Enteroviral Central Nervous System Infections in Children of the Region of Monastir, Tunisia: Diagnosis, Laboratory Findings of Cerebrospinal Fluid and Clinical Manifestations

Raida EL Hiar · Samir Haddad · Hela Jaïdane · Didier Hober ·
Manel Ben M'hadheb-Gharbi · Maria Gullberg · Mohamed Neji-Guediche ·
A. Michael Lindberg · Jawhar Gharbi · Mahjoub Aouni

Received: 28 May 2012 / Accepted: 14 August 2012 / Published online: 4 September 2012
© Indian Virological Society 2012

Abstract Human enteroviruses (HEV) are one of the major causes of central nervous system (CNS) infections in pediatrics. A prospective study was conducted to assess the epidemiological, clinical, and laboratory characteristics of enterovirus (EV) infections of the CNS in children under 15-years-old, suspected of having viral CNS infections and admitted to the Pediatric Department of Monastir University Hospital, Tunisia. Enteroviral RNA was detected by 5' NCR nested RT-PCR assay in 33 % (20 out of 60) of cerebrospinal fluid specimens, whereas only six samples (10 %) were EV positive in cell culture. EV-positive patients were clustered according to their clinical manifestations, predominantly diagnosed as aseptic meningitis (65 %) and meningoencephalitis (20 %). Fever, headache, vomiting, and neck stiffness were the most pronounced symptoms. Pleocytosis with the predominance of lymphocytes was observed in 60 % of EV positive specimens. Although patients suffering from EV infections were encountered throughout the year, most occurred during

spring and summer months. Using VP1-2A nested RT-PCR and sequence analysis, three of the 20 positive HEV were identified as Echovirus (E)-9. This is the first report of a cluster of aseptic meningitis cases caused by E-9 in Monastir.

Keywords Enterovirus · Cerebrospinal fluid · Children · Molecular typing · Epidemiology

Introduction

Human enteroviruses (HEV) are small, single-stranded RNA viruses, belonging to the large genus *Enterovirus* of the *Picornaviridae* family and classified into four distinct species according to their molecular properties: HEV-A, B, C, and D [26]. Each species is itself composed of a growing number of different serotypes/types, as new (sero)types are continuously being discovered. Currently, more than 100 (sero)types are recognized by the International Committee on Taxonomy of Viruses: HEV-A (at least 17 serotypes), HEV-B (more than 58 serotypes), HEV-C (at least 22 serotypes, including the three poliovirus serotypes) and HEV-D (three serotypes) [26].

Although enterovirus (EV) infections are frequently either asymptomatic or causing only mild febrile illness, non-polio enteroviruses (NPEV) are also the most common viral pathogens responsible for aseptic meningitis in patients of all age groups [28, 49]. They have also been linked to other neurological manifestations such as encephalitis, paralysis, febrile seizures, Guillain-Barré syndrome, transverse myelitis, cerebellar ataxia, and peripheral neuritis [17, 21].

To diagnose these enteroviral infections, a standard method involves virus isolation followed by serotyping using neutralizing reference antisera. Such process is,

R. EL Hiar (✉) · H. Jaïdane · M. Ben M'hadheb-Gharbi ·
J. Gharbi · M. Aouni
Laboratory of Infectious Diseases and Biological Agents
LR99-ES27, Faculty of Pharmacy, University of Monastir,
Monastir, Tunisia
e-mail: raidahiar@yahoo.fr

S. Haddad · M. Neji-Guediche
Pediatric Department, Fattouma Bourguiba Hospital,
5000 Monastir, Tunisia

D. Hober
Laboratory of Virology EA3610, Faculty of Medicine, CHRU
Lille, University Lille 2, 59120 Loos-lez-Lille, France

M. Gullberg · A. M. Lindberg
School of Natural Sciences, Linnaeus University, 391 82,
Kalmar, Sweden

however, time consuming, and results are difficult to interpret, especially with some EV serotypes that either grow poorly or not at all in cell cultures. Furthermore, some samples, such as cerebrospinal fluid (CSF), tend to have virus titers too low to be detected by standard methods [10].

Molecular techniques based on the amplification by reverse transcription polymerase chain reaction (RT-PCR) of conserved parts within the 5' non coding region (5' NCR) of the EV genome are highly sensitive and currently the golden standard to detect small RNA amounts of enteroviral RNA directly from clinical samples. Most molecular methods are used as diagnostic tools [9, 25, 27, 30, 38, 46, 56, 59]. In addition, molecular typing and molecular characterization systems may be used for epidemiological purposes and for the improvement of diagnostic strategies [35]. In order to identify all the serotypes of HEV, more recent studies target the region encoding capsid proteins such as the VP1 [4, 8, 39, 41, 42, 55], or the VP2 [23]. These results concord with those based on seroneutralization methods [37].

EVs are the pathogens associated most commonly with acute meningitis worldwide and can cause sporadic cases, outbreaks, and epidemics. A 2-year study of epidemiology of pediatric EV infections carried out in Monastir, Tunisia, has revealed that the leading viral cause of aseptic meningitis was EV [17]. Moreover, epidemiological studies from Tunisia have reported that echoviruses were the most frequently isolated serotypes [3, 17]. In Tunisia, it will be important to maintain ongoing epidemiological EV surveillance to understand the present and changing patterns of EV infection and disease association. Such knowledge will help in the control of enteroviral infectious diseases. The present report complements previously collected data [17], with the aim of evaluating HEV incidence over a whole calendar year (2007) as reported through clinical manifestations and CSF laboratory findings and establishing potential seasonal patterns of infection in the Monastir region. In addition, two RT-PCR assays, one based on 5' NCR and a second based on VP1-2A region, were studied for rapid detection and typing of EV directly on CSF samples.

Materials and Methods

Patients and Clinical Samples

Sixty CSF samples from children (under 15-years-old) admitted to the Pediatric Department of Monastir University Hospital (from January to December 2007), Tunisia, with clinical suspicion of viral central nervous system (CNS) infection were collected by the examining physician

and immediately sent to the Virology Laboratory (Faculty of Pharmacy, Monastir) for EV detection. Upon receipt, the samples were stored at -70°C until processed. An epidemiological record for each sample including clinical diagnosis and laboratory parameters was filed by the physician. Microscopic and biochemical analysis of the CSF samples namely fungi and bacteria cultures and determination of glucose and proteins levels were carried out. The presumptive diagnosis of the patients was based on (a) clinical symptoms indicative of neurological infections (fever, vomiting, photophobia, and headache), (b) CSF pleocytosis (>5 white blood cells/ mm^3), and (c) the lack of an alternative diagnosis (i.e., negative cultures for bacteria and fungi).

Cell Culture

Cerebrospinal fluid (CSF) samples were tested in cell cultures. Culture was performed using two different cell lines: human epithelial cervical cancer cells (HeLa) and African green monkey kidney cells (Vero), since some EV are able to grow only in either one of the two. Cells were grown in Eagle's minimum essential medium (MEM, Invitrogen) supplemented with 10 % fetal bovine serum (Invitrogen), 2 mM L-glutamine (Invitrogen), 100 U/ml penicillin (Invitrogen), 100 $\mu\text{g}/\text{ml}$ streptomycin (Invitrogen), and 5 $\mu\text{g}/\text{ml}$ of fungizone (Invitrogen). Maintenance medium was Eagle's basal medium (BME, Invitrogen) with 2 % serum substitute.

Two hundred microliters of each CSF specimen were inoculated to monolayers of Vero or HeLa cell lines in 24-well plates. After 1 h of incubation, maintenance cell culture medium was added and cells were incubated at 37°C in a CO_2 incubator. Uninfected cells were used as negative controls. Cells were observed daily for EV cytopathic effect (CPE). Cultures became positive for the majority of specimens between the 2nd and 7th day. For cultures exhibiting no CPE after 7 days, a blind passage was performed and the cells were grown for another 7 days period before being reported as negative. Cultures showing CPE suggestive for EV after the first blind passage were passed once more in the same type of cell line for confirmation.

RNA Extraction and Diagnostic 5' NCR Nested RT-PCR Assay

Viral RNA was extracted from 140 μl of CSF using the QIAamp viral RNA kit (Qiagen) according to manufacturer's instructions. The extracted RNA was either used immediately or stored at -70°C until RT-PCR amplification analysis could be performed. EV diagnosis was performed by RT-nested PCR of a conserved 297-bp

fragment of the 5' NCR region. Primers used were published inner and outer primers [27] of the conserved 5' NCR region of EV genome (Table 1).

The synthesis of cDNA was performed in a 20 µl reaction volume of a mixture of 50 mM Tris-HCl (pH 8.3), 75 mM KCl, 3 mM MgCl₂, 10 mM dithiothreitol, 1 mM deoxynucleoside triphosphate, containing 100 U of Murine Moloney Leukemia Virus reverse transcriptase (Invitrogen), 40 pmol of antisense primer K1, 10 U of RNasin (Promega) and 10 µl of either the RNA containing solution extracted from the CSF or control specimens. The mixture was incubated at 42 °C for 30 min. After elongation, tubes contents were inactivated at 95 °C for 5 min.

The first round of PCR amplification was carried out in a 50 µl reaction volume containing: 2 µl of the cDNA product, 50 mM Tris-HCl (pH 8.4), 50 mM KCl, 2 mM MgCl₂, 0.2 mM deoxynucleoside triphosphate mixture, 1.25 U of Taq DNA polymerase (Invitrogen) and 40 pmol of each sense and antisense outer primers (K2 and K1). The final volume was completed to 50 µl with sterile nuclease-free distilled water. The reaction mixture underwent a first denaturation step for 5 min at 94 °C. DNA was amplified for 35 cycles in a programmable thermal cycler (Mastercycler, Eppendorf) by using the following conditions: 94 °C for 30 s, 45 °C for 60 s, 72 °C for 60 s, followed by a final extension step at 72 °C for 10 min. Two microliters from the first PCR were further amplified with sense and antisense inner primers (K3 and K4) in 50 µl of the reaction mixture described above. The mixture was subjected to a first denaturation step for 5 min at 94 °C. The second round of amplification was performed as follows: 94 °C for 30 s, 54 °C for 60 s and 72 °C for 60 s, followed by a final extension step at 72 °C for 10 min.

To prevent occurrence of amplicon contamination, the different PCR assay steps (extraction, mixture preparation, amplification, purification, and revealing of PCR products)

were performed in separate rooms, and negative controls (water samples) were included in all experiments, in accordance with molecular biology usual standard operation procedures. CV-B4-JVB prototype strain was kindly provided by J. W. Almond, Whiteknights, United Kingdom and used as the positive control for the assays.

VP1 Nested RT-PCR: Amplification of the VP1-2A Region Directly from CSF Specimens

Virus specimen typing was carried out for each PCR positive sample, with VP1 nested RT-PCR. Generated amplicons were 609 bp of the VP1-2A enteroviral genome region [10]. Table 1 shows the sequences and the relative positions of VP1 primers in Echovirus 9 strain Barty. Before running the nested PCR, a reverse transcription step was performed. The RT reaction mixture contained, as recommended by the manufacturer, 200 U of SuperScript[®] III Reverse Transcriptase (Invitrogen), 50 ng of random primers and 40 U of RNase Inhibitor (Invitrogen) in a 20 µl volume. The mixture was incubated at 50 °C for 60 min. For amplification, a total of 5 µl of cDNA was added to a tube containing a PCR bead system (Amersham Biosciences) in a 25 µl reaction volume composed of 100 pmol of VP1-1S and VP1-1A outer primers, as recommended by the manufacturer. The amplification step consisted of 40 PCR cycles (denaturation for 30 s at 94 °C, primer annealing for 60 s at 50 °C and elongation for 60 s at 72 °C). After the first round of PCR, 1 µl of amplified products was added to a tube containing a PCR bead as above, except that inner primers VP1-2S and VP1-2A replaced outer primers. Thirty cycles were performed consisting of denaturation (94 °C for 30 s), primer annealing (50 °C for 60 s) and extension (72 °C for 60 s). PCR amplicons were purified using the gel extraction kit (Qiagen).

Table 1 Enterovirus genome 5' NCR and VP1 primers used for nested RT-PCR assays

Oligonucleotide	Sequence ^a (5'–3')	Positions ^{b,c}	Reference
Outer primer K1 (sense)	ATTGTCACCATAAGCAGCCA	5' NCR 577–596	Kuan et al. [27]
Outer primer K2 (antisense)	ACCTTTGTACGCCTGTT	5' NCR 67–83	Kuan et al. [27]
Outer primer VP1-1S (sense)	GGTTYGAYITGGARITACITTYGT	VP1 2,764–2,809	Casas et al. [10]
Outer primer VP1-1A (antisense)	TGIGAYTGRYCTIKYKGGRTARTA	VP1 3,612–3,637	Casas et al. [10]
Inner primer K3 (sense)	AAGCACTTCTGTTCCC	5' NCR 166–182	Kuan et al. [27]
Inner primer K4 (antisense)	ATTCAAGGGGCCGAGGA	5' NCR 447–463	Kuan et al. [27]
Inner primer VP1-2S (sense)	ARWTWATGTAYRTICCCIGGIG	VP1 2,874–2,898	Casas et al. [10]
Inner primer VP1-2A (antisense)	CCIGTKKWRCAIYKRCAYCTIGC	VP1 3,507–3,529	Casas et al. [10]

^a Degenerate positions I: deoxyinosine, Y: T/C, R: A/g, W: T/A, K: T/g

^b Relative positions of VP1 primers in Echovirus 9 strain Barty

^c Relative positions of 5' NCR primers in CV-B5

Serotype Identification by Sequencing in the VP1-2A Genomic Region

The nucleotide sequences were determined with an ABI Prism 3130 automated sequencer (Applied Biosystems) by a primer-walking strategy on both strands using BigDye chemistry (ABI Prism BigDye Terminator cycle sequencing ready-reaction kit, version 1.1; Applied Biosystems). Sequences were analyzed using the Sequencher, software package (version 4.6, Gene Codes Corporation). The obtained sequences were submitted to the GenBank sequence database under accession numbers JQ315047–JQ315049.

To determine the EV type, the obtained consensus sequences of 609 bp for VP1-2A region were compared to all the corresponding HEV sequences available in GenBank for this region by using BLAST software (National Center for Biotechnology Information; <http://www.ncbi.nlm.nih.gov/Blast>). As previously proposed by Oberste et al. [43], nucleotide sequence identity of at least 75 % was required for assignment to a certain previously recognized serotype.

Statistical Analysis

Cerebrospinal fluid (CSF) Laboratory findings were analyzed using the SPSS statistics software (version 10 for Microsoft windows). Student's *t* test was used to compare continuous variables and to investigate the statistical significance between groups. The difference was considered significant for *p* values below 0.05. Results are presented as mean $\pm$ SD.

Results

Virus Isolation, RT-PCR Results

A total of 60 CSF specimens from children with suspected viral CNS infection were analyzed. Enteroviral RNA was detected by 5' NCR nested RT-PCR assay in 20 (33 %) of the 60 CSF specimens, whereas six samples (10 %) were EV positive in cell culture (see Fig. 1). All CSF specimens from which EV were isolated were positive by 5' NCR RT-PCR. Both cell lines used (Vero and HeLa) presented the same rate of isolation. No EV could be isolated after three blind passages in 14 specimens out of the 20 specimen's detected positive by PCR. We were able to identify the EV type by employing molecular typing of the VP1-2A in three samples. In the remaining EV positive by screening the 5' NCR, no VP1-2A product could be amplified.

Sequence Analysis

The VP1-2A positive amplicons were sequenced and compared with previous known EV genomic sequences.

Blast analyses were carried out and each sample was assigned the type that gave the highest VP1 identity score. All three VP1-2A positive specimens were identified as containing E-9 genomic sequences. All sequences were closely related to each other (≥ 96 %) and demonstrated a 96 % sequence identity to a previously described Greek E-9 strain, isolated during a meningitis outbreak in 2007 [31].

Epidemiological Characteristics

This study was performed in children under 15. EV-positive patients' ages ranged from newborns to 12-years-old. As shown in Fig. 2, the highest incidence among EV-positive patients was observed in the infants age group (<2-years-old) with a 45 % incidence and in young children (2–5 years old), with a 35 % incidence for that age group. Infected children were predominately males (70 %) with a male-to-female ratio of 2.33.

Figure 3 shows the seasonal distribution of EV infections cases, confirmed by 5' NCR nested RT-PCR. Although some cases were notified throughout the year, they mostly occurred in two peaks during spring and summer months; the first one in April and May (35 %) and the second one from June to August (30 %).

Clinical Signs and Symptoms

Clinical information's were obtained for all patients with positive EV infections. The dominant clinical symptom upon admission was fever (80 %). Vomiting was noted in 50 % of the patients, headache in 45 %, neck stiffness in 35 %, febrile seizures, disturbed consciousness, and meningeal signs (positive Brudzinski or Kernig signs) each one in 30 %. No fatalities, serious complications or sequela occurred among the hospitalized patients.

Electroencephalogram Findings

Four children underwent an electroencephalogram (EEG) at the time of, or shortly after, admission to the hospital. EEG abnormalities with slow spike and wave discharges, compatible with encephalitis, were observed in two cases.

Laboratory Findings in CSF

Cerebrospinal fluid (CSF) laboratory findings for the EV-positive and -negative patients are shown in Table 2. As mentioned above, 20 children were positive for EV. The mean CSF white blood cell (WBC) count was $170 \pm 267/\text{mm}^3$ and this is significantly higher compared to the EVs-

Fig. 1 Enterovirus detection in CSF samples by both enterovirus cell culture and RT-PCR assays. **a** EVs-positive isolates induced obvious enterovirus cytopathic effects on Vero cells. **1** uninfected Vero cell monolayer; **2** Vero cells infected with an EV-positive isolate. **b** Representative agarose gel electrophoresis of 5' NCR nested-RT-PCR-amplified products (294 bp band) for enteroviral RNA carried out on RNA extracted from CSF samples. *Lane M* 100 bp DNA ladder. *Lanes 1, 3, 4 and 5* CSF negative samples. *T-* negative control. *T+* positive

control (CV-B4 JVB). *Lane 2* CSF positive sample. **c** Representative agarose gel electrophoresis of VP1 nested-RT-PCR-amplified products (609 bp band) for enteroviral RNA carried out on RNA extracted from CSF samples. *Lane M* Ladder marker for a size from 200 to 10,037 bp. *Lanes 1, 2, and 4* CSF negative for VP1 nested-RT-PCR. *T-* negative control. *T+* positive control (CV-B4 JVB). *Lane 3* CSF positive for VP1 nested-RT-PCR

Fig. 2 Age group distribution of enterovirus infections positive cases as determined by 5' NCR nested RT-PCR

negative group ($p = 0.035$). Lymphocytes were predominant in 16 children (80 %) of the EVs-positive group. The mean lymphocyte percentage was 62 ± 36 which is also significantly higher than in the EVs negative group ($p = 0.001$). CSF pleocytosis (>5 white blood cells/mm³), was noted in 12 children (60 %) of the EVs-positive group. No significant difference between EV-positive and -negative groups was found regarding CSF glucose and protein levels that were within the normal range in both groups.

Fig. 3 Monthly distribution of enterovirus infections positive cases as determined by 5' NCR nested RT-PCR

Etiologies

Aseptic meningitis was diagnosed in 13 of the 20 EVs positive patients (i.e., 65 % of them), and meningoencephalitis in four of them (20 %). Febrile seizures, cerebellitis and ischemic stroke were each diagnosed in 5 % of the cases (see Fig. 4).

Table 2 CSF laboratory findings for enterovirus positive and negative cases

Enterovirus PCR results			
CSF laboratory findings	Positive <i>n</i> = 20	Negative <i>n</i> = 40	<i>p</i>
WBCs (nbr/mm ³) <i>m</i> ± <i>SD</i>	170 ± 267	33 ± 69	0.035
Lymphocytes (%) <i>m</i> ± <i>SD</i>	62 ± 36	27 ± 37	0.001
Proteins (g/l) <i>m</i> ± <i>SD</i>	0.41 ± 0.48	0.31 ± 0.23	0.239
Glucose (nmol/l) <i>m</i> ± <i>SD</i>	3.42 ± 1.08	3.42 ± 0.97	0.986

m mean; *SD* standard deviation

Fig. 4 Distribution of enterovirus infections positive cases as determined by 5' NCR nested RT-PCR according to their etiologies

Discussion

Since the introduction of vaccines and the control of poliovirus infection in much of the world, attention has been focused on NPEVs, which accounts for the majority of aseptic meningitis cases in infants [2].

The present study describes the epidemiological, clinical, and laboratory characteristics of pediatric CNS associated EV infections in the region of Monastir (central costal area of Tunisia), between January and December 2007. Variations in the overall epidemiological and etiological pattern of enteroviral neurological infections were reported. The majority of previous studies have shown that EV are responsible for high percentages of all aseptic meningitis cases, ranging from 44 to 83 % [17, 24, 44, 48]. There are, however, other studies reporting that EV associated meningitis seems to be less prevalent, detecting incidences between 16 and 27 % [32, 54]. The present study displayed that EVs were responsible for 33 % of cases of pediatric CNS infections suspected of having viral etiology. These results do not seem out of line with previous findings on EV prevalence in Tunisia [1, 3]. In 2002, EV was diagnosed by RT-PCR in 63 % of children hospitalized with neurological diseases in the region of

Monastir [17]. The decreased rate of EV detection in our study compared to the previous one can be explained by the impact of hygiene improvement.

The previous conventional virological diagnostic method for detection of EV is virus isolation in cell culture followed by serotyping with a panel of neutralizing antisera. This procedure is hampered by a low sensitivity of (65–75 %) [47]. Moreover, failure of detection of an EV serotype can sometimes be attributed to the presence of a small amount of infectious virus in CSF specimens [5, 45]. The use of molecular techniques described in this report improved the diagnosis of EV meningitis in our laboratory [22, 58]. Previous studies have shown that the PCR techniques including nested PCR are less time consuming and more sensitive than viral culture based techniques in the diagnosis of enteroviral meningitis [27, 52]. Our results confirm that PCR based methods are superior to cell culture based detection of EV [19].

In the present study, EV were detected by cell cultures in 10 % of samples from patients with neurological diseases, whereas they were detected by 5' NCR nested RT-PCR in 33 % of cases. The low rate of HEVs isolation by tissue culture compared to RT-PCR could be due either to the limited number of cell cultures (two cell lines) or to inadequate sample collection, handling, and processing or even more likely to the presence of non cultivable Coxsackievirus group A serotypes in clinical samples.

Children are the predominant targets of enteroviral associated CNS infections [11, 34]. The present study showed that the age of children with EV neurological infections ranged from 1 day to 12 years. These results are consistent with other previous findings [17, 24].

A higher prevalence of enteroviral meningitis in males versus females has been reported in the present study (2.33:1) and in others but there is no clear explanation [15, 17, 24].

In temperate climates, EV infections predominate in the summer and fall seasons. In the current study, sporadic cases of enteroviral meningitis were observed throughout the year even in winter months (a year-round EV distribution), with a peak in spring and summer which is in agreement of previous reports [15, 17, 24].

In the present study, the major NPEV-related CNS disorders were aseptic meningitis (65 %) occurring mainly in young infants and newborns, followed by meningoencephalitis (20 %) which was common in children under 2-years-old. This is in agreement with the pattern of data in our previous studies in Monastir [17]. Febrile seizures, cerebellitis and ischemic stroke are less commonly reported manifestations of EV infections that were observed in 5 % of all cases in our study [7, 12, 21, 53].

The clinical picture of enteroviral associated meningitis in neonates and young children was commonly dominated

by fever and headache followed by stiff neck, gastrointestinal or respiratory symptoms [6, 13]. In the present study, the patients' clinical signs and symptoms were consistent with those previously described. Fever was the most consistent clinical finding (80 %), followed by vomiting (50 %) and headache (45 %). Stiff neck occurred only in 35 % of cases, as did meningeal signs.

In aseptic meningitis, pleocytosis is always present in the CSF, and the WBCs usually range between 100 and 1,000/mm³ with a predominance of lymphocytes [16, 40]. In the current study, 60 % of EVs positive cases had elevated leukocyte counts in the CSF. In accordance with previous reports, the absence of pleocytosis was frequent in children under 2-years-old [18, 20, 29, 36, 50]. Lymphocytes were predominant in 80 % of EVs positive patients. With regards to CSF protein and glucose levels, their mean values were found to be similar to those described previously [14, 51]. EEG abnormalities and disturbed consciousness associated or not with seizure were strong predictors of encephalitis.

It has been shown that a VP1 nucleotide sequence identity of 75 % or more correlates with serotype classification [41–43]. This study was conducted with a nested RT-PCR assay described previously [10]. The major advantage of RT-PCR assay is that EV detection and characterization done directly on CSF samples overcomes some of the difficulties associated with virus cultures such as: the inoculation of specimens into non-susceptible cell lines, the inability of virus conjugated with antibodies to infect susceptible cells, and the failure of some EV to grow in cell cultures, as is the case for most of the group A Coxsackieviruses. However, this approach allowed amplification of the VP1-2A region in only three of the 20 cases detected positive with the 5' NCR nested RT-PCR. In the other 17 samples, we were unable to generate the VP1-2A amplicon necessary for virus typing, suggesting either an amount of viral RNA below the detection limit or mispairing of the primers with the viral genome. Sequence analysis of the amplified product displayed that the three EV belonged to the EV-B species, and were assigned to E-9. These findings are consistent with other reports, which described that E-9 is among the most common virus typed in the majority of EV CNS infection cases observed in patients under 15-years-old [17, 33, 57]. Echovirus-9 was already detected several times over a 12-year period in Tunisia [3]. Interestingly, in the present study, Echovirus 9 was identified in a cluster of three cases in a short-time period in the same hospital.

In conclusion, enteroviral infections were diagnosed thanks to the detection of viral RNA in CSF by 5' NCR nested RT-PCR. Amplification of VP1-2A by nested RT-PCR allowed molecular typing directly from CSF samples, and displayed a cluster of three E-9 cases in a short-time

period in Monastir. Further studies are needed to determine the extent of E-9 infection in Tunisia.

Acknowledgments This work was supported by the Ministère de l'Enseignement Supérieur et de la Recherche Scientifique (LR 99 ES 27), University of Monastir, Tunisia. We want to express our gratitude to the staff of the Pediatrics Department of Fattouma Bourguiba Hospital of Monastir. The authors are grateful to Rachel Décor (B.C.T. Consulting, Rue du 13 Août 1956, 5000 Monastir, Tunisia) for the English revision of this manuscript.

References

1. Abdallah H, Rezig D, Bahri O, Ben Yahia A, Kechrid A, Khaldi F, Barsaoui S, Bousnina S, Kanoun F, Bouzouia N, Triki H. Place des entérovirus dans les méningites aseptiques en Tunisie. *La Tunisie médicale*. 2003;81:919–25.
2. Absug MJ, Rotbart HA. Enterovirus infections of neonates and infants. *Sem Ped Infect Dis*. 1999;10:169–76.
3. Bahri O, Rezig D, Ben Nejma-Oueslatin B, Ben Yahia A, Ben Sassi J, Hogga N, Sadraoui A, Triki H. Enteroviruses in Tunisia: virological surveillance over 12 years (1992–2003). *J Med Microbiol*. 2005;54:63–9.
4. Bailly JL, Beguet A, Chambon M, Henquell C, Peigue-Lafeuille H. Nosocomial transmission of echovirus 30: molecular evidence by phylogenetic analysis of the VP1 encoding sequence. *J Clin Microbiol*. 2000;38:2889–92.
5. Berlin LE, Rorabaugh ML, Heldrich F, Roberts K, Doran T, Modlin JF. Aseptic meningitis in infants <2 years of age: diagnosis and etiology. *J Infect Dis*. 1993;168:888–92.
6. Bernit E, de Lamballerie X, Zandotti C, Berger P, Veit V, Schleinitz N, de Micco P, Harlé JR, Charrel RN. Prospective investigation of a large outbreak of meningitis due to echovirus 30 during summer 2000 in Marseilles, France. *Medicine*. 2004;83:245–53.
7. Bottner A, Daneschnejad S, Handrick W, Schuster V, Liebert UG, Kiess W. A season of aseptic meningitis in Germany: epidemiologic, clinical and diagnostic aspects. *Pediatr Infect Dis J*. 2002;21:1126–32.
8. Caro V, Guillot S, Delpeyroux F, Crainic R. Molecular strategy for “serotyping” of human enteroviruses. *J Gen Virol*. 2001;82:79–91.
9. Casas I, Tenorio A, Echevarria JM, Klapper PE, Cleator GM. Detection of enteroviral RNA and specific DNA of herpesviruses by multiplex genome amplification. *J Virol Methods*. 1997;66:39–50.
10. Casas I, Palacios GF, Trallero G, Cisterna D, Freire MC, Tenorio A. Molecular characterization of human enteroviruses in clinical samples: comparison between VP2, VP1, and RNA polymerase regions using RT nested PCR assays and direct sequencing of products. *J Med Virol*. 2001;65:138–48.
11. Cherry JD. Enteroviruses. In: Remington JS, Klein JO, editors. *Infections in the fetus and newborn infant*. Philadelphia: WB Saunders; 1995. p. 374–7.
12. Ciardi M, Giacchetti G, Fedele CG, Tenorio A, Brandi A, Libertone R. Acute cerebellitis caused by Herpes Simplex Virus Type 1. *Clin Infect Dis*. 2003;36:e50–4.
13. Connolly KJ, Hammer SM. The acute aseptic meningitis syndrome. *Infect Dis Clin North Am*. 1990;4:599–622.
14. Dommergues MA, Harzic M, Gobert ME, Landre C, De Truchis A, Charara O, Foucaud P. Seasonal outbreak of enteroviral meningitis during summer 2005: experience of a French pediatric unit. *Arch Pediatr*. 2007;14:964–70.

15. Dumaïdi K, Frantzidou F, Papa A, Diza E, Antoniadis A. Enterovirus meningitis in Greece from 2003–2005: diagnosis, CSF laboratory findings, and clinical manifestations. *J Clin Lab Anal.* 2006;20:177–83.
16. Elmore JG, Horwitz RI, Quagliarello VJ. Acute meningitis with a negative Grams stain: clinical and management outcomes in 171 episodes. *Am J Med.* 2000;100:78–84.
17. Gharbi J, Jaïdane H, Ben M'hadheb M, El Hiar R, Chouchene C, Gueddiche MN, Aouni M. Epidemiological study of non-polio enterovirus neurological infections in children in the region of Monastir, Tunisia. *Diagn Microbiol Infect Dis.* 2006;54:31–6.
18. Graham AK, Murdoch DR. Association between cerebrospinal fluid pleocytosis and enteroviral meningitis. *J Clin Microbiol.* 2005;43:1491.
19. Guney C, Ozkaya E, Yapar M, Gumus I, Kubar A, Doganci L. Laboratory diagnosis of enteroviral infections of the central nervous system by using a nested RT-polymerase chain reaction (PCR) assay. *Diagn Microbiol Infect Dis.* 2003;47:557–62.
20. Henquell C, Chambon M, Bailly JL, Alcaraz S, De Champs C, Archimbaud C, Labbé A, Charbonné F, Peigue-Lafeuille H. Prospective analysis of 61 cases of enteroviral meningitis: interest of systematic genome detection in cerebrospinal fluid irrespective of cytologic examination results. *J Clin Virol.* 2001;21:29–35.
21. Hosoya M, Sato M, Honzumi K, Katayose M, Kawasaki Y, Sakuma H, Kato K, Shimada Y, Ishiko H, Suzuki H. Association of nonpolio enteroviral infection in the central nervous system of children. *Pediatrics.* 2001;107:1–5.
22. Hosoya M, Sato M, Honzumi K, Katayose M, Sakuma H, Ishiko H, Shimada Y, Kato K, Suzuki H. Application of polymerase chain reaction and subsequent phylogenetic analysis to the diagnosis of enteroviral infection in the central nervous system. *J Clin Virol.* 2002;25:S27–38.
23. Ishiko H, Shimada Y, Yonaha M, Hashimoto O, Hayashi A, Sakae K, Takeda N. Molecular diagnosis of human enteroviruses by phylogeny-based classification by use of the VP4 sequence. *J Infect Dis.* 2002;185:744–54.
24. Jaidane H, Chouchane C, Gharbi J, Chouchane S, Merchaoui Z, Ben Meriem C, Aouni M, Guediche MN. Entéroviroses neuro-méningées en Tunisie: profil épidémiologique, clinique et évolutif. A propos de 26 cas pédiatriques. *Med Mal Infect.* 2005;35:33–8.
25. Kämmerer U, Kunkel B, Korn K. Nested PCR for specific detection and rapid identification of human picornaviruses. *J Clin Microbiol.* 1994;32:285–91.
26. Knowles NJ, Hovi T, Hyypää T, King AMQ, Lindberg M, Pallansch MA, Palmenberg AC, Simmonds P, Skern T, Stanway G, Yamashita T, Zell R. *Picornaviridae*. In: King AMQ, Adams MJ, Carstens EB, Lefkowitz EJ, editors. *Virus taxonomy: classification and nomenclature of viruses: ninth report of the International Committee on Taxonomy of Viruses*. San Diego: Elsevier; 2011. p. 855–80.
27. Kuan MM. Detection and rapid differentiation of human enteroviruses following genomic amplification. *J Clin Microbiol.* 1997;35:2598–601.
28. Kupila L, Vuorinen T, Vainionpää R, Hukkanen V, Marttila RJ, Kotilainen P. Etiology of aseptic meningitis and encephalitis in an adult population. *Neurology.* 2006;66:75–80.
29. Landry ML. Frequency of normal cerebrospinal fluid protein level and leukocyte count in enterovirus meningitis. *J Clin Virol.* 2005;32:73–4.
30. Lina B, Pozzetto B, Andreoletti L, Beguier E, Bourlet T, Dussaix E, Grangeot-Keros L, Gratacap-Cavallier B, Henquell C, Le-grand-Quillien MC, Novillo A, Palmer P, Petitjean J, Sandres K, Dubreuil P, Fleury H, Freymuth F, Leparç-Goffart I, Hober D, Izopet J, Kopecka H, Lazizi Y, Lafeuille H, Lebon P, Aymard M. Multicenter evaluating of a commercially available PCR assay for diagnosing enterovirus infection in a panel of cerebrospinal fluid specimens. *J Clin Microbiol.* 1996;34:3002–6.
31. Logotheti M, Pogka V, Horefti E, Papadakis K, Giannaki M, Pangalis A, Sgouras D, Mentis A. Laboratory investigation and phylogenetic analysis of enteroviruses involved in an aseptic meningitis outbreak in Greece during the summer of 2007. *J Clin Virol.* 2009;46:270–4.
32. McIntyre JP, Keen GA. Laboratory surveillance of viral meningitis by examination of cerebrospinal fluid in Cape Town, 1981–9. *Epidemiol Infect.* 1993;111:357–71.
33. Mistchenko AS, Viegas M, Latta MP, Barrero PR. Molecular and epidemiologic analysis of enterovirus B neurological infection in Argentine children. *J Clin Virol.* 2006;37:293–9.
34. Morens DM. Enteroviral disease in early infancy. *J Pediatr.* 1978;92:374–7.
35. Muir P, Kämmerer U, Korn K, Mulders MN, Poyry T, Weissbrich B, Kandolf R, Cleator GM, van Loon AM. Molecular typing of enteroviruses: current status and future requirements. The European Union Concerted Action on Virus Meningitis and Encephalitis. *Clin Microbiol Rev.* 1998;11:202–27.
36. Mulford WS, Buller RS, Arens MQ, Storch GA. Correlation of cerebrospinal fluid (CSF) cell counts and elevated CSF protein levels with enterovirus reverse transcription-PCR results in pediatric and adult patients. *J Clin Microbiol.* 2004;42:4199–203.
37. Nasri D, Bouslama L, Omar S, Saoudin H, Bourlet T, Aouni M, Pozzetto B, Pillet S. Typing of human enterovirus by partial sequencing of VP2. *J Clin Microbiol.* 2007;45:2370–9.
38. Nicholson F, Meetoo G, Aiyar S, Banatvala JE, Muir P. Detection of enterovirus RNA in clinical samples by nested polymerase chain reaction for rapid diagnosis of enterovirus infection. *J Virol Methods.* 1994;48:155–66.
39. Norder H, Bjerregaard L, Magnius L. Homotypic echoviruses share aminoterminal VP1 sequence homology applicable for typing. *J Med Virol.* 2001;63:35–44.
40. Nowak DA, Boehmer R, Fuchs HH. A retrospective clinical, laboratory, and outcome analysis of 43 cases of acute aseptic meningitis. *Euro J Neurol.* 2003;10:271–80.
41. Oberste MS, Maher K, Kilpatrick DR, Flemister MR, Brown BA, Pallansch MA. Typing of human enterovirus by partial sequencing of VP1. *J Clin Microbiol.* 1999;37:1288–93.
42. Oberste MS, Maher K, Kilpatrick DR, Pallansch MA. Molecular evolution of the human enteroviruses: correlation of serotype with VP1 sequence and application to picornavirus classification. *J Virol.* 1999;73:1941–8.
43. Oberste MS, Maher K, Flemister MR, Marchetti G, Kilpatrick DR, Pallansch MA. Comparison of classic and molecular approaches for the identification of untypeable enteroviruses. *J Clin Microbiol.* 2000;38:1170–4.
44. Ponka A, Pettersson T. The incidence and aetiology of central nervous system infections in Helsinki in 1980. *Acta Neurol Scand.* 1982;66:529–35.
45. Rotbart HA. Enteroviral infections of the central nervous system. *Clin Infect Dis.* 1995;20:971–81.
46. Rotbart HA, Sawyer MH, Fast S, Lewinski C, Murphy N, Keyser EF, Spadoro J, Kao SY, Loeffelholz M. Diagnosis of enteroviral meningitis by using PCR with a colorimetric microwell detection assay. *J Clin Microbiol.* 1994;32:2590–2.
47. Rotbart HA, Ahmed A, Hickey S, Dagan R, McCracken GH Jr, Whitley RJ, Modlin JF, Cascino M, O'Connell JF, Menegus MA, Blum D. Diagnosis of enterovirus infection by polymerase chain reaction of multiple specimen types. *Pediatr Infect Dis J.* 1997;16:409–11.
48. Rubio G, Mintegui S, Gaztelurrutia L, Sánchez J. Meningitis by enterovirus in pediatrics. Clinical characteristics and virologic diagnosis. *Enferm Infecc Microbiol Clin.* 1998;16:14–8.

49. Santti J, Hyypiä T, Kinnunen L, Salminen M. Evidence of recombination among enteroviruses. *J Virol*. 1999;73:8741–9.
50. Sato M, Hosoya M, Honzumi K, Watanabe M, Ninomiya N, Shigeta S, Suzuki H. Cytokine and cellular inflammatory sequence in enteroviral meningitis. *Pediatrics*. 2003;112:1103–7.
51. Sawyer MH. Enterovirus Infections: diagnosis and treatment. *Sem Ped Infect Dis*. 2002;13:40–7.
52. Shoja ZO, Tabatabai H, Sarijloo M, Shahmahmoodi S, Azad TM, Nategh R. Detection of enteroviruses by reverse-transcriptase polymerase chain reaction in cell culture negative stool specimens of patients with acute flaccid paralysis. *J Virol Methods*. 2007;142:95–7.
53. Steinlin M, Wehrli E, Berne. L'accident vasculaire ischémique en pédiatrie. Quand y penser-quoi faire !*. *Paediatrica*. 2009;20: 22–6.
54. Tardieu M, Dussaix E, Lebon P, Landrieu P. Prospective study of 59 cases of viral meningitis in children. Clinical and virologic diagnosis. *Epidemiology and physiopathology. Arch Fr Pediatr*. 1986;43:9–14.
55. Thoelen I, Lemey P, Van Der Donck I, Beuselinck K, Lindberg AM, Van Ranst M. Molecular typing and epidemiology of enteroviruses identified from an outbreak of aseptic meningitis in Belgium during the summer of 2000. *J Med Virol*. 2003;70: 420–9.
56. Thoren A, Widell A. PCR for the diagnosis of enteroviral meningitis. *Scand J Infect Dis*. 1994;26:249–54.
57. Trallero G, Avellon A, Otero A, De Miguel T, Pérez C, Rabella N, Rubio G, Echevarria JE, Cabrerizo M. Enteroviruses in Spain over the decade 1998–2007: virological and epidemiological studies. *J Clin Virol*. 2010;47:170–6.
58. Verstrepen WA, Bruynseels P, Mertens AH. Evaluation of a rapid real-time RT-PCR assay for detection of enterovirus RNA in cerebrospinal fluid specimens. *J Clin Virol*. 2002;25:S39–43.
59. Yerly S, Gervaix A, Simonet V, Caflisch M, Perrin L, Wunderli W. Rapid and sensitive detection of enteroviruses in specimens from patients with aseptic meningitis. *J Clin Microbiol*. 1996; 34:199–201.