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Prospective research of human parechovirus and cytokines in cerebrospinal fluid of young children less than one year with sepsis-like illness: Comparison with enterovirus

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ABSTRACT

Background: Human parechovirus (PeV) and enterovirus are important pathogens that cause viral infection and aseptic meningitis in young children. We aimed to investigate the rate of HPeV and enterovirus detection, and to characterize cytokine profiles in the cerebrospinal fluid (CSF) of young infants with sepsis-like illness or meningitis/encephalitis.

Study design: This was a prospective cohort study. CSF samples were collected from 90 infants less than 1 year of age. PeV and enterovirus detection was performed using reverse transcription polymerase chain reaction. Fifteen cytokines in the CSF were measured simultaneously by using multiplex immunoassays.

Results: PeV (PeV-group) and enterovirus (EV-group) were detected in 10 (11.1%) and 12 (13.3%) CSF samples, respectively. Other aseptic meningitis (AM-group) was diagnosed in 22 (24.4%) patients. Forty-six (51.1%) patients exhibited non-central nervous system infection (Ngroup). The PeV-group had the lowest CSF leukocyte (2.1 ± 3.5/mm3, p = 0.022) and blood leukocyte (7,953 ± 4,583/mm3, p = 0.046) count and Creactive protein levels (0.2 ± 0.1 mg/dL, p = 0.036), than did those in the EV- and AM-groups. CSF leukocyte count and protein levels were not significantly different between the PeV- and N-groups. The levels of interleukin (IL)-1β, IL-5, IL-6, IL-12, and IL-17 were higher in the EVgroup; conversely, IL-2, IL-4, IL-7, and IL-13 were higher in the PeVgroup.

Conclusions: Examinations to detect PeV in the CSF may help identify the etiological basis of undiagnosed febrile illness in young children. Significant differences in CSF and blood laboratory findings were observed between PeV- and enterovirus-infected children.

1. Background

Meningitis is one of the most common central nervous system (CNS) disorders of childhood; this disease leads up to 12% of deaths, global, in children of < 14 years of age [1]. The majority of aseptic meningitis is viral infections; most are caused by enteroviruses (EVs) and human parechoviruses (PeVs) [2]. The clinical presentation and populations affected by these two pathogens overlap to such an extent that both infections are considered in the differential diagnosis for neonates and infants with symptoms of a CNS infection or sepsis [3,4]. This is most likely due to the growing recognition of PeV as a potential cause of sepsis and febrile seizures from recent investigations of outbreaks [5]. Clinical manifestations of PeV meningitis in infants and young children are diverse, including sepsis-like illness, meningitis, or a nonspecific

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febrile illness [6]. However, it's not been studied whether the blood and cerebrospinal fluid (CSF) findings between PeV meningitis and non-CNS febrile illness may have any difference or not.

Developments of sensitive and specific molecular tests for PeV RNA have recently improved PeV detection and typing, and better understanding of PeV epidemiology. The mainstream of PeV infections occur very early in life, mostly before 1 year of age [4,7]. However, thus far, few clinical laboratories have applied PeV molecular tests as routine tests in combination with EV [7,8]. The absence of CSF pleocytosis is nearly common (up to 90%) in patients with PeV-3 [8]. Data from the literature advocates that the release of specific cytokines, such as interleukin (IL)-1, IL-8, tumor necrosis factor (TNF)-α, and interferon (IFN)-γ, may be responsible for meningeal inflammatory infiltration in bacterial and aseptic meningitis [9]. In viral meningitis, proinflammatory cytokines, including IL-6, IL-8, and IFN-γ, were detected at relatively higher levels in the CSF [10]. Wang et al revealed a significant association between CSF levels of IL-1β, IL-6 and IFN-γ and the severity of enterovirus 71 brainstem encephalitis, and IFN-γ seems to play a more prominent role in the overwhelming disease process caused by enterovirus 71 [11]. It has not yet been documented in the literature that cytokine profiles in CSF of infants with PeV or EV meningitis may have any different values.

The aim of this study was to investigate the rates of PeV and EV detection in CSF of young infants with symptoms of sepsis-like illness or meningitis/encephalitis, and to compare the clinical and laboratory findings and CSF cytokine profiles of PeV meningitis with those of EV meningitis, other aseptic meningitis, as well as non-CNS febrile illness.

2. Study design

2.1. Participants

A prospective cohort study from a single center was conducted at the emergency department affiliated with Pusan National University Children's Hospital. The study was performed between March 2016 and February 2017. Criteria for inclusion were neonates and infants aged 0–12 months, with fever (≥38 °C) and clinical signs and symptoms of suspected sepsis or meningitis/encephalitis (headache, irritability, lethargy, nausea/vomiting, nuchal rigidity, hypothermia, poor feeding, and/or rash). Exclusion criteria were as follows: (a) combined severe poor condition (e.g., shock, severe malnutrition, congestive heart failure, or renal failure), (b) prior diagnosis of chromosomal anomaly, inborn error of metabolism, or epilepsy, and/or (c) chronic intake of other medication(s) within the prior 3 months.

This study was approved by the Institutional Review Board of Pusan National University Yangsan Hospital (04-2015-009). Informed consent was obtained from the parent or guardian of each participant included in the study.

2.2. Sample acquirement

Following enrollment in the study, blood, urine, and nasal specimens were collected for laboratory studies, bacterial and viral culture, and reverse-transcriptase real-time polymerase chain reaction (RT-PCR) for herpes simplex virus-1 and 2, Epstein-Barr virus, cytomegalovirus, rubella virus, and *Mycobacterium tuberculosis*. A lumbar puncture (LP) was performed to obtain CSF of the neonate/infant. Routine CSF analysis was performed, including white blood cell (WBC) count, glucose and protein levels, bacterial and viral cultures, and RT-PCR for EV, PeV, herpes simplex virus-1 and 2, and *M. tuberculosis*. CSF pleocytosis was defined as the presence of elevated WBC count for the age in the CSF (patient's age of < 4 weeks, WBC of > 22/mm^3; 4–7 weeks, > 15/mm^3; ≥ 8 weeks, > 5/mm^3) [12]. CSF samples contaminated with red blood cells were excluded. The remaining CSF samples were centrifuged and frozen for subsequent use in this study. None of EV- and PeV-positive samples underwent additional genotyping. We considered patients with CSF RT-PCR-positive results for PeV or EV to have PeV or EV meningitis, respectively. Patients with lack of CSF pleocytosis and all RT-PCR negative results were defined as non-CNS infection.

2.3. RT-PCR

GeneXpert Enterovirus Assays (GXEA, Cepheid, Sunnyvale, California, USA) [13] were used for EV RT-PCR assays. The primers and probes were from the portion of the EV genome that codes for the 5′-nontranslated region. Primer sequences in GXEA were 5′-CCC TGA ATG CGG CTA ATC C-3′(F) and 5′-ATT GTC ACC ATA AGC AGC CA-3′(R). Probe sequence was 5′-AAA CAC GGA CAC CCA AAG TAG TCG G-3′. The sensitivity for EV detection of GXEA has been found to be 98 − 100% [13]. ViroReal® Parechovirus (Ingenetix, Vienna, Austria) kits is based on the amplification and detection of the polyprotein gene of PeV using RT-PCR (detection in FAM channel, 530 nm) [14]. PCR-platforms were performed in the ABI PRISM® 7500 instrument (Applied Biosystems), Mx3005P® QPCR System (Agilent) and LightCycler® 1.2/1.5/2.0/480 instruments (Roche). ViroReal® Parechovirus has a sensitivity of about 10–20 RNA copies/PCR and is specific for human parechovirus [14]. For the diagnosis of meningitis, PeV RT-PCR has revealed a sensitivity of 100% with CSF, stool, and blood samples [15].

2.4. Multiplex immunoassay (CSF cytokine analysis)

The levels of CSF cytokines/chemokines were measured simultaneously for 15 different cytokines and chemokines: IL-1β, IL-2, IL-4, IL-5, IL-6, IL-7, IL-8, IL-10, IL-12 (p70), IL-13, IL-17,IFN-γ, TNF-α, granulocyte colony-stimulating factor (G-CSF), and granulocyte monocyte colony-stimulating factor (GM-CSF). All concentrations were determined using the Bio-Plex Cytokine Assay System (Bio-Rad Laboratories Inc., Hercules, CA, USA), in accordance with the manufacturer's instructions. A total of 50 μL of each CSF supernatant, along with cytokine standards (Bio-Rad), were added to a 96-well plate. The plate was analyzed using the Bio-Plex Array Reader (Bio-Rad). The cytokine levels were calculated with reference to the standard curves for each cytokine. We used the Bio-plex Manager 4.0 software (Bio-Rad) for analysis; we defined the target values as within 10% for the intra-assay coefficients of variation. We performed concurrent assays of all samples to avoid inter-assay variability.

2.5. Data collection

Clinical and laboratory data were obtained from the medical records, which included sex, age, symptoms and signs, interval period from the onset of initial symptoms to LP, results of blood and CSF studies, results of virological testing of the CSF, and neurological outcomes.

2.6. Statistical analysis

Statistical analyses were performed using SPSS 19.0 software (SPSS, Inc., Chicago, IL, USA) with raw scores. The results of the immunoassay measurements were presented as median, minimum, and maximum levels; nonparametric tests were used for statistical analysis. A two-tailed chi-squared test or the Fisher's exact test was used for the analysis of categorical data. For comparisons between the two groups, Student's *t*-test was used for comparison of continuous variables with normal distributions, while the Mann-Whitney test was used for comparison of continuous variables without a normal distribution. Analysis of variance (ANOVA) was used to compare continuous variables among three groups. In all analyses, p-values <0.05 was considered to indicate statistical significance.

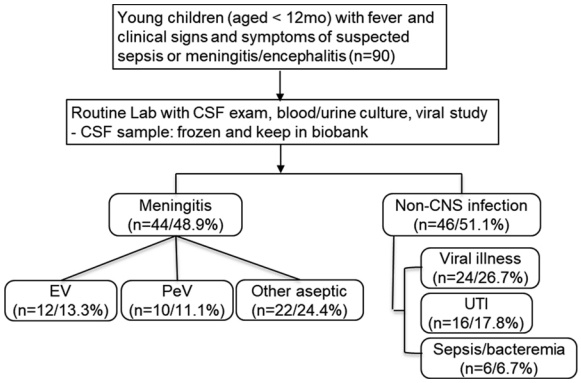

Fig. 1. Patients enrollment and proportions of causative disorders. (EV, enterovirus; PeV, human Parechovirus; CNS, central nervous system; UTI, urinary tract infection).

3. Results

3.1. Demographic and laboratory data

The patient population was divided into four groups by their clinical diagnosis (Fig. 1). Of these 90 patients, 44 (48.9%) exhibited meningitis and 46 (51.1%) exhibited non-CNS infection (N-group). Of the 44 patients with meningitis, 12 (13.3%) were in the EV-group (EV meningitis), 10 (11.1%) were in the PeV-group (PeV meningitis), and 22 (24.4%) were in the AM-group (clearly demonstrated by CSF analysis, although specific viral etiologies were not confirmed in the AM samples). Virus isolation by CSF culture was demonstrated in 58.3% (7/12) of EV-group and none of PeV-group. Final diagnoses for the N-group included systemic viral illness (respiratory or gastrointestinal infection, n = 24), *Escherichia coli* urinary tract infection (n = 16), and sepsis/bacteremia of unknown origin (n = 6).

The proportion of boys in the three groups of meningitis patients was similar (Table 1). The mean age did not significantly differ among the AM-, EV-, and PeV-groups (2.5 ± 1.3 vs. 2.1 ± 0.9 vs. 2.1 ± 1.0 months, $p = 0.606$). A history of fever was noted in all patients, consistent with the inclusion criteria of this study. Subjective symptoms, such as headache, were detected by their verbal expressions or behavior (touching their head); these were present in one patient in the AM-group. Notably, seizures were observed in two patients in the AM-group (2/22, 9.1%).

The mean interval period from the onset of symptoms to LP (2.0 ± 2.4 vs. 1.3 ± 0.6 vs. 1.0 ± 0.5 days, $p = 0.493$) was similar among the three groups. Patients in the PeV-group had the lowest CSF leukocyte count (2.1 ± 3.5/mm^3); these significantly differed from those in the AM- and EV-groups (17.7 ± 24.1/mm^3, $p = 0.019$; 11.9 ± 29.8/mm^3, $p = 0.048$) (Table 1). CSF non-pleocytosis was observed in 90% (9/10) of patients in the PeV-group and 83.3% (10/12) of patients in the EV-group ($p = 0.723$). The mean level of CSF protein was the lowest in the PeV-group (40.5 ± 14.2 mg/dL), not significantly different from those in the AM- and EV-groups (78.4 ± 89.0 mg/dL, $p = 0.487$; 52.6 ± 20.3 mg/dL, $p = 0.967$). PeV-group also showed the lowest peripheral leukocyte count (7953 ± 4,583/mm^3) and C-reactive protein (CRP, 0.2 ± 0.1 mg/dL) levels; these significantly differed from those in the AM- (12,817 ± 6,308/mm^3, $p = 0.042$; 1.5 ± 1.1 mg/dL, $p = 0.045$) and EV-groups (9675 ± 6,154/mm^3, $p = 0.048$; 1.8 ± 2.3 mg/dL, $p = 0.042$) (Table 1).

The demographic and clinical features of both PeV- and EV-groups did not significantly differ from those of the N-group, with the exception of mean age (PeV vs. N, 2.1 ± 1.0 vs. 3.0 ± 2.1 months, $p = 0.044$; EV vs. N, 2.1 ± 1.0 vs. 3.0 ± 2.1 months, $p = 0.045$). The mean peripheral leukocyte count (7953 ± 4,583/mm^3 vs. 13,883 ± 7,636/mm^3, $p = 0.022$) and CRP levels (0.2 ± 0.1 vs. 4.5 ± 5.8 mg/dL, $p < 0.001$) were significantly lower in the PeV-group than in the N-group. CSF leukocyte count and protein levels were not significantly different between the PeV- and N-groups. EV-group had significant lower CRP levels (1.8 ± 2.3 vs. 4.5 ± 5.8 mg/dL, $p = 0.016$) and higher CSF leukocyte count (11.9 ± 29.8/mm^3 vs. 2.2 ± 2.7/mm^3, $p = 0.048$) than those of the N-group (data not shown).

3.2. Cytokine profile of cerebrospinal fluid

Among the three groups of meningitis patients, the levels of IL-1β, IL-2, IL-4, IL-5, IL-6, IL-7, IL-12, IL-13, IL-17, and GM-CSF significantly differed in CSF, particularly between the EV-group and each of the other two groups (EV vs. PeV, and EV vs. AM) (Fig. 2). Of these 10 cytokines, IL-1β, IL-5, IL-6, IL-12, IL-17, and GM-CSF were significantly higher in the CSF of the EV-group than in the CSF of the PeV- or AM-groups; conversely, IL-2, IL-4, IL-7, and IL-13 were significantly higher in the CSF of the PeV- or AM-groups, compared with the CSF of the EV-group. Levels of IL-8, IL-10, IFN-γ, TNF-α, and G-CSF were similar among the three groups. All examined cytokines levels were similar in both PeV- and AM-groups (Fig. 2).

Furthermore, most CSF cytokine profiles were similar between PeV- and N-groups. Levels of IL-7 and IL-17 were significantly higher in the CSF of the PeV-group than in that of the N-group, whereas the level of G-CSF was significantly lower in the CSF of the PeV-group than in that of the N-group. Between EV- and N-groups, the levels of IL-1β, IL-5, IL-

Table 1
Comparison of demographic and laboratory data among neonates and infants with central nervous system infections.

	AM (n = 22)	EV (n = 12)	PeV (n = 10)	*p* (PeV vs AM)	*p* (PeV vs EV)	*p* (EV vs AM)	*p* (all)
Boy	17 (77.3%)	7 (58.3%)	7 (70.0%)	0.660	0.571	0.247	0.512
Age (months)	2.5 ± 1.3	2.1 ± 0.9	2.1 ± 1.0	0.658	0.988	0.730	0.606
Symptoms							
Fever	22 (100.0%)	12 (100.0%)	10 (100.0%)	–	–	–	–
Headache	1 (4.5%)	0 (0.0%)	0 (0.0%)	0.49	–	0.45	0.600
Vomit	2 (9.1%)	1 (8.3%)	1 (10.0%)	0.93	0.89	0.94	0.991
Seizure	2 (9.1%)	0 (0.0%)	0 (0.0%)	0.32	–	0.28	0.351
Interval period	2.0 ± 2.4	1.3 ± 0.6	1.0 ± 0.5	0.259	0.915	0.450	0.493
CSF exam							
WBC (/mm^3)	17.7 ± 24.1	11.9 ± 29.8	2.1 ± 3.5	0.019*	0.048*	0.076	0.022*
Protein (mg/dL)	78.4 ± 89.0	52.6 ± 20.3	40.5 ± 14.2	0.487	0.967	0.623	0.441
Glucose	61.8 ± 11.1	61.2 ± 6.8	60.2 ± 10.1	0.904	0.972	0.982	0.912
Glucose ratio	0.6 ± 0.2	0.6 ± 0.1	0.6 ± 0.1	0.823	0.993	0.727	0.701
Lab (blood)							
WBC (/mm^3)	12817 ± 6308	9675 ± 6154	7953 ± 4583	0.042*	0.048*	0.211	0.046*
CRP (mg/dL)	1.5 ± 1.1	1.8 ± 2.3	0.2 ± 0.1	0.045*	0.042*	0.846	0.036*

AM: patients with other aseptic meningitis, EM: patients with enteroviral meningitis, PeV: patients with human parechovirus meningitis, CSF: cerebrospinal fluid, WBC: white blood cell, CRP: C-reactive protein. *$p < 0.005$.

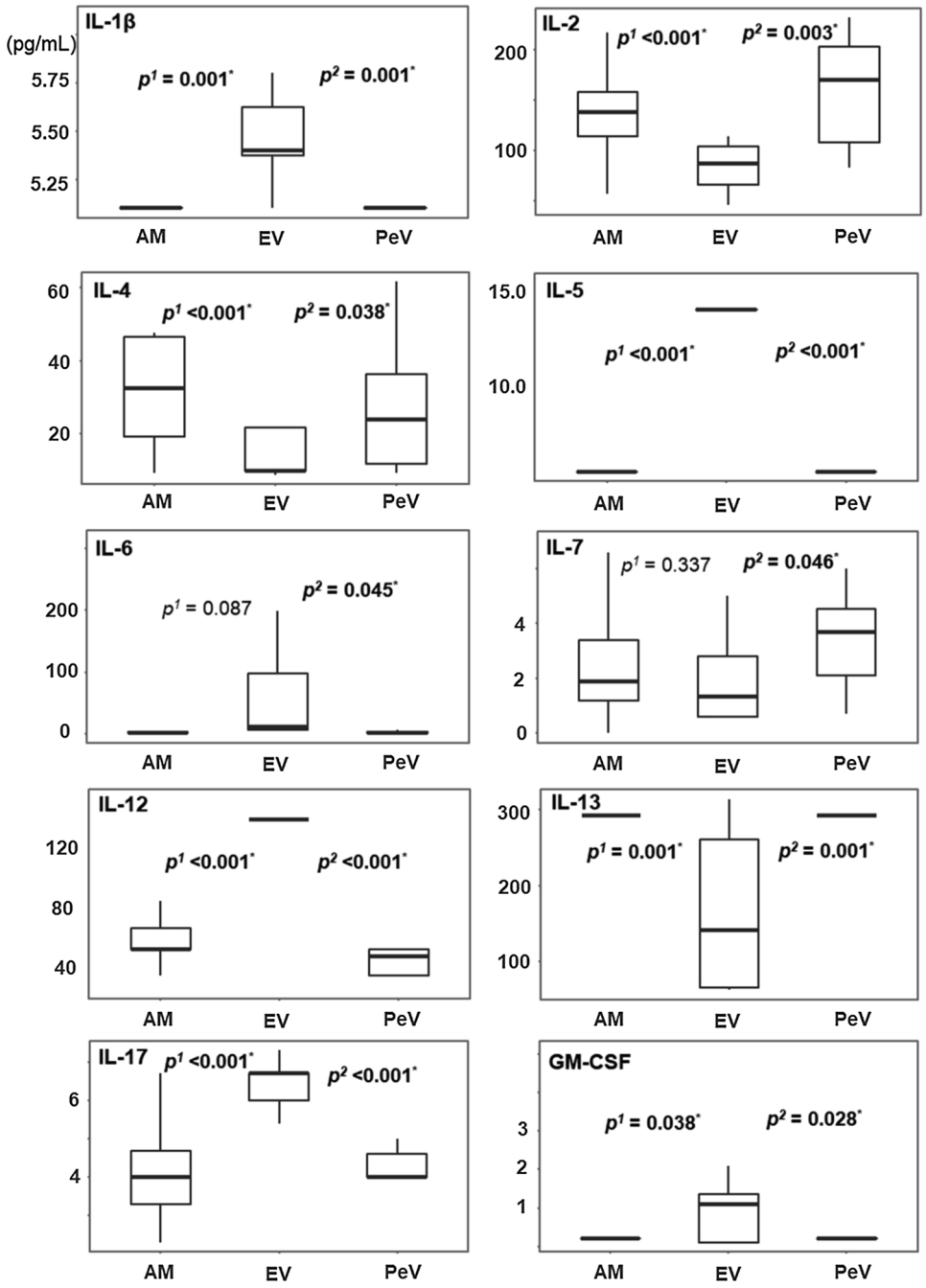

Fig. 2. Comparison of cerebrospinal fluid cytokine profiles among children with enteroviral meningitis (EV), human parechovirus meningitis (PeV), and other aseptic meningitis (AM) (p^1 = AM vs. EV; p^2 = EV vs. PeV; *$p < 0.05$). (GM-CSF, granulocyte monocyte colony-stimulating factor; IL, interleukin).

6, IL-12, IL-17, and GM-CSF were significantly higher in the CSF of the EV-group than in that of the N-group, whereas the level of IL-2, IL-4, and IL-13 were significantly higher in the CSF of the N-group than in that of the EV-group (data not shown).

4. Discussion

In this study, we investigated the rates of PeV and EV infection and characterized the CSF cytokine profiles of 90 young children (<12 months of age) with sepsis-like illness. PeV and EV were detected in 10 (11.1%) and 12 (13.3%) CSF samples, respectively. The PeV-group had the lowest levels of CSF leukocytes (2.1 ± 3.5/mm^3, p = 0.022), blood leukocytes (7953 ± 4,583/mm^3, p = 0.046), and CRP (0.2 ± 0.1 mg/dL, p = 0.036); these were significantly different from those of EV- and AM-groups. Levels of CSF leukocytes and protein were not significantly different between the PeV- and N-groups. The levels of IL-1β, IL-5, IL-6, IL-12, and IL-17 were higher in the EV-group than in the PeV-group; conversely, most CSF cytokine profiles were similar between the PeV- and N-groups. This is one of few studies involving analysis of CSF cytokines and clinical features between pediatric patients with PeV and EV infections [16].

The incidence of PeV infection has been underrated; however, recent data show that PeVs are increasingly recognized as a causative factor in meningoencephalitis in children [17,18]. The 11.1% rate of PeV detection in CSF of young infants observed in this study is similar to that in previous research [4,7,18]. Remarkably, a lack of CSF pleocytosis in PeV meningitis frequently occurs [19]. The present study found that CSF non-pleocytosis was frequently present in PeV- and EV-

groups (90% and 83.3%, $p = 0.723$). Verboon-Maciolek et al. [3] observed significantly higher serum CRP and CSF protein levels in 21 infants with EV infection than in 11 children with PeV infection. We revealed that blood CRP levels were lower in the PeV-group than in the EV-group; the mean CSF protein level in the PeV-group was normal.

Whereas the patient population and clinical symptoms are similar for these two picornavirus infections (EV and PeV), their pathogenicities and interactions with the immune system may differ. EV infects a variety of CNS parenchymal cells, including neurons [20]. The pathogenesis of PeV-3 CNS infections is not well understood; however, recent studies have identified PeV-3 in the smooth muscle cells of leptomeningeal blood vessels [21]. Limitation of CNS PeV infection to smooth muscle cells of leptomeningeal blood vessels may alter CNS pathways of immune activation in the presence of PeV infection, as these viruses are known to infect neural-glial tissue [20,21]. Separate tissue tropisms for PeV and EV may play a role in the immune responses to these viruses; for PeVs, there are no significant increases in levels of IFN or downstream type I IFN pathway effectors. Most chemokines/cytokines that were elevated in the CSF of the EV group remained within the reference range in the CSF of PeV positive patients, including IL-15, fractalkine/CXCL1, IP-10/CXCL10, IFN-2, and IL-1R [19]. Whereas the mechanisms of PeV immune elusion are not understood, many PeV-mediated processes may be involved in producing the markedly muted innate immune response to PeV, compared with EV [22]. Our study also showed that CSF cytokine profiles significantly differed between EV- and PeV-groups, although CSF non-pleocytosis was frequently observed in both groups. Various cytokines were elevated in the CSF of the EV-group, compared with those of the PeV-group. Moreover, most cytokine levels of patients in the PeV-group were similar to those of patients in the N-group. This inconsistent PeV immune profile can potentially lead to diverse clinical ranges, which can be difficult to diagnose. Additionally, in vitro studies have indicated that PeV-3 has specific neuronal tropism [23]. This may explain the mechanisms by which PeV-3 CNS infection results in tissue damage, CSF non-pleocytosis, and relatively low viral loads in CSF [24].

This study has several limitations. First, it involved a relatively low number of patients. Second, the levels of CSF cytokine profiles were not investigated with respect to the EV or PeV genotypes due to the limitation of the amount of CSF, which precludes a correlation between specific genotypes and cytokine expression. Third, the serum fecesRT-PCR for EV and PeV were not routinely tested. We had a difficulty in collecting their feces during hospitalization. However, CSF, blood, and feces have the highest sensitivity for detecting an EV or PeV [15]. Finally, the methods used may have caused a selection bias in the observed cytokine/chemokine profiles. Additional chemokines, known as major attractants of immune cells to sites of infection (e.g., CCL2, CCL3, CCL5, and CXCL10) were not investigated.

In our experience with infants admitted for sepsis-like illness or meningitis/encephalitis, the detection of PeV CNS infection is not uncommon, despite a lack of CSF pleocytosis and the presence of normal serum laboratory findings. These data support the need for a focus on PeV testing in young infants. While no specific therapy is available, testing for PeV in CSF obtained from young children who present with unusual fever and acute illness is worth consideration, even if typical markers of sepsis are practically normal. Discovery of PeV in such clinical situations with unknown etiologies could reduce the lengths of hospital stays, reduce usage of antibiotics, and minimize overall care expenses.

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We have no financial relationships relevant to this article.

Author contribution

Yun-Jin Lee: Conceptualization, Methodology, Data curation, Formal analysis, Writing original draft, Review & Editing, Supervision, Project Administration; Su Eun Park: Conceptualization, Methodology, Data curation, Writing original draft, Review & Editing, Project Administration; Duyeal Song: Methodology, Investigation, Data curation, Writing original draft, Review & Editing, Project Administration; Kyunghwa Shin: Methodology, Investigation, Formal analysis, Review & Editing; Sang Ook Nam: Data curation, Review & Editing, Supervision, Project Administration; Ara Ko: Data curation, Formal analysis, Review & Editing, Supervision; JuHyun Kong: Data curation, Review & Editing, Supervision; Young Mi Kim: Resources, Writing Review & Editing, Supervision; Gyu Min Yeon: Resources, Writing Review & Editing, Supervision

Declaration of Competing Interest

No competing financial interests exist.

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