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SHORT COMMUNICATION

Enteroviruses Associated with Aseptic Meningitis in Poland, 2011–2014

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Abstract

A 4-year study (2011–2014) of patients with meningitis was performed. Out of the 686 cerebrospinal fluid samples, 465 (67.8%) were positive for eneteroviruses using RT-PCR and out of 334 clinical samples, 216 (64.7%) were positive for enteroviruses using cell culture methods. The highest detection rate was observed in the summer and autumn. In total, 185 enteroviruses were identified by using neutralization test. Echovirus 6 and 30 were the most common (41.7% and 37.5% respectively). The highest frequency of neurological infections (32.7%) occurred in children aged 5–9 years, mostly males (63.9%).

Key words: aseptic meningitis, cerebrospinal fluid, diagnostic PCR, enteroviruses

Human enteroviruses (HEVs) are members of the *Picornaviridae* family, a large and diverse group of small RNA viruses characterized by a single-positive-strand genomic RNA. They are classified in four species: enteroviruses A, B, C and D. More than 100 serotypes are described. They affect millions of people worldwide each year, and are often found in the respiratory secretions and stool of an infected person. Infection can result in a wide variety of symptoms ranging from mild respiratory illness (common cold), through hand, foot, and mouth disease, acute haemorrhagic conjunctivitis, and myocarditis, to severe neonatal sepsis-like disease and acute flaccid paralysis, but the most common neurological manifestation is aseptic meningitis.

In temperate climates enteroviral meningitis is more than 5 times more common in summer than in winter and spread is predominantly through the faecal-oral route. More than 90% of infected people remain asymptomatic. However, since most HEV infected individuals are asymptomatic, it is difficult to prevent further spread of the virus. The majority of symptomatic patients develop only mild febrile illness, less than 5% develop meningitis. Enteroviral meningitis may affect all age groups and usually is self-limiting. However, some patients show complications such as seizure, coma, and movement disorders. The predominant serotypes identified in enteroviral meningitis outbreaks are echovirus (E) 6, E9, E11, E13, E19 and E30 (Hayashi *et al*., 2012).

Enteroviral meningitis is confirmed by either virus isolation followed by identification of virus by neutralization assay using type-specific antisera or polymerase chain reaction (PCR) using cerebrospinal fluid (CSF). Molecular methods are faster and more sensitive than viral cell culture. The 5'UTR (5' untranslated region) is the most conserved region among EVs and is therefore targeted in many diagnostic tests (Richter *et al*., 2006). Early diagnosis is optimal for patient management because it helps to avoid unnecessary antibiotic treatment.

This paper examines the epidemiology of HEVs in Poland over a four-year period with special attention to serotypes associated with meningitis.

A total of 686 CSF samples that were sent to the National Polio Laboratory in Warsaw for analysis between January 2011 and December 2014, were included in this study. Most of the samples were obtained from patients with viral meningitis of suspected enteroviral etiology, hospitalised in neurological and infectious disease departments of hospitals mainly in two voivodships podlaskie and mazowieckie (> 90% of all samples). The clinical samples were tested with diagnostic pan-enterovirus RT-PCR (EV PCR). Viral RNA was extracted from 140 µl of sample using spin columns (Qiagen) following the manufacturer's instructions. RT-PCR was carried out using Pan-enterovirus primers for enterovirus detection based on the WHO manual (WHO, 2004). This set of primers produces a product

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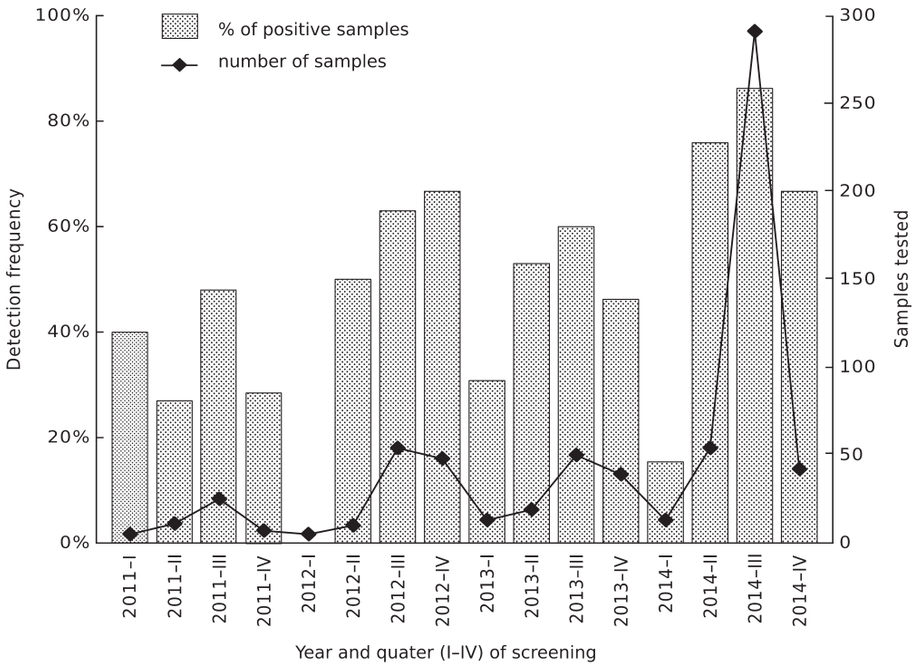

Fig. 1. Detection frequency of enterovirus in cerebrospinal fluid samples, 2011–2014

of 114 bp and has been designed to detect and amplify a genome segment present at the 5'-UTR of the enterovirus genomes. RT-PCR amplification was performed: one cycle of reverse transcription at 45°C for 20 min; one cycle of denaturation at 94°C for 2 min; 30 cycles of denaturation at 94°C for 30 s, annealing at 55°C for 30 s, elongation at 70°C for 30 s followed by one cycle of elongation at 70°C for 7 min. Reaction mixtures were then held at 4°C. Amplification products were analysed in 2% agarose gels, GelRed-stained and examined under a UV DNA trans-illuminator. When the volume of CSF collected was sufficient positive samples were inoculated onto cell cultures.

In clinical samples that were tested with diagnostic pan-enterovirus RT-PCR, enteroviral RNA was detected in 465 (67.8%) of the 686 CSF specimens. EVs were detected throughout the four-year study period: 19 of 48 (39.6%) in 2011, 71 of 117 (60.7%) in 2012, 53 of 121 (43.8%) in 2013 and 322 of 400 (80.5%) in 2014. Figure 1 shows the seasonal distribution of EV infections cases, confirmed by PCR. Although some cases were notified throughout the year, they mostly occurred during summer and autumn months (III and IV quarter of year).

EV-positive patients' ages range from newborns to 73-years-old. As shown in Fig. 2, the highest rate among positive patients was observed in children 5–9 years old. Infected children were predominately males (63.9%) with a male-to-female ratio of 1.77:1, with 297 male and 168 female cases.

For virus isolation in cells, total of 334 clinical samples (cerebrospinal fluids – 127, stools – 202, throat swabs – 5), obtained from patients with viral meningitis, were analysed for enterovirus (EV) isolation in cell cultures during a 4-year period (2011–2014). Viruses have been isolated from cerebrospinal fluid (CSF), throat swabs and stool specimens by conventional cell culture methods using WHO recommendations. Viral isolation was performed on RD-cells (human rhabdomyosarcoma). In order to exclude the involvement of polioviruses L20B-cells (transgenic mouse cell with the human poliovirus receptor) were also used for virus cultivation. RD and L20B cells were cultivated in minimal essential medium (MEM) supplemented with 10% foetal bovine serum (FBS). A volume of 200 µl of sample was inoculated into tubes with RD and L20B cells. The tubes were incubated at 36°C and were examined daily. After 7 days, the tubes were frozen and thawed and repassaged, and another 7-day examination was performed. Each specimen underwent two passages in RD and L20B cells. Samples demonstrating viral cytopathic effect (CPE) were identified by neutralization assay using specific antisera (National Institute for Public Health and the Environment, the Netherlands).

A total of 334 clinical samples were analysed for enterovirus by isolation in cell cultures. The study found that 216 (64.7%) samples were positive (78 – cerebrospinal fluid, 134 – stool, 4 – throat swabs). Out of the 334 samples analysed by cell culture isolation, 177 came from patients with positive EV PCR results,

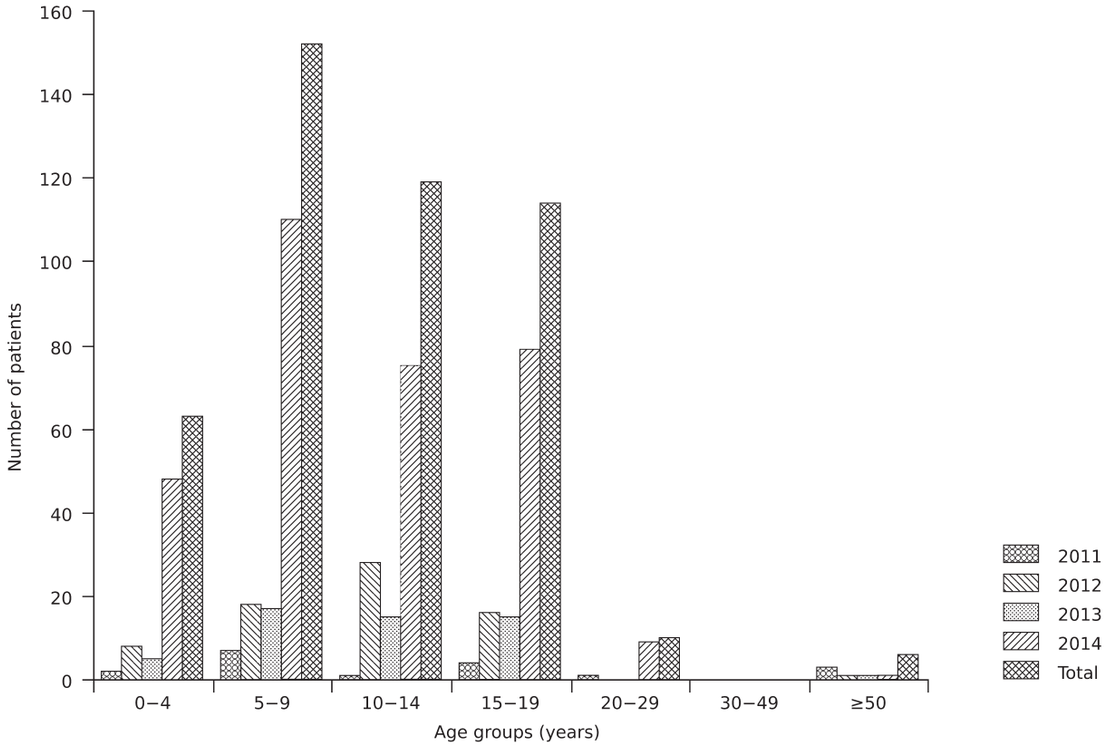

Fig. 2. Age distribution of patients with a cerebrospinal fluid sample positive for enterovirus, 2011–2014

51 came from patients with negative EV PCR results and 106 samples were not analyzed earlier for enteroviruses. Among the samples from patients with positive EV PCR results, 135 (76.3%) were positive in cell culture isolation, but also among the 51 samples from patients with negative EV PCR results, 14 (27.4%) were positive in cell culture isolation. Out of the 106 samples not analyzed earlier for enteroviruses, 67 (63.2%) were positive in cell culture isolation.

Table I shows the number and types of enteroviruses detected during 2011–2014. During these 4 years,

Table I
Enterovirus serotypes detected in viral culture by year the specimen was received at NPL – Warsaw; January 1, 2011 to December 31, 2014; Poland

Enterovirus	2011	2012	2013	2014	Total
CVA9	–	–	2	–	2
CVB	–	–	3	1	4
E6	7	55	22	6	90
E7	–	–	–	1	1
E11	2	1	2	2	7
E30	–	3	33	45	81
Untyped enteroviruses	–	2	2	–	4
Not done	–	–	–	27	27
Total enteroviruses/year	9	61	64	82	216
Total specimens tested/year	33	72	113	116	334

the positive EV isolation ratio was fluctuated between 27.3% in 2011 and 84.7% in 2012. Two hundred and sixteen enteroviruses were isolated. Serotyping was performed on 189 isolates, and serotypes of E6, E7, E11, E30, CVA9 and CVB were revealed from 185 isolates, while the other 4 isolates could not be typed by serological method. The most common were echovirus 6 (E6) (90, 41.7%) and echovirus 30 (E30) (81, 37.5%). Together these two genotypes represented 79.2% of all EVs isolated. These predominant strains circulated in each year of the study with the exception of E30 in 2011. In 2011, echovirus 6 was predominant and E11 was also represented. In 2012, E6 constituted a high percentage (90.2%) of enteroviruses detected, and E11 and E30 were also represented. In 2013, E30 and E6 were predominant and Coxsackievirus A9 (CVA9), CVB and E11 were also represented. In 2014, E30 constituted the highest percentage (54.9%) of enteroviruses detected, and CVB, E6, E7 and E11 were also represented.

The present study describes the epidemiological and laboratory characteristics of enteroviral aseptic meningitis cases in two regions in Poland (mazowieckie and podlaskie), between January 2011 and December 2014. The majority of previous studies have shown that HEVs are responsible for high percentages of all aseptic meningitis cases, ranging from 43 to 83% (Gharbi *et al*., 2006; Kumar *et al*., 2013; Papadakis *et al*., 2013). Others studies, however, demonstrated that enteroviral

meningitis is less prevalent and ranged between 16% and 27% (Tao *et al*., 2014). The incidence of the enteroviruses varies globally according to different populations and methodologies. The present study displayed that EVs were responsible for 67.8% (from 39.6% in 2011 to 80.5% in 2014) of cases of CNS infections suspected of having viral etiology.

In temperate climates, EV infections predominate in the summer and fall seasons. In the current study, PCR results for EV meningitis demonstrate a clear seasonality. Positive results were reported mainly in III and IV quarter of year. However, infections were also detected at other times of year, indicating year-round sporadic infections.

Among the 465 enterovirus positive patients, 385 (82.8%) were 5–19 years of age. Surveillance data from several countries have shown that approximately 29% to 44% of CNS-associated EV infections occur in young children under the age of one year (Harvala *et al*., 2014). For example, in the United State, the peak age for children with aseptic meningitis is reported to be <1 year old. The age distribution of meningitis cases varies, possibly due to different causative agents. Others studies described a large proportion of teenagers and young adults infected during previous E30 outbreaks (Khetsuriani *et al*., 2006; Savolainen-Kopra *et al*., 2011; Takamatsu *et al*., 2013).

Aseptic meningitis is generally commoner in males with a male-to-female ratio of 1.2–2.3:1, although the exact reason for this in unknown. In the current study the male-to-female ratio was 1.77:1.

Isolation of the enterovirus in cell culture is the traditional method used to identify the causative agent of viral meningitis, and it also makes serotyping of the isolated enterovirus possible, but it usually has low sensitivity, ranging from 60 to 75%. In our study, among the samples from patients with positive EV PCR results, 23.7% were negative in cell culture isolation, but also among the samples from negative patients, 27.4% were positive in cell culture isolation. The presence of enteroviruses does not necessarily generate positive PCR results. Negative results are probably a consequence of the presence of compounds that inhibit RT or PCR (Kopecka *et al*., 1993). Obtaining false negative results in cell culture can be due to the presence of noninfectious virus particles or slow growing enteroviruses, lack of sensitivity of cell line, low titre of virus in the specimens and toxic factors. Not all enteroviruses cause cytopathic effects in cell lines and thereby PCR assay detect a wider variety of viruses than cell culture method. A number of studies have demonstrated that RT-PCR is more sensitive than cell culture for enterovirus detection. Sensitivity and specificity of enterovirus RT-PCR are estimated at >95%. It should also be considered that in years with high activity of types growing well in cell culture, the sensitivity of cell culture can be higher than in years with lower enterovirus activity (Roth *et al*., 2007). Nevertheless, a combination of cell culture methods and detection by RT-PCR is more sensitive for detection of enteroviruses than either method alone.

The three predominant genotypes identified were E30, E6, and E11, all of which are members of the species Human Enterovirus B (HEV-B). Members of HEV-B have been widely described as the most common cause of aseptic meningitis cases and outbreaks worldwide (Gharbi *et al*., 2006; Mirand *et al*., 2008; dos Santos *et al*., 2011). Some serotypes of enteroviruses are more commonly associated with aseptic meningitis than others. E6 and E30 were among the most frequently reported serotypes in the United States in 1970 to 2005 (Khetsuriani *et al*., 2006), and in Europe in 2000s (Antona *et al*., 2007; Blomqvist *et al*., 2008; Kapusinszky *et al*., 2010; Milia *et al*., 2013). E30 is the most commonly associated with aseptic meningitis. Increases in echovirus 30 activity are characterized by global spread and large-scale aseptic meningitis outbreaks (Martinez *et al*., 2012; Hyeon *et al*., 2013; Xiao *et al*., 2014; Nougairede *et al*., 2014).

This study focused on EV epidemiology in Poland over a 4-year period. The results described in this study provide valuable information on the circulation of different EV types in the context of limited EV surveillance in Poland. Enterovirus surveillance is important not only for monitoring the changing epidemiology these infections but also for the rapid identification of spread of emerging EV types.

Acknowledgments

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