Enterovirus Meningitis in Greece From 2003–2005: Diagnosis, CSF Laboratory Findings, and Clinical Manifestations

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Enteroviruses (EVs) are the most commonly identified cause of aseptic meningitis. Rapid detection and characterization of EV meningitis is essential in making decisions for patient management and treatment. A total of 52 cases of acute aseptic meningitis that occurred from March 2003 to April 2005 were investigated for EVs using viral culture and/or molecular methods directly in the cerebrospinal fluid (CSF). EVs were detected in 21 out of 52 (40.4%) patients using reverse transcription-PCR (RT-PCR) and/or tissue culture. EVs were isolated from six out of 37 (16.2%) cultured specimens, while 20 out of 52 (38.4%) specimens yielded positive results when 5'non-coding region (5'NCR) RT-PCR assay was used. One specimen that was culture-positive was RT-PCR-negative. Using the VP1-2A RT-PCR and sequence analysis, 14 of the 21 positive

EVs were identified as: four strains of Coxsackie virus B5, five echovirus 11, two echovirus 9, one echovirus 5, one echovirus 14, and one Coxsackie virus A9. Fever, headache, vomiting, and stiff neck were the most pronounced symptoms. Pleocytosis with the predominance of lymphocytes and mild elevated protein levels characterized the CSF specimens. Coxsackie virus B5 and echovirus 11 were the predominant serotypes during the study period. Although there was seasonal enteroviral activity (April–November), cases also occurred in the cold months. The 5'NCR and VP1-2A RT-PCR with sequence analysis were found to be superior to conventional methods for direct diagnosis and the typing of EVs. *J. Clin. Lab. Anal.* 20:177–183, 2006. © 2006 Wiley-Liss, Inc.

Key words: aseptic meningitis; echovirus; Coxsackie virus; RT-PCR; CSF

INTRODUCTION

Human enteroviruses (HEVs) belong to the family *Picornaviridae*. They are major human pathogens with more than 70 serotypes divided into five human enterovirus species (HEV A to D and poliovirus) (1). Most enteroviral infections cause a benign, self-limited disease; however, they can also produce severe and sometimes fatal illness such as aseptic meningitis, meningoencephalitis, encephalitis, myocarditis, “hand, foot and mouth disease,” neonatal multi-organ failure, and acute flaccid paralysis (2).

Non-polio enteroviruses (NPEVs) are the most commonly identified cause of aseptic meningitis and account for over 80% of the cases in which the etiological agent is identified (3). In temperate climates, these infections occur during the summer and fall (4). Sporadic cases of infections are also reported through-

out the year, but only a few winter outbreaks have been reported (5,6). Although young children are the most common victims, enteroviral meningitis is not uncommon in adults (3,7,8). In previous years, several epidemic outbreaks of aseptic meningitis have been reported worldwide, the majority of which were caused by enteroviruses if an etiological agent was identified (9). The outcome of enteroviral meningitis is usually favorable in otherwise healthy and immunocompetent patients. Specific diagnosis of enterovirus meningitis has

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many advantages in patient management (10). Traditionally, laboratory diagnosis of enterovirus infection is based on virus isolation in cell cultures and serotyping by a neutralization test, which are labor-intensive and time-consuming (11). In an attempt to improve the speed, sensitivity, and specificity of the laboratory diagnosis of enterovirus infections, multiple reverse transcription-PCR (RT-PCR) assays have been developed based on the amplification of a highly conserved 5'non-coding region (5'NCR) in the enterovirus RNA genome (12). A major drawback of 5'NCR-based RT-PCR assays is their inability to identify the enterovirus type, but a correlation between the nucleotide sequence of the highly variable gene encoded for the major capsid protein, VP1, and the serotype has now been established (13–15). However, in many instances the enteroviral infections are not detected quickly enough (or at all) to provide sufficient means for an initial diagnosis. Therefore, the distinction between the enteroviral aseptic meningitis and bacterial or other viral infections is based on clinical signs and laboratory analysis of the cerebrospinal fluid (CSF) and serum specimens (16).

The main objectives of this study were the diagnosis of enterovirus and the investigation of the most common enteroviruses that cause aseptic meningitis in respect to clinical manifestations and CSF laboratory findings in Greece, during the period between March 2003 and April 2005. In addition, two RT-PCR assays, one based on 5'NCR and a second based on VP1-2A region, were studied in comparison with conventional methods for rapid detection and typing of enteroviruses directly on CSF samples.

MATERIALS AND METHODS

Patients

During the period from March 2003 to April 2005, 52 sporadic cases of aseptic meningitis were investigated for enteroviral infection. Forty-one CSF samples were sent to our laboratory from hospitals located in the northern part of Greece (Thessaloniki 33, Drama 2, Serres 4, Bolos 1, and Larisa 1), and 11 samples were received from Patras located in the southern part of the country. Patients' clinical history, epidemiological data, and the result of the CSF analysis were derived from hospital files. The presumable diagnosis of aseptic meningitis was based on the Centers for Disease and Control and Prevention (CDC) definitions (17).

Virus Isolation and Neutralization Test

Two cell lines, Hep-2 (human epidermoid carcinoma) and RD (rhabdomyosarcoma) cells were used for virus isolation, according to the recommendations given by

the World Health Organization (18). An amount of 200 μ L of each CSF specimen were inoculated into two tubes of each cell line. After incubation at 36°C for 7 days, the inoculated tubes that showed cytopathic effects (CPEs), suggestive of enteroviruses, were passed through the same cell lines one more time for confirmation. The cells were scratched, harvested, and stored at –70°C for further investigation. The isolates were typed by a neutralization test, using the RIVM antiserum pools (National Institute of Public Health and the Environment, Bilthoven, The Netherlands). For cultures exhibiting no CPE by the end of the first incubation period, blind passage was performed for another 7 days before they were reported as negative.

Extraction of Viral RNA

Viral RNA was extracted from 140 μ L CSF and/or positive cell culture supernatant using QIAamp viral RNA mini kit (Qiagen GmbH, Hilden, Germany), according to the manufacturer's instructions with a little modification concerning the volume of elution for the CSF samples (30 μ L was used instead of 70 μ L).

RT-PCR Assays

The reverse transcription and the subsequent amplification of the cDNA were carried out in one tube to minimize the risk of contamination. Enteroviral RNA was amplified by PCR as described previously (19), using the primers EV1 (forward primer, 5'-CAAG CACTTCTGTTTCCCCGG-3') position 160–180 and EV2 (reverse primer, 5'-ATTGTCACCATAAGCAGC CA-3') position 580–560, yielding a 435-bp product. The primers' binding sites are located within the 5'NCR of the enteroviral genome, thus facilitating the detection of the majority of human enteroviruses. In cases of a positive PCR result, a second nested RT-PCR assay was used to amplify a 609-bp product in the VP1-2A region of the viral RNA using VP1-1S and VP1-1A in the first run and VP1-2S and VP1- 2A in the second run as forward and reverse primers, respectively, according to the RT-PCR method described elsewhere (20). A volume of 10 μ L of each PCR product was analyzed by using 1.5% agarose gel electrophoresis stained with ethidium bromide in tris-boric acid-EDTA (TBE) buffer. In each RT-PCR assay, one negative control of sterile distilled water and one positive control (Coxsackie virus B5 10^{–3} dilution in a negative CSF sample) were included.

The 609-bp amplicon was purified using the gel extraction kit (Qiagen GmbH) and was sequenced using the Thermo Sequence Cy 5.5 Dye Terminator Cycle Sequencing Kit (Amersham Biosciences, UK) and OpenGene system (Visible Genetics, Little Chalfont Buckinghamshire, UK) according to the manufacturers'

instructions. The nucleotide sequence of the amplified VP1-2A region for each sample was analyzed using the BLAST program of NCBI (National Center for Biotechnology Information; <http://www.ncbi.nlm.nih.gov/BLAST>). The genotype determined by the PCR-sequence method was based on the homology of our sequence data with published sequences in GenBank.

Statistical Analysis

Statistical Package for Social Science (SPSS, IL) was used to analyze the collected data; the calculated values included: mean, median, and range. In addition, because the data concerning the white blood cell counts (WBCs) were not normally distributed, statistical comparisons between groups were performed using the nonparametric Mann-Whitney test. For the clinical signs and symptoms, chi-square tests used to investigate the statistical significance between groups.

RESULTS

CSF was collected from 32 children (aged 21 days to 14 years) and 20 adults (aged 15–73 years) with acute aseptic meningitis. Quantitative and qualitative cytological examinations of CSF revealed WBCs of 10–1,300/mm³, with a predominance of lymphocytes, protein levels of 18–180 mg/dL, and normal glucose levels. The results of bacterial and fungal investigations were negative in all cases.

Virus Isolation, RT-PCR, and Typing Results

Overall, 21 out of 52 (40.4%) tested samples were found to be positive for enteroviruses by viral cultures and/or RT-PCR. Standard isolation procedures were only applied on 37 samples when a sufficient volume of CSF was available. Enterovirus was isolated in cell cultures in six out of 37 CSF samples (16.2%). Four isolates came from children and two from adults. All isolates were typed by a neutralization test, except one. Molecular typing was achieved in 14 samples. In the other seven samples, no VP1-2A product was amplified, suggesting either an amount of viral RNA below the detection limit or mispairing of the primers with the viral genome. Month and year of diagnosis, sex, age, location, results of culture and/or RT-PCR assays, and typing are shown in Table 1.

Twenty out of 52 (38.4%) cases were positive for enterovirus by 5'NCR RT-PCR, including 14 children (43.7%) and six adults (30%). Five of them had a positive culture. One CSF with a positive culture was negative by both RT-PCR assays. In the latter situation, the virus was detected by PCR when the cell culture supernatant was used as a sample.

Laboratory Findings

CSF laboratory findings for the EV-positive and -negative patients are shown in Table 2. Fourteen children aged 21 days to 14 years (median 9.5 years)

TABLE 1. Cases of enteroviral meningitis in Greece 2003–2005

Patient number	Date of diagnosis	Sex	Age (years)	Location	Culture	RT-PCR	Neutralization test (NT)	Sequence of VP1-2A region
4/03	03/03	M	32	Thessaloniki	Negative	Positive		
11/03	06/03	M	4	Thessaloniki	Negative	Positive		
12/03	06/03	F	12	Thessaloniki	Negative	Positive		COX A9
14/03	08/03	M	30	Thessaloniki	Negative	Positive		
18/03	09/03	F	12	Thessaloniki	Positive	Positive	Untypeable	ECHO 9
27/03	11/03	M	62	Thessaloniki	Positive	Negative	COX B	COX B5
28/03	11/03	M	41	Thessaloniki	Negative	Positive		
33/03	12/03	M	11	Thessaloniki	Positive	Positive	COX B	COX B5
35/04	01/04	F	22	Thessaloniki	Negative	Positive		
37/04	01/04	M	11	Thessaloniki	Negative	Positive		
38/04	01/04	M	7.5	Thessaloniki	Negative	Positive		
30A/04	04/04	M	45	Thessaloniki	Positive	Positive	COX B	COX B5
46/04	07/04	M	14	Thessaloniki	Negative	Positive		ECHO 11
48/04	07/04	M	14	Thessaloniki	Positive	Positive	COX B	COX B5
52/04	08/04	M	8	Thessaloniki	Negative	Positive		ECHO 11
64A/04	10/04	M	12	Drama	Positive	Positive	ECHO 11	ECHO 11
82A/04	10/04	M	0.06	Patra	ND	Positive		ECHO 14
63A/04	11/04	F	0.17	Drama	ND	Positive		ECHO 11
74A/04	12/04	F	2.5	Patra	ND	Positive		ECHO 11
110A/05	04/05	F	0.097	Larisa	ND	Positive		ECHO 5
115A/05	04/05	M	56	Bolos	ND	positive		ECHO 9

ND, not done; COX, coxsackievirus; ECHO, echovirus.

TABLE 2. Laboratory profile of 52 patients with aseptic meningitis

Patient profile				CSF laboratory profiles				
Group	No.	Results of EV investigation	Age/years m/(r)	CSF WBCs m (r)	Lymphocytes $\geq 50\%$ no. (%)	Neutrophils ≥ 50 no. (%)	Protein mg/dL (m)	Glucose mg/dL (m)
Children	32	+14 (43.8%)	7.73 (0.6–14)	462* (102–1300)	12/13 (92.3)	1/13 (7.7)	76* (23–122)	53 (33–85)
		–18 (56.2%)	6.15 (0.38–14)	185* (10–606)	8/13 (61.5)	5/13 (38.5)	54* (18–180)	59 (45–95)
Adults	20	+7 (35%)	41.1 (22–62)	454 (38–1200)	4/5 (80)	1/5 (20)	81 (40–100)	60 (36–101)
		–13 (65%)	39.6 (23–73)	194 (50–480)	11/12 (91.6)	1/12 (8.4)	95 (30–169)	56 (37–66)
Total	52	+21 (40.4%)	18.8 (0.06–62)	459 (38–1300)	16/18 (88.9)	2/18 (11.1)	78 (23–122)	55 (33–101)
		–30 (59.6%)	21.1 (0.38–73)	189 (10–606)	19/24 (76)	6/25 (24)	71 (18–180)	58 (37–95)

* $P < 0.05$.

r, range; m, mean.

were positive for enterovirus. The total number of WBCs/mm³ in the CSF ranged between 102–1,300 (median 405), and this is significantly higher compared to the negative EVs group ($P \leq 0.05$). Lymphocytes were predominant in 12 of the 13 children in which the CSF differential of WBCs was available, whereas they were predominant in eight of the 13 children negative for enterovirus. CSF protein levels were found to be significantly higher in EV-positive children than in the negative group. No significant differences were found between EV-positive and -negative children regarding age and glucose levels. Seven adults aged 22–62 years (median 41 years) were EV positive. The total number of WBCs in CSF ranged from 38–1,200/mm³ and no significant difference was found between the EV-positive and the EV-negative group. Lymphocytes were predominant in four out of five samples in which the differential of WBCs was available. However, lymphocytes were also predominant in 11 of the 12 adults negative for EVs. No significant differences were observed between the EV-positive adults and the negative group regarding age and the protein and glucose levels. In general, CSF levels of protein were mildly elevated in most cases, whereas glucose levels were within the normal range.

Clinical Signs and Symptoms

Clinical information was obtained for 50 of the 52 studied cases. The majority of the aseptic meningitis cases presented with typical signs and symptoms. No significant differences in clinical signs and symptoms were observed between children and adult groups with positive EV infections in comparison to the negative group. Both children and adults with positive EV infections tended to have high fever (96%), headache (96%), nausea/vomiting (75%), photophobia (40%), and stiff neck (40%). Only one child and one adult with

EV infections presented with mental disturbance. The severity of the aseptic meningitis in our study was mild to moderate and the final outcome was favorable with no reported deaths or sequelae.

DISCUSSION

To the best of our knowledge this is the first report of the commonest enteroviruses that circulate and cause aseptic meningitis in Greece with respect to CSF laboratory findings and clinical manifestations.

Children are the primary victims of enterovirus meningitis, but the illness is not uncommon in the adult population (3,7,8,12,21). In the present study, 21 out of 52 (40.4%) cases of aseptic meningitis were found positive for enteroviruses. Fourteen of them (66.6%) were children less than 15 years old and seven (33.3%) were adults.

A higher prevalence of enteroviral meningitis in males vs. females has been reported by several studies (7,22). The findings in our study were consistent with these studies and the male:female ratio was found to be 2.5:1.

In temperate climates enteroviral meningitis appears with a peak in the summer and fall seasons, while in tropical areas there is a high incidence of this disease throughout the whole year (23). In this study, 15 cases (71.4%) occurred during the period from April to November and six (28.6%) cases in the cold months. Several previous studies showed an atypical pattern of enteroviral circulation and occurrence of aseptic meningitis during the winter season (24,25).

The clinical picture of enteroviral meningitis in neonates and young children are mostly nonspecific, with fever and headache being the most common symptoms followed by stiff neck, photophobia, gastrointestinal or respiratory symptoms, and rash. In older children or adults, the signs of the central nervous system such as headache and stiff neck are more

TABLE 3. The performance of enterovirus RT-PCR assay vs. combined viral cell cultures and clinical diagnosis

Specimen (n = 37) and PCR result	Culture and clinical diagnosis		Sensitivity (%)	Specificity (%)	PPV (%)
	Positive	Negative			
PCR-positive	5	10	83	68	33
PCR-negative	1	21			

PPV, positive predictive value.

pronounced (9,22,16,26). In the present study, the clinical signs and symptoms of the patients were consistent with those described previously.

In aseptic meningitis, pleocytosis is always present in the CSF, and the WBCs usually range between 100–1,000/mm³ with a predominance of lymphocytes (27,28). Predominance of neutrophils within the first 24 hr was observed in one study, followed by a shift to lymphocytes (29). In our study, all 52 patients had elevated leukocyte counts in the CSF. Lymphocytes were predominant in all EV-positive patients except in two cases. CSF protein levels were elevated in most EV-positive cases, but in comparison to the EV-negative groups, significant differences were only observed in children. In general, the mean values of protein and glucose were found to be similar to those described previously (22,28).

In this study, the effectiveness of two RT-PCR assays was evaluated in comparison to conventional methods. When compared with viral culture, 5'NCR RT-PCR detected the virus in five out of six (83.3%) of the CSF samples with positive culture and in 10 out of 31 (32.2%) of the cases with negative culture. The PCR method missed one culture-positive sample. This suggests that the RNA may have degraded during the preparation or perhaps there were inhibitors of the PCR process in the sample. The low number of EVs isolated by tissue culture compared to RT-PCR could be due to the limited number of cell cultures used (two cell lines) or due to inadequate collection, handling, and processing of the samples.

Overall, enterovirus RNA was detected in five out of six culture-positive, 10 out of 31 culture-negative, and five out of 15 non-cultured samples. The sensitivity, specificity, and positive predictive value of the PCR vs. combined culture and clinical diagnosis were 83%, 68%, and 33% respectively (Table 3). Using this approach, Verstrepen et al. (30) found that the sensitivity and the specificity of real time PCR was 72.6% and 57.4%, respectively, while Guney et al. (31) found that the sensitivity and specificity of PCR compared to virus isolation was 89% and 66%, respectively.

It has been shown that a VP1 nucleotide sequence identity of 75% or more correlates with the serotype's

classification (13,32,33). In this study, we followed a nested RT-PCR assay described previously (20). The major advantage of this RT-PCR assay for the detection and characterization of the enteroviruses directly from the CSF overcomes some of the difficulties associated with a virus culture such as the inoculation of specimens into non-susceptible cell lines, the inability of virus conjugated with antibodies to infect susceptible cells, virus aggregation preventing neutralization, and the failure of some enteroviruses to grow in cell cultures such as most of the group A Coxsackie viruses. By using this approach, we managed to amplify the VP1-2A region in 14 of the 21 cases, with positive 5'NCR RT-PCR. Sequence analysis of the amplified product showed that all 14 enteroviruses belong to human enterovirus group B, including six different serotypes. Echoviruses accounted for 64.3% and coxsackieviruses 35.7%. Similar epidemiological studies carried out from 1999–2003 in Europe and the United States reported the predominance of echoviruses (echovirus 30, 13, 11, 6, and 9 and Coxsackie virus B5) (15,24,25,34). Although the number of the examined samples is relatively small and the duration of the study covers only two enteroviral seasons without the occurrence of an outbreak, the results of molecular typing suggest that coxsackievirus B5 was predominant in 2003–2004 and echovirus 11 in 2004–2005. In Greece, some previous studies conducted in south and central Greece and the island of Crete showed that Coxsackie virus B5 and echovirus 6 and 13 were the most common types that circulated and caused sporadic or epidemic outbreaks of aseptic meningitis, during the period 1994–2001 (35–37). It would be of great interest to determine if there is an epidemiological association between the strains that were isolated or detected in this study and those isolated previously in Greece, Europe, and other countries (study in progress).

In conclusion, this study showed that six different serotypes of non-polio enteroviruses were present and caused sporadic cases of aseptic meningitis during the period of 2003–2005 in Greece. The most commonly detected enteroviruses were Coxsackie virus B5 and echovirus 11. Additionally, the clinical picture of our cases, characterized by the typical clinical signs and

symptoms and the predominance of the lymphocytes in the CSF, was confirmed. This study supports the seasonality of enterovirus infections from April to November, as well as the continuous circulation of EVs in the cold months. The 5'NCR RT-PCR assay used for the direct detection of enteroviral RNA in CSF is an efficient, accurate, and rapid means for diagnosis. The rapid detection and characterization of EVs in cases of meningitis improves patient management and treatment, avoiding the use of antibiotic or antiherpetic medication for prolonged periods and also reduces hospital inpatient time. Amplification of the VP1-2A by nested RT-PCR permits molecular typing directly on CSF, which has significant clinical and epidemiological implications.

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