,5–7,22,31,32], we showed that CNS-associated EV infection cases were more prevalent during the warm season (peaks in June or July). Positive subjects were mostly neonates and infants under the age of three

Table 2
Clinical characteristics of pediatric and adult subjects enrolled in Geneva with a diagnosis of EV meningitis.

		Pediatric		Adults	
Age (median)		1 month		29 y.o	
Sex (male:female ratio)		1.6:1		0.6:1	
C-reactive protein (mg/L)	$p = 0.0203$	Non viremic (mean): 10 Viremic (mean): 16.3		Non viremic (mean): 17.9 Viremic (mean): 22.8	n.s
Cerebrospinal fluid cell count (leucocytes/μL)	$p = 0.0002$	Non viremic (mean): 657.8 Viremic (mean): 150.6		Non viremic (mean): 271.5 Viremic (mean): 184.7	n.s
Duration of symptoms before lumbar punctures (median, days)		Median: 0	$p = 0.0015$	Median: 1	
Clinical presentation					
Fever (%)		100		78.3	
Fever (median max temperature in °C)		38.9	$p < 0.0001$	38.1	
Headache (%)		≥3 y.o: 100		100	
Vomiting (%)		10.8		47.8	
Neck stiffness (%)		≥3 y.o: 16.7		30.4	
Photophobia (%)		≥3 y.o: 83.3		43.5	
Phonophobia (%)		≥3 y.o: 33.3		26.1	
Nausea (%)		≥3 y.o: 16.7		17.4	
Irritability (%)		<1 y.o: 61.3			
Feeding difficulty (%)		<1 y.o: 48.4			

n.s: not significant; y.o: years old.

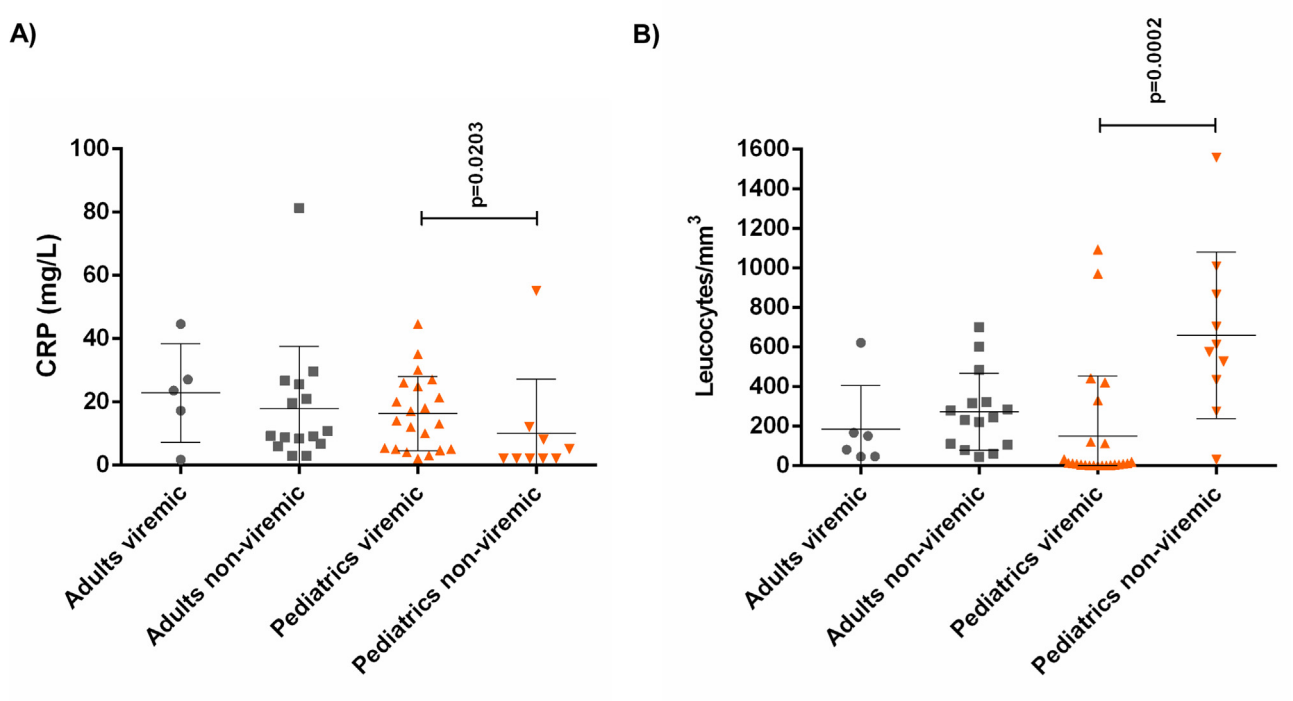

Fig. 3. Comparison of the blood C-reactive protein and the number of leucocytes/mm^3 in the cerebrospinal fluid. Comparative analysis of the C-reactive protein (CRP) level (panel a) and the number of leucocytes/mm^3 in the cerebrospinal fluid (panel b) between viremic and non-viremic subjects enrolled at the University Hospitals of Geneva. *p* Values are displayed only when considered statistically significant.

months, as well as young adults (all ≤40 years old), thus supporting that EV-associated meningitis is largely related to close contact with children in the latter population. Nevertheless, EV-associated meningitis can be observed also in elderly patients [22,33]. Transplacental infections and transmissions at delivery were not investigated in this study as blood specimens from the mothers were not collected.

The ability of many EV genotypes to infect the CNS has been previously described [2]. All belonged to the EV-B species, except one case that belonged to the EV-A species, thus confirming the predominance in Europe of EV-B species in EV-associated CNS infections [5,7,34,35]. Our data show that a large panel of EV genotypes co-circulate and are responsible for CNS infection in neonates and infants compared to children ≥3 years old and adults where EV genotypes appear to be more restricted, with a predominance of E30 and CVB5. This suggests a different susceptibility between these populations with regard to the diverse EV genotypes. E30 is the most prevalent genotype detected in adults and children ≥ 3 years old with a lower representation in neonates and infants. This is in agreement with recent findings [5]. CVB5, a genotype known to cause EV meningitis outbreaks [36], represents the most frequent genotype detected in our pediatric population. Importantly, we did not observe any association between clinical signs/symptoms and the presence of specific EV genotypes, nor between clinical signs/symptoms and the presence/absence of viremia in the pediatric and adult populations.

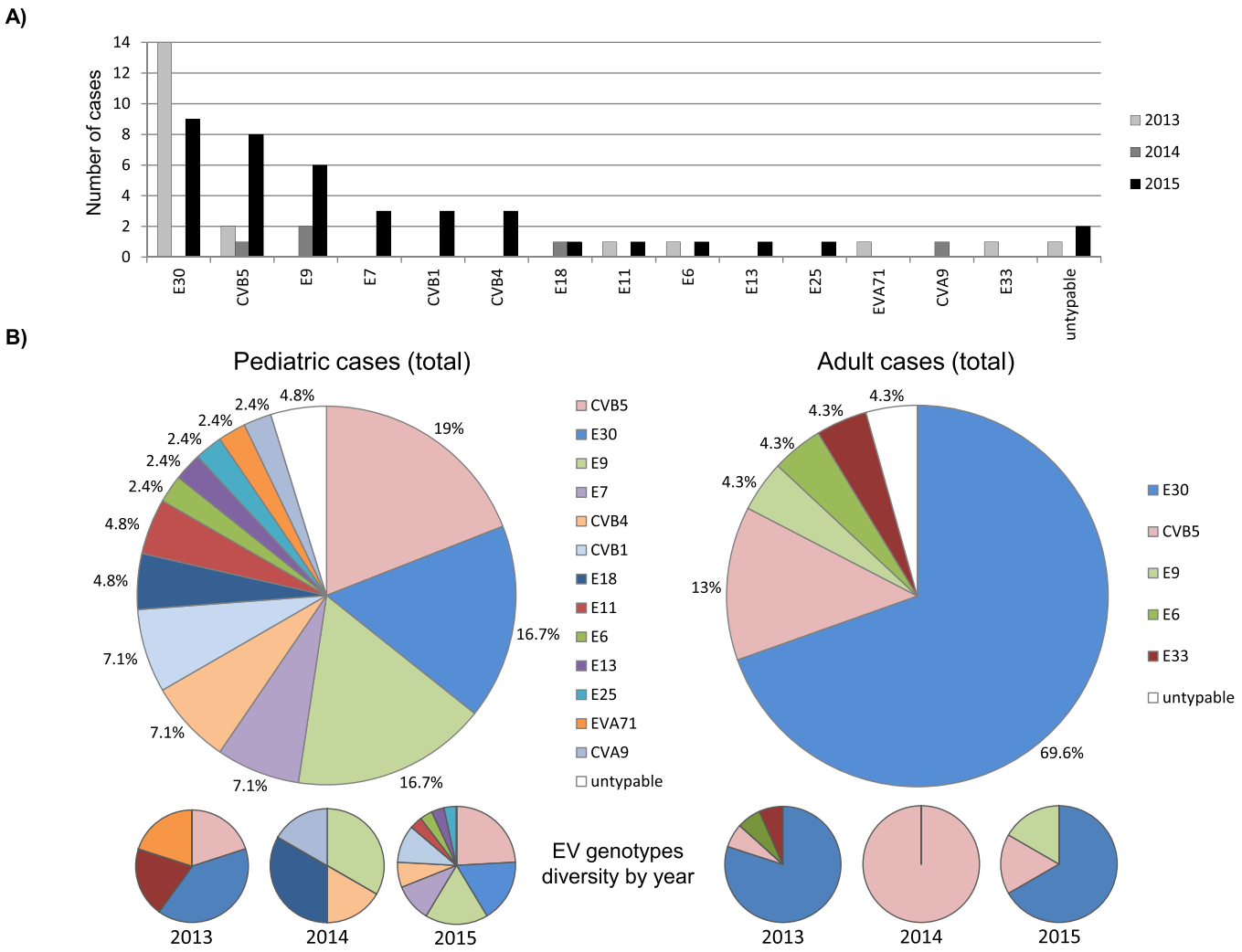

Fig. 4. Distribution of enterovirus genotypes. Pediatric subjects analyzed are from the University Hospitals of Geneva and the University Hospital Center in Lausanne; adults are all from the University Hospitals of Geneva. Distribution of enterovirus genotypes over a 3-year period (panel a). Distribution of enterovirus genotypes in pediatric and adult patients (panel b).

The comparative analysis of the EV detection rate in the different putative sites of replication in pediatric and adult subjects revealed the presence of two distinct viral shedding patterns. While low positive rates were observed for urine and respiratory samples in both populations, confirming that these types of specimens represent poor predictors in the case of suspicion of EV meningitis, stool and blood showed a high positive rate for the pediatric population, but remained low in adults. This observation was confirmed when viral shedding was compared in specific patients for whom CSF, blood and stool were obtained. Up to 62.5% of pediatric cases were positive in all three specimens (positivity rate reached 96.9% when considering positivity in stool or blood, in addition to CSF; Fig. 1), thus supporting the presence of disseminated EV infections, while this was not the case for any adult in our study. This observation seems not correlated to the uneven distribution of EV genotypes between the two populations, although this statement still needs to be confirmed by large comparative studies. Our results are in agreement with previous findings focusing either on pediatric EV-positive specimens [37] or both populations [38]. This suggests that a systematic screening of EV in stool and blood samples by molecular assays as the first line of investigation may either increase the yield of detection of CNS-associated EV infection in pediatric subjects or may be useful when lumbar puncture is contraindicated or unavailable. Our analysis of the VP1 and VP4/VP2 regions confirmed the presence of disseminated EV infection as the same EV genotype was constantly present in all three specimens. This is a relevant point as EV can replicate during several weeks in the gastrointestinal tract [39], meaning that the detection of EV in stool may not necessarily reflect the ongoing infection leading to CNS infection.

The analysis of viral load in EV-positive pediatric and adult CSF samples shows no significant difference between both populations. This confirms previous findings [35], with median values similar to those obtained in our study (Fig. 2a). By contrast, the viral load was significantly higher for EV-positive blood specimens in pediatric subjects. Although not significantly different, a similar trend was observed for positive stool specimens ($p = 0.0593$; Fig. 2a). Interestingly, EV-positive blood specimens in adult subjects were systematically associated with E30 genotype. The higher viral load in the pediatric population was observed when all EV-positive blood specimen were considered (uneven distribution of EV genotypes between the pediatric and adult populations), while it was not statistically significantly different ($p = 0.3939$) when considering only E30-positive cases in both populations. This result supports that the higher viral load observed in blood specimen in the pediatric population reflects infections with different EV genotypes.

Our results strengthen the hypothesis of two different patterns of EV viral shedding in pediatric and adult subjects, with

the presence of a high replication level in the gastrointestinal tract with sustained viremia observed in pediatric cases. The most likely hypothesis for this observation is the presence of different immune profiles between the pediatric and adult populations. In contrast to children for whom the primary immune response seems to be mostly engaged, disseminated infections are infrequent in adult subjects since the EV infections is likely partially controlled by some degree of immune memory acquired from previous EV exposures. Thus, the CNS invasion may directly result from a sustained viremia with a direct cross of viral particles through the blood–brain barrier in pediatric subjects. Alternatively, viral CNS invasion might occur differently in adults, and could be achieved through the retrograde transport pathway as proposed for other enteroviruses [40].

In conclusion, our study highlights the importance and the need for extensive investigation to assess the differences in EV viral shedding and genotype sensitivity between pediatric and adult subjects leading to CNS-associated EV infection. This would be valuable information for the clinician as it has the potential to significantly modify the current practices of patient care.

Conflict of interest statement

None declared.

Funding

This study was supported by the Swiss National Science Foundation (grant 32003B_146993 to L.K.).

Ethical approval

Written informed consent was obtained from all adult patients, parents or legal guardian prior to study participation. The study was approved by the research ethics committees of the University Hospitals of Geneva (project no. 13-074) and of the University Hospital Center in Lausanne (project no. 76/14).

Acknowledgments

We are grateful to Chantal Gaille (University Hospitals of Geneva) for technical assistance, and Rosemary Sudan for editorial assistance.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.jcv.2017.01.008.

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