Clinical and laboratory characteristics of enteroviral meningitis in children, including qRT-PCR and sequencing analysis

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Aims. Enteroviruses (EVs) are the most common agents of aseptic meningitis. Some serotypes can cause serious neuroinfection leading to death. The aim of this study was to determine the representation of EVs in the etiology of aseptic meningitis in children and to analyze the demographic, clinical, laboratory, and epidemiological characteristics of patients with EV meningitis.

Patients and Methods. This was a prospective study including 147 patients in three groups: EV meningitis, tick-borne encephalitis, and aseptic meningitis with unidentified agent.

Results. Boys with EV meningitis predominated over girls. The average patient age was 11 years. Compared to the control group, these patients suffered more from stiff back (P=0.010), vomiting and nausea (P=0.009). They had shorter symptom duration (P<0.001), higher C-reactive protein in blood (P<0.001), higher predominance of polynuclears (P=0.026), and greater lactate (P=0.003) in cerebrospinal fluid (CSF). The serotype seen most frequently (68%) was ECHO virus (ECV) 30.

Conclusions. Enteroviruses play the most important role in the differential diagnosis of aseptic meningitis. Short symptom duration, slightly higher inflammatory parameters in blood, predominance of polynuclears, and elevated CSF lactate have predictive value in diagnosing this disease. ECV 30 (frequently the agent of epidemics in the Czech Republic) was the aseptic meningitis agent most often seen.

Key words: enterovirus, meningitis, children, ECHO virus 30

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INTRODUCTION

Enteroviruses cause a broad range of illnesses worldwide. Originally, they were divided into five groups: polioviruses, Coxsackie viruses A (CV A), Coxsackie viruses B (CV B), ECHO viruses (ECV), and new numbered EVs (ref.[1]). Human EVs are newly distributed into four classes (HEVs A–D) according to their structural properties[1]. Occurrences of EV infections increase from summer to autumn in regions with temperate climates. The viruses are mostly transmitted fecal–orally but rarely also through respiratory secretions[2].

Enteroviral infections have benign outcomes in most immunocompetent patients. They cause mild respiratory and exanthematous diseases (e.g., hand-foot-mouth disease [HFMD] or herpangina). More serious infections (meningitis, encephalitis, paralytic diseases, myocarditis, acute transversal myelitis, acute cerebral ataxia, benign intracranial hypertension, respiratory failure, neonatal sepsis, or chronic infection) occur in some patients[3,4]. Several EV serotypes (e.g., CV A4, 7, 21, 24; EV 71, 76, 89) are associated with serious forms of diseases, including serious neuroinfection, paralytic diseases, and respiratory illnesses[3-11].

The typical cerebrospinal fluid (CSF) profile is pleocytosis with a predominance of mononuclears (MN), usually with 10 to 1000 white blood cells per microliter, normal or mildly elevated total protein, and normal glycorrhachia[12,13]. Three methods are commonly available to detect EV: viral culture, serology tests, and nucleic acid amplification. Sensitivity of the EV cultivation is relatively low[12,14]. Laboratories currently offer universal enzyme-linked immunosorbent assay (ELISA) kits for detecting specific antibodies[3]. The detection method most used is acid amplification by polymerase chain reaction (PCR) (ref.[15-17]).

Most of these infections are self-limiting and do not require specific therapy. The prognosis of EV neuroinfections is poorer in high-risk groups and in patients infected by virulent serotypes. Pleconaril is currently the only causal drug against EV (presently it is in phase III–IV clinical trials) (ref.[18,19]). Ribavirin can inhibit EV replication, but

it cannot be used in the treatment of EV meningitis due to its poor ability to cross the blood–cerebrospinal fluid barrier. RNA inhibitors, monoclonal antibody reactive against the N-terminal region of EV 71 VP1 capsid protein, and Verapamil have been tested as therapeutics[20-22]. In China, vaccination against the virulent serotype EV 71 has been possible since 2015 (ref.[23]).

The aims of this study were first to determine the representation of EVs in the etiology of aseptic meningitis in children and second to evaluate the demographic (age, gender), clinical (symptoms, length of hospitalization, symptoms duration before sampling), and laboratory (inflammatory blood parameters, cytological and biochemical parameters in CSF, detection of EV RNA and specific antibodies from CSF, EV genotyping) characteristics of the infected patients compared with those not infected by EV. Sequential analysis contributed to acquisition of epidemiological data, including the most common serotypes in pediatric patients and representation of virulent serotypes in the region. This prospective study was carried out at the Department of Children's Infectious Diseases (Faculty of Medicine and University Hospital, Masaryk University, Brno) in the Czech Republic from May 2012 to February 2016. The study was approved by the ethical committee.

MATERIALS AND METHODS

Patients, inclusion and exclusion criteria

Altogether, 147 children were screened (90 patients with EV meningitis, 15 patients with tick-borne encephalitis [TBE], and 42 patients with aseptic meningitis of unidentified etiology). Patients with TBE and aseptic meningitis of unidentified etiology comprised the control group. All pediatric patients with EV meningitis under 18 years of age were included into the study. EV meningitis was defined as detection of EV RNA or IgM antibodies in CSF. Patients with co-infection (another detected agent in the same period) were excluded from the statistical analysis.

Investigative tests

C-reactive protein from blood (CRP; normal range: 0-5 mg/L) was examined in all patients (n=147). CSF was examined cytologically (pleocytosis was regarded as >5 cells/μL) and biochemically (total protein [normal: 0.15-0.45 g/L], lactate [normal: 1.1-2.8 mmol/L], glucose [normal: 2.2-3.9 mmol/L]). Serum CRP levels were determined by immunoturbidimetry. Photometry was used in assessing total protein, lactate, and glycorrhachia in CSF. Analyses were made using a Cobas Integra 400 device by Roche. The following examinations were made in all 147 patients: EVs (PCR, serology); TBE (serology); herpes simplex virus 1, 2 (PCR); human herpetic virus 6 (PCR), varicella zoster virus (PCR), and borrelia (serology). TBE was diagnosed by detecting specific immunoglobulin M (IgM) and immunoglobulin G (IgG) antibodies in sera and IgM in CSF by routine capture ELISA test (TestLine Clinical Diagnostics, Brno, Czech Republic). Lyme neuroborreliosis was evaluated using antibody indices (AIs) for IgM and IgG in all CSF samples. An AI expresses the ratio of pathogen-specific antibodies in the CSF to specific antibodies in blood serum in relation to the condition of the blood–CSF barrier and concentration of total immunoglobulins in the CSF and serum. AIs were calculated using Antibody Index Software and calibration curves based upon AI standards (TestLine, Clinical Diagnostics, Brno, Czech Republic). An AI value >1.4 for IgM and IgG antibodies against recombinant antigens was considered positive. Other viruses (Epstein-Barr virus, cytomegalovirus, paramyxoviruses, adenovirus, influenza viruses, parainfluenza viruses, human immunodeficiency virus, etc.) were excluded in a nonstandard manner depending upon the symptoms of the patient. CSF was cultivated in cases where bacterial meningitis was suspected. Autoimmune antibodies were not tested, because there were no symptoms of autoimmune encephalitis.

Enterovirus was detected by quantitative reverse-transcription real-time PCR (qRT-PCR) using an ENTEROVIRUS R-gene™ kit (Argene SA, France). Detection was performed from blood (130 samples) and CSF (144 samples). Human parechoviruses (e.g., formerly ECV 22, 23) are not part of the ENTEROVIRUS R-gene™ kit. Specific antibodies were analyzed in CSF (115 samples), in particular immunoglobulins A (IgA), IgM, and IgG using a VIROTECH-ELISA kit (Sekisui Virotech, Germany).

Genotyping of EVs was made using frozen nucleic acid isolates at the National Reference Laboratory for Enteroviruses in Prague (67 samples). Classic PCR was used in molecular control detection and typing. The span of noncode region 5´RNA was chosen for genotyping. Samples were amplified using specific primers (E1-M13F, E2-M13R from GeneriBiotech, Hradec Králové, Czech Republic) and the SuperScript III Platinum OneStep™ qRT-PCR kit (Invitrogen, USA) (ref.[24]). Electrophoresis and first cleaning were made later. The amplifications using these primers were then repeated and a second cleaning followed. The isolates were sequenced using an ABI PRISM 3130 xL genetic analyzer (Applied Biosystems, USA). Sequence editing and analysis were performed using the Lasergene™ program (DNASTAR, Madison, WI, USA). The results were evaluated by the GenBank central sequencing database using BLAST algorithms[25].

Mann–Whitney test, Fisher's exact test, and t-test were used depending on character of the data. Software R version 3.3.3 was used for statistical analysis[26]. Level of significance was considered $P<0.05$.

RESULTS

Demographic data

In total, 147 children were afflicted with aseptic meningitis. EV caused aseptic meningitis in 90 patients (61%). Fifteen patients (10%) suffered from TBE. The etiology was unknown in 42 children (29%).

Patients with EV meningitis were 3 to 17 years of age (median 11 years). There was no difference between

Table 1. Basic demographic data, duration of symptoms before sampling.

Viruses detected	Mean age ± SD[a] (years)	Gender (male/female)	Median symptoms duration at presentation in days (range)
EV[b] (n=90)	11 ± 4.0 (*P*[c]=0.943)	64/26	1 (1–14) (*P*[d]<0.001)
TBE[e] (n=15)	11 ± 4.5	9/6	14 (5–20)
AM[f] (n=42)	10 ± 4.4	30/12	4 (1–17)
EPI 1[g] (n= 6)	10 ± 4.4 (*P*[c]=0.884)	11/5	1 (1–5)
EPI 2[h] (n=8)	10 ± 4.4 (*P*[c]=0.884)	5/3	2 (1–5)

[a]Standard deviation (SD). [b]Enteroviral meningitis (EV). [c]Statistical significance: $P<0.05$ (*t*-test). [d]Statistical significance: $P<0.05$. [e]Tick-borne encephalitis (TBE). [f]Aseptic meningitis – unidentified agents (AM). [g]Epidemic 1 – patients from the center of Brno (EPI 1). [h]Epidemic 2 – swimming pool (EPI 2).

patients with EV meningitis and the control group ($P=0.943$). Boys with EV meningitis predominated over girls (64; 71%). Two small epidemics were registered, one in each of the summers of 2014 and 2015. The first outbreak occurred in the center of Brno. Sixteen patients suffered from EV meningitis. The second outbreak was reported from a region just north of Brno, in the Czech Republic's district of Blansko. Eight children were infected at a swimming pool there (Table 1).

Clinical signs and symptoms

Patients with EV meningitis suffered from headache (n=89; 99%); fever (n=86; 96%); nuchal rigidity (n=83; 92%); tiredness (n=81; 90%); stiff back (n=79; 88%); vomiting and nausea (n=71; 79%); photophobia (n=46; 51%); abdominal pain or diarrhea (n=20; 22%); exanthema (HFMD; n=4; 4%); phonophobia (n=3; 3%) and changes of consciousness or behavior, including apathy, somnolence, and/or febrile seizures (n=3; 3%). Stiff back ($P=0.01$), vomiting, and nausea ($P=0.009$) occurred more in children with EV meningitis (Table 2). Patients from the control group suffered more from tiredness ($P=0.013$) and changes of consciousness or behavior ($P<0.001$). The patients with EV meningitis were admitted 1 to 14 days after onset of symptoms (median 1 day). The duration of symptoms was significantly shorter in patients with EV meningitis ($P<0.001$). None of the patients received antiviral treatment. Two children received ceftriaxone due to suspected bacterial meningitis (antibiotic treatment was discontinued upon negative result of the CSF cultivation). Hospitalization lasted for 7 to 14 days (median 12 days). All children recovered and were discharged.

Laboratory and epidemiological findings

CRP was significantly higher in children with EV meningitis (56; 62%; <50 mg/L in 51 patients; 57%) ($P<0.001$). Cytological and biochemical results of CSF examination are described in Tables 3 and 4. There were no difference in pleocytosis between patients with EV meningitis and those in the control group ($P=0.818$). Mononuclears predominated in 57 (63%) cases ($P=0.31$) and polynuclears (PN) in 33 (37%) cases ($P=0.026$). The difference in the number of CSF PN among children with EV meningitis and among the control group was found to be just barely statistically significant (17 from 33 samples with predominance of PN were taken on the first day of symptoms). No pleocytosis was present in 2 patients (4 and 12 years of age; EV RNA detected in CSF). Glucose in CSF was higher in patients from the first outbreak in Brno ($P=0.002$). Although the number of patients with elevated lactate was insignificant (14/90; 16%), patients with EV meningitis had higher lactate in CSF in comparison with the control group ($P=0.003$). Fig. 1 demonstrates the correlation between patients with elevated lactate and predominance of PN in CSF (children with EV meningitis) ($P<0.001$).

Enterovirus was detected in blood only in 30 of 77 patients (39%, qRT-PCR; sensitivity 40%, specificity 100%; $P<0.001$) and in CSF in 83 of 90 patients with EV meningitis (92%; qRT-PCR, sensitivity 92.2%, specificity 100%; $P<0.001$). IgA and IgM antibodies were detected in CSF only in 14 of 71 patients (20%; 5 results borderline; ELISA; sensitivity 19.7%, specificity 97.7%; $P=0.008$). IgM antibodies were found in CSF only in 4 patients with detected EV RNA in CSF (5%; 1 result borderline).

Genotyping was performed from 67 isolates of nucleic acid from CSF. The following serotypes were detected: ECV 30 (n=49), 13 (n=2), 6 (n=2), 11 (n=1), and 16 (n=1); CV B4 (n=2); EV 71 (n=1); and EV B (n=1). Genotyping was not successful for 6 samples. The serotype most often detected was ECV 30 (68%), which was

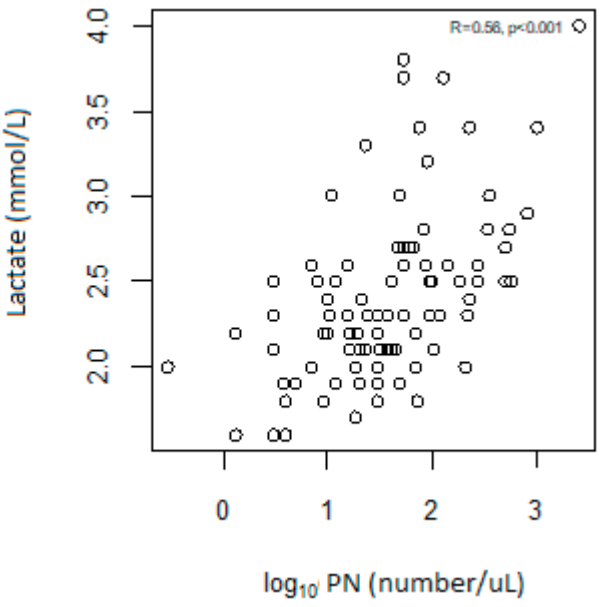

Fig. 1. Correlation between patients with elevated lactate and predominance of polynuclears in cerebrospinal fluid (patients with enteroviral meningitis, Spearman's $R=0.56$, $P<0.001$).

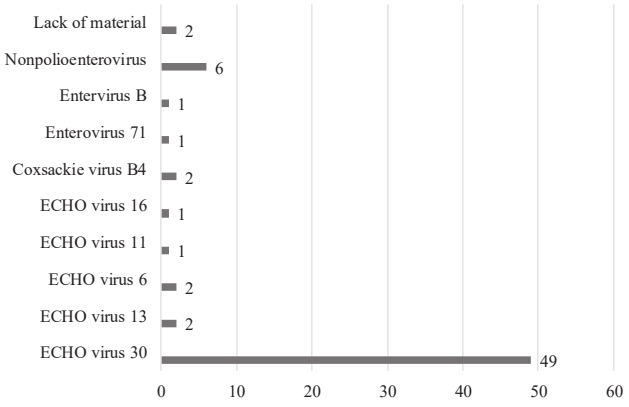

Fig. 2. Genotyping of enteroviruses by means of nucleic acids from cerebrospinal fluid (n=67); number of samples.

the etiology of the outbreak in the center of Brno and infection affecting visitors to the swimming pool. One of the virulent serotypes was detected in 1 boy (EV 71; symptoms and laboratory findings for that case did not differ significantly from others) (Fig. 2).

DISCUSSION

Representation of EVs in the etiology of aseptic meningitis (n=90; 61%) is consistent with the fact that EVs are the most frequently seen agents of viral meningitis worldwide. Owing to progress in molecular biology and diagnostic methods, it is now known that EVs currently cause 85-95% of these diseases[1,3,15]. Lower detection of EV RNA could be due to our using ENTEROVIRUS R-gene™ kits, because the manufacturer indicates the kit has lower sensitivity to some serotypes (in particular, CV A, EV 68, 71, 95-120). The group of cases with unidentified etiology occurred due to the high number of agents of aseptic meningitis in existence (not all of which were tested for).

Other studies have reported younger patients with EV meningitis[3,15,27-32]. There were no neonates or infants included in this study (only 2 toddlers; 2%). No such patients were present in other departments of the University Hospital. These differences might reflect that the main circulating serotype within the Czech Republic may have an affinity for older children. A Finnish study reported an outbreak of aseptic meningitis caused by ECV 30 in adolescents[33]. A predominance of boys among the patients has been reported also by other studies[33,34]. The seasonal distribution of patients corresponded to the characteristic occurrence in areas having temperate climate (summer and autumn).

The clinical presentation of patients with EV men-

Table 2. Symptoms in children (n=147).

Symptoms	EV[a]	CTR[b]; TBE[c]/AM[d]	OR[e]	*P*[f]
Headache	89 (99%)	53 (93%); 14/39	6.72	0.074
Fever[g]	86 (96%)	56 (98%); 14/42	0.38	0.649
Nuchal rigidity	83 (92%)	51 (89%); 12/39	1.39	0.566
Tiredness	81 (90%)	57 (100%); 15/42	NA	0.013
Stiff back	79 (88%)	40 (70%); 12/28	3.05	0.010
Vomiting and nausea	71 (79%)	33 (58%); 11/22	2.72	0.009
Photophobia	46 (51%)	26 (46%); 8/18	1.25	0.612
Abdomen pain, diarrhea	20 (22%)	8 (14%); 2/6	1.75	0.282
Exanthema	4 (4%)	4 (7%); 0/4	0.62	0.711
Other symptoms[h]	3 (3%)	16 (28%); 4/12	0.09	<0.001
Phonophobia	3 (3%)	4 (7%); 2/2	0.46	0.431

[a]Enteroviral meningitis, n = 90 (EV). [b]Control group (CTR; CTR = AM + TBE). [c]Tick-borne encephalitis, n=15 (TBE). [d]Aseptic meningitis, n=42 (AM). [e]Odds ratio (OR). [f]Statistical significance: $P<0.05$. [g]Temperature > 38 °C. [h]Apathy, somnolence, febrile seizures.

Table 3. Results from the cerebrospinal fluid in patients with enteroviral meningitis (n=90).

Indicator	Mean	Median	Minimum	Maximum	Standard deviation
Pleocytosis	225	126.5	4.3	2560	329.5
Mononuclears	100	56	2	590	114
Polynuclears	124.7	36	0	2440	298.4
Protein (g/L)	0.38	0.37	0.12	0.72	0.15
Lactate (mmol/L)	2.4	2.3	1.6	4	0.5
Glucose (mmol/L)[a]	3.5	3.4	2.7	5.1	0.5

[a]Fifteen patients had hyperglycemia. Q_{glu} (glucose quotient): 0.49-1.1 (median 0.62, standard deviation 0.17, six patients Q_{glu}>0.65). Physiological value around 0.6. Pathological value of Q_{glu}<0.45 (differential diagnosis of purulent meningitis).

Table 4. Comparison of inflammatory parameters from blood and indicators from cerebrospinal fluid between patients with enteroviral meningitis and control group (n=147); two epidemics (n=24).

Indicator	EV[a] (n=90)	CTR (n=57)	*P*[b]	EPI 1[c] (n=16)	EPI 2[d] (n=8)	*P*
CRP[e] (mg/L)	7.4 (1.0–131.0)	2.8 (1.0–64.2)	< 0.001	9.4 (1.0–76.8)	6.2 (2.6–64.6)	0.926
Pleocytosis (number/uL)	126.5 (4.3–2560.0)	120.0 (7.4–1500.0)	0.818	133.5 (12.3–1173.0)	134.5 (36.0–340.0)	0.806
Mononuclears (number/uL)	56 (2–590)	66 (3–1457)	0.31	52 (2–167)	88 (14–336)	0.374
Polynuclears (number/uL)	36 (0–2440)	20 (0–627)	0.026	60 (0-1006)	26 (4–97)	0.098
Protein (g/L)	0.37 (0.12–0.72)	0.39 (0.10–3.57)	0.358	0.32 (0.23–0.68)	0.37 (0.16–0.64)	0.736
Lactate (mmol/L)	2.3 (1.6–4.0)	2.0 (1.2–3.8)	0.003	2.7 (1.9–3.7)	2.5 (1.6–3.8)	0.803
Glucose (mmol/L)	3.4 (2.7–5.1)	3.4 (2.3–6.1)	0.219	3.9 (3.0–5.1)	3.3 (2.9–3.6)	0.002

[a]Enteroviral meningitis (EV). [b]Statistical significance: $P<0.05$. [c]Epidemic 1 – patients from the center of Brno (EPI 1). [d]Epidemic 2 – swimming pool (EPI 2). [e]C-reactive protein from blood (CRP). Presented as median (range)

ingitis was also typical. The symptoms most frequently seen were headache (99%), fever (96%), and nuchal rigidity (92%). A Greek study described positivity of meningeal signs only in 60% of cases during 2007 (ref.[35]). Uncomplicated febrile seizures occurred in one child (3 years of age) in our patients group, whereas more cases have been reported by others[2,35,36]. Some patients were admitted with symptoms lasting for 14 days. It was very difficult to determine if the symptoms might not be due to another cause (e.g., other viral infection, migraines). Hospitalization of patients with EV meningitis lasted longer than described in other studies[31,35,37], but it is standard practice of the Department to discharge children with aseptic meningitis after 10-14 days.

Pleocytosis was present in most children with EV meningitis. Only two patients without pleocytosis and with EV RNA detected in CSF occurred in this study (2%). Other studies have reported cases of EV RNA being detected in CSF without pleocytosis (infants, toddlers) (ref.[15,27,30,31,38,39]). Mechanisms to explain the lack of pleocytosis are various. One theory explains that in the initial stage of illness inflammatory cells have not yet penetrated into the CSF (ref.[30,35]). Another theory describes immaturity of specific immunity against EVs in young children[37]. The first theory provides the more probable explanation in relation to these patients (4 and 12 years of age). The predominance of PN in children with EV meningitis may be explained by the use of lumbar puncture for examining CSF in the initial stage of illness. A few studies have suggested that a rapid shift from predominance of PN to MN occurs in aseptic meningitis after 8 to 48 h (ref.[13,40]). Patients with EV meningitis had higher lactate in CSF than did the control group. Lactate can be slightly elevated in cases of aseptic meningitis, but it is several times higher in cases of purulent meningitis[41]. A predominance of PN occurred in half of children with elevated lactate in CSF (7/14). Differences in total protein (P=0.45) and glucose (P=0.21) in CSF were not significant. Elevated total protein and glucose in CSF are not good predictive indicators of EV meningitis[27,38]. A few studies have reported more frequently elevated total protein in CSF among neonates and infants with EV meningitis[15,35]. This cannot be confirmed in this study due to the lack of such patients.

Detection of EV RNA in CSF by qRT-PCR was the most adequate diagnostic assay. EV was found in the majority of patients from CSF (92%). Detection of specific antibodies (by ELISA) can be helpful in cases of diagnostic problems. Many studies have confirmed the importance of EV RNA detection by PCR in diagnosing EV meningitis in terms of faster diagnosis, avoiding unnecessary antibiotic therapy, and reducing the costs and duration of hospitalization[2,3,12,17,30,31,35].

The serotype most often detected was ECV 30 (68%). This serotype has been reported for the past 3 years and has caused small outbreaks in the Czech Republic (aseptic meningitis, encephalitis, HFMD, myocarditis, gastroenteritis, and others) (ref.[25]). ECV 30 has very often been the agent of viral meningitis worldwide in this period[28,42-44]. In the USA, however, it has been reported only in 5% of cases since 2009 (ref.[45]). There was no evidence in this study of death or more complicated diseases caused by ECV 30. A Brazilian study, however, has described 5 cases of death in children infected by this serotype[42]. A few studies have reported high affinity of ECV 30 for older children and adults, and this can be a reason for the patients being older in this study[33,45]. Other serotypes were detected only sporadically in this study. EV 71 occurred in 1 case. This serotype is associated with more serious forms of neuroinfection. Several children infected with EV 71 have died of serious bulbar encephalitis in Asia[5,10,11]. A different clinical presentation appeared in Australian children (Guillain–Barré syndrome, acute transversal myelitis, acute cerebral ataxia, benign intracranial hyper-

tension, febrile seizures) (ref.[36]). Lower sensitivity when using a kit for this serotype could cause the lower incidence of detecting EV 71 inasmuch as the manufacturer indicates this to be the case by its own data. Genotyping was not successful for some samples (possibly due to freezing). Human parechoviruses (e.g., formerly ECV 22, 23) were not tested for because these were not indicated by the symptoms observed[32,39]. Most of the circulating serotypes detected in the Czech Republic in recent years have belonged to HEV B, and these are the most frequently detected agents of aseptic meningitis[25]. A study from Northern Italy based on sampling children younger than 5 years of age has reported the same conclusion[46].

CONCLUSION

Although most infections are uncomplicated, aseptic meningitis caused by EVs currently plays an important role in the differential diagnosis of neuroinfections and paralytic diseases. The symptoms most frequently observed were headache, fever, and nuchal rigidity. The patients with EV meningitis suffered from vomiting, nausea, and stiff back more often than did the control group. Patients had significantly shorter duration of symptoms before sampling, slightly elevated inflammatory parameters in blood, predominance of PN, and elevated lactate in CSF. These features can have significant predictive value in diagnosing this disease. Detection of EV RNA by qRT-PCR in CSF was adequate. Virulent serotypes were not often detected in this period. ECV 30, which is frequently the agent of epidemics in the Czech Republic, was most often the agent of aseptic meningitis in this study.

ABBREVIATIONS

AI, Antibody index; CRP, C-reactive protein; CSF, Cerebrospinal fluid; CV, Coxsackie virus; ECV, ECHO virus; ELISA, Enzyme-linked immunosorbent assay; EV, Enterovirus, Enteroviral; HEV, Human enterovirus; HFMD, Hand-foot-mouth disease; IgA, Immunoglobulin A; IgG, Immunoglobulin G; IgM, Immunoglobulin M; MN, Mononuclears; PCR, Polymerase chain reaction; PN, Polynuclears; qRT-PCR, quantitative reverse transcription real-time polymerase chain reaction; RNA, Ribonucleic acid; TBE, Tick-borne encephalitis; WBC, White blood cells.

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