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Original Article

Diagnosing Enteroviral Meningitis Using Real-time RT-PCR with Cerebrospinal Fluid and Stool Specimens

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Abstract Enteroviral infections are common among children and are a main cause of meningitis. Cerebrospinal fluid (CSF) analysis is important for diagnosing meningitis, although CSF sampling is difficult and time-consuming. In contrast, stool testing provides simpler sampling and a higher positive rate. We performed polymerase chain reaction testing for paediatric patients (≤18 years old) who were admitted to Cheonan Dankook University Hospital during 2011-2015 for suspected meningitis (942 patients, 1,884 specimens). The stool specimens exhibited the highest positive rate, and 114 patients exhibited positive CSF and stool specimens. Fourteen patients had positive CSF specimens and negative stool specimens, while 101 patients had positive stool specimens and negative CSF specimens. CSF analysis combined with stool testing may provide a more efficient and accurate diagnosis of enteroviral meningitis, compared to only CSF or stool testing.

Key words Enterovirus; Meningitis; Polymerase chain reaction

Introduction

Enteroviruses (EV) belong to the *Picornaviridae* family and exhibit >70 different serotypes, which include coxsackieviruses.[1] In Korea, EV infections typically occur between early spring and summer.[2,3] Large-scale EV infections have been reported worldwide,[4] although EV infections occur more frequently in children, compared to adults.[5] Once infected, the patient may exhibit various clinical symptoms that range from mild upper respiratory tract infection and Guillain-Barre syndrome to severe symptoms of paralysis that are associated with transverse myelitis.[6,7] Furthermore, EV infections are a major source of central nervous system infections in children and infants.[8] Moreover, 85% of aseptic meningitis cases are caused by EV.[7,9,10] However, it is difficult to differentiate between bacterial meningitis and viral meningitis that is caused by the herpes simplex virus, and this difficulty can result in unnecessary hospitalisation, excess treatment costs, and antibiotic misuse.[11] Thus, it is important to accurately diagnose the meningitis, in order to prevent unnecessary diagnostic testing and treatment.[12]

The traditional method for confirming a diagnosis of aseptic meningitis is cerebrospinal fluid (CSF) analysis and culture to detect the virus. However, this procedure is invasive, can cause patients anxiety,[13] requires a prolonged detection period, and can prevent early diagnosis and treatment based on the difficulty of obtaining CSF culture results. In addition, low virus titers are common in clinical specimens, which can create low sensitivities for many

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serotypes, including the group A coxsackieviruses.[14-16] Thus, polymerase chain reaction (PCR) testing has been developed as an accurate and rapid diagnostic test for aseptic meningitis.[17] Both CSF and stool specimens are typically used for PCR testing, as a CSF specimen is essential for diagnosis,[18] although it has a lower positive rate than PCR stool testing[2,18] and has been associated with diagnostic issues that are related to the sampling period.[18] Thus, it is difficult to confirm a diagnosis using PCR with only a CSF specimen. Moreover, very few studies have directly compared the efficacy of single or repeated testing. Therefore, the present study aimed to evaluate the usefulness of real-time reverse-transcription PCR (real-time RT-PCR) with CSF and stool specimens that were obtained from children at our center during the last 5 years. We believe that the results of this analysis may provide the basis for fast and accurate diagnosis of aseptic meningitis.

Methods

1. Patients

This retrospective study analysed real-time RT-PCR results from CSF and stool specimens that were being tested for EV infection. The specimens were obtained from children (≤18 years old) who were admitted to Cheonan Dankook University Hospital for suspected aseptic meningitis between January 2011 and October 2015. This study received ethical approval from the Dankook University institutional ethics review board (2015-11-011).

2. Experimental Methods

Specimens were collected from patients with symptoms of meningitis during a single hospital admission. CSF specimens were collected via lumbar puncture, and stool specimens were also collected at approximately the same time. The specimens were subjected to same-day nucleic acid extraction, or were stored at 4°C for next-day nucleic acid extraction. The CSF and stool nucleic acid extractions were performed using an automated QIAcube system with the QIAamp DNA Mini Kit (Qiagen, Hilden, Germany), according to the manufacturer's protocol. The real-time RT-PCR was performed using 40 μL of the eluent from the QIAamp Spin column. The extracted RNA was dissolved in 50 μL of nuclease-free water and stored at -80°C until testing.

The real-time RT-PCR assay was performed according to the manufacturer's instructions using an AccuPower® Enterovirus real-time RT-PCR Kit (Bioneer, Daejeon, Korea), which contains primers and probes for the highly-conserved 5'-nontranslated region of the human EV genome. Positive specimens were subjected to semi-nested RT-PCR in the VP1 coding region for molecular typing, as previously described,[19] and the VP1 amplicons were sequenced using internal primer sets.

All data were expressed as median and range. The chi-square test was used to analyse categorical data. A p-value of <0.05 was considered statistically significant.

Results

A total of 942 patients were included in this study, and both CSF and stool specimens were obtained from each patient (total: 1,884 specimens). Among the 942 patients, 229 patients (24.3%) tested positive for EV, 128 CSF specimens tested positive, and 215 stool specimens tested positive (Table 1). Male patients were most likely to exhibit positive CSF and stool results (Figure 1), although the difference was not statistically significant (p=0.23). The average age was 1.87 years, and children who were <1 year old accounted for 49.2% of the CSF specimens and 54.4% of the stool specimens; similar results were observed for patients who were <1 year old and ≥1 year old (Figure 2). During the 5-year study period, the lowest number of positive specimens was detected during 2012, and the highest numbers were detected during 2011 and 2015 (Figure 3). Monthly comparisons revealed that the positive rates were highest during June-August, and a gradual decline was observed during late fall.

A comparison of the CSF and stool specimen results revealed that 713 patients had negative results for both specimens, and 114 patients had positive results for both specimens. Fourteen patients had a positive CSF specimen and a negative stool specimen, and 101 patients had a

Table 1 Analysis of the PCR results

		Positive		
		Patients (%)	CSF specimens (%)	Stool specimens (%)
All patients	942	229 (24.3)	128 (13.6)	215 (22.8)
Male patients	549	138 (25.1)	72 (13.1)	131 (23.9)
Female patients	393	91 (23.2)	56 (14.3)	84 (21.4)

PCR: polymerase chain reaction; CSF: cerebrospinal fluid

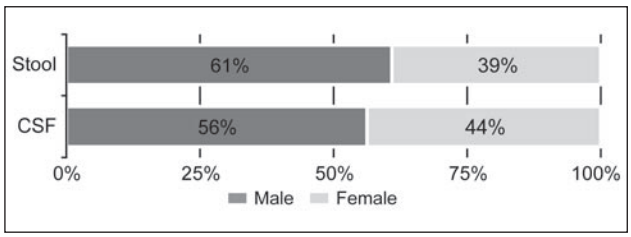

Figure 1 Sex ratios for positive cerebrospinal fluid (CSF) and stool specimens.

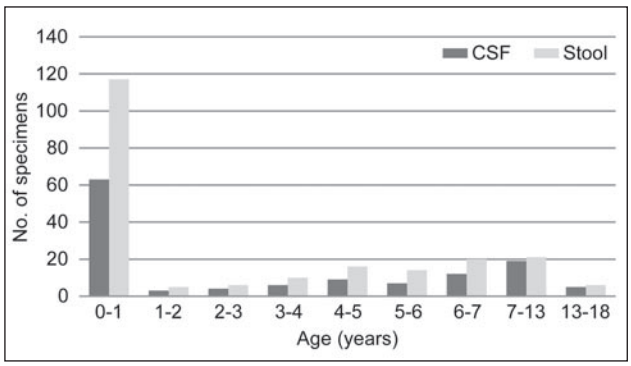

Figure 2 Positive specimen rates according to age group.

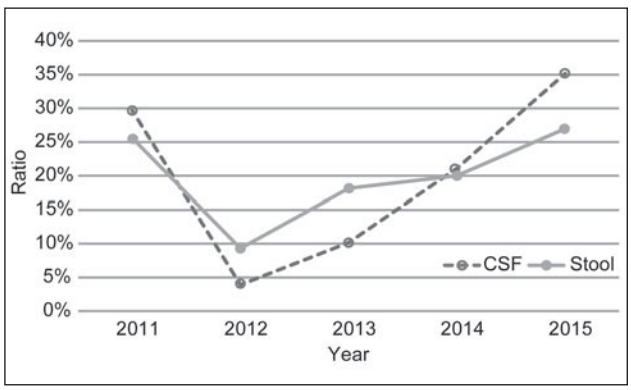

Figure 3 Annual positive rates for cerebrospinal fluid (CSF) and stool specimens.

Table 2 Comparing the CSF and stool results

		Stool	
		Positive	**Negative**
CSF	Positive	114	14
	Negative	101	713

CSF: cerebrospinal fluid

negative CSF specimen and a positive stool specimen (Table 2). A higher positive rate was observed among stool specimens, compared to CSF specimens (Figure 4).

Discussion

In the present study, we analysed the use of CSF and stool specimens for real-time RT-PCR testing to diagnose aseptic meningitis that was secondary to EV infection. Both the CSF and stool results revealed that male patients had a higher positive rate, compared to female patients, although this difference was not statistically significant (p=0.23). Similarly, previous studies have reported higher positive rates among male patients (vs. female patients),[18,20,21] and some have even reported that the positive rate was two-fold higher among male patients.[21] This difference may be related to sex-based differences in immunity[22] that arise from differences in genetic and endocrine functions.[23]

When we compared the annual positive rates for EV during the past 5 years, both the CSF and stool results exhibited similar increasing and decreasing trends. For example, the highest positive rates were observed in 2011 and 2015. Moreover, examination of the entire 5-year period revealed that the EV infection rate appeared to exhibit a significant quadrennial increase. This is similar to a previous study that found that a national EV pandemic occurred approximately every 3 years.[20]

The monthly EV positive rates during the 5-year period revealed that positive CSF and stool specimens were most frequently detected during June-August, and that the number of positive specimens gradually decreased during the fall. This result is similar to the findings of a study regarding EV infection in Chungnam during 2005-2006,[24] and is also

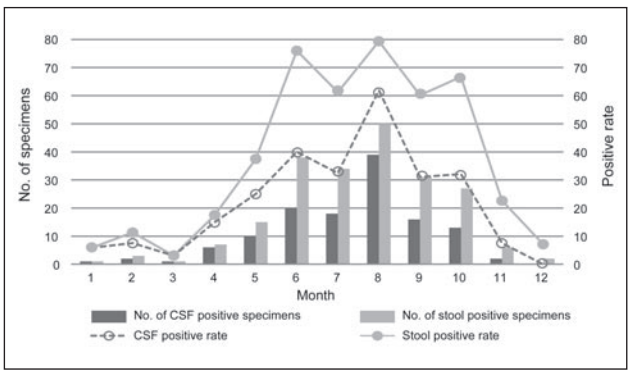

Figure 4 Monthly numbers of positive specimens.

consistent with findings from other studies that reported higher frequencies of EV infection in the summer and fall.[19,25,26] These findings are likely related to the high temperature and humidity during the summer, as previous studies have found that EV infection is common during the summer and fall in temperate regions, but occurs year-round in tropical regions.[21] Moreover, this trend for EV infections was also observed during 2007-2009.[8] Positive CSF and stool specimens were also most frequent among patients who were <1 year old, and this finding is similar to those of past studies,[18,24] which suggests that EV infection is more likely among younger patients.

Stool specimens were more frequently positive for EV, compared to CSF specimens. Furthermore, there were a greater number of cases with negative CSF specimens and positive stool specimens, compared to cases with positive CSF specimens and negative stool specimens. These results suggest that stool testing was associated with a higher positive rate, compared to CSF testing. However, there is controversy regarding whether CSF and/or stool specimens should be used to diagnose aseptic meningitis, as varying positive rates are observed in clinical practice. For example, one study found a higher positive rate in stool specimens, compared to CSF specimens,[18,27,28] and our findings validate that difference. This difference may be explained by the nature of EV, which is stable in an acidic environment and easily reaches the lower gastrointestinal tract.[21] Thus, once an infection has developed, it may take 4-8 weeks (or even up to 11 weeks) for the virus to be cleared from the stool.[28] Moreover, other studies have suggested that there is a high possibility of past EV infections causing aseptic meningitis, based on the prolonged clearance time, although it is difficult to evaluate this theory.[18]

Kim et al have reported that there is a sharp decline in positive rates that is based on the sampling time.[18] For example, positive RT-PCR results were observed in approximately 50% of their specimens that were obtained within 2 days, compared to 4.2% in specimens that were obtained after 2 days. In contrast, stool specimens provided an average positive rate of 90.5%, even after 7 days. This may be because EV meningitis is caused by viremia during the early EV infection period, and viral detection in CSF specimens may only be possible during a short period after the infection. Moreover, EV replication persists in the gastrointestinal tract after infection, which led those authors to conclude that sampling time was an important factor.[18]

This study has several limitations that warrant consideration. First, we only analysed results from a single institution in the Cheonan area during 2011-2015. Second, we used a retrospective design, which is associated with several well-known risks of bias. Third, it is possible that we did not identify all cases of EV infection, based on potential procedural variations that may have introduced RNA denaturation during the specimen freezing and thawing.[29]

In conclusion, our comparison of real-time RT-PCR results from CSF and stool specimens during the past 5 years revealed similar trends in the sex-specific, annual, and monthly positive rates. Furthermore, we did not observe any significant source-specific differences, unlike the differences in the age-specific positive rates (highest among patients who were <1 year old). Thus, we propose that the co-analysis of CSF and stool specimens during the early disease stage may provide a faster and more accurate diagnosis. This is especially important because differences in the source-specific positive rates and sampling times may complicate the diagnosis of EV infection. Therefore, our results may help to reduce the extended hospitalisation, medical costs, and antibiotic misuse that are related to incorrect diagnosis in cases of EV infection.

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Declaration of Interest

The authors have no conflicts of interest.

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