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Short communication

Detection and genetic characterization of enterovirus strains circulating among children with acute gastroenteritis in Japan during 2014–2016

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ABSTRACT

Of 972 stool samples collected from infants and children with acute gastroenteritis in six different regions (Hokkaido, Tokyo, Shizuoka, Kyoto, Osaka, and Saga) of Japan during 2-year period from July 2014 to June 2016, 63 samples (6.5%) determined to be positive for enterovirus by multiplex RT-PCR were subjected to genotype determination based on the partial VP1 region using the CODEHOP method. Sixty-two strains were succeeded in genotyping and seventeen EV types were identified. The majority of the studied strains belonged to EV-A (30 of 62; 48.4%) and EV-B (31 of 62, 50%) species, and the remaining strain was of the EV-D species. The most frequently detected type was Coxsackievirus A5 (CV-A5) in 2014–2015 while was CV-B5 in 2015–2016. This study provides an insight into the genetic diversity of EV with the predominance of EV-A and EV-B species in Japanese infants and children with acute gastroenteritis during 2014–2016.

List of abbreviations

Bp	Base pair
CODEHOP	Consensus degenerate hybrid oligonucleotide primer
CV	Coxsackievirus
EV	Enterovirus
RV	Rhinovirus
RT-PCR	Reverse transcription- Polymerase chain reaction

1. Introduction

Enterovirus (EV) belongs to the *Picornaviridae* family and consists of 13 species, 10 EV species (EV-A to -J) and 3 Rhinovirus (RV) species (RV-A to -C) (http://www.picornaviridae.com). Of these, seven distinct species comprising 4 EVs (EV-A to -D) and 3 RVs affect human. EV is a small, non-enveloped, single-stranded positive sense RNA virus composed of 4 structural proteins, namely VP1, VP2, VP3, and VP4. The VP1 is located at the external side and contains a type-specific antigenic neutralization site, the BC-loop (Reimann et al., 1991). Complete or partial sequencing of the VP1 has been widely used for the identification of EV types (Caro et al., 2001; Casas et al., 2001; Oberste et al., 1999a; Oberste et al., 1999b; Pallansch and Roos, 2007). Up to date, 118 types of human EV classified into EV-A, -B, -C, and -D species have been described (http://www.picornaviridae.com). Human EV has been associated with a wide variety of acute and chronic diseases which were related to disease on gastrointestinal or respiratory tracts and central nervous systems (Lee et al., 2013; Pallansch and Roos, 2007). Most infections are asymptomatic, however, EV can cause severe disease with high morbidity and mortality in children (Pallansch and Roos, 2007).

Acute gastroenteritis is one of the most common diseases in infants and children. Viruses such as rotavirus, norovirus, astrovirus, and adenovirus have been identified as the most common cause of a child diarrhea (Goodgame, 2001). EV infection on diarrheal disease and molecular characteristics of EV has been recently reported (Chansaenroj et al., 2017; Kumthip et al., 2017; Patil et al., 2015; Phan et al., 2005; Rao et al., 2013). The purpose of this study was to identify the circulating genotypes of EV isolated from children with acute gastroenteritis in Japan during 2014–2016 by genetic analysis.

2. Materials and methods

2.1. Clinical specimens

Of 972 stool samples collected from Japanese pediatric outpatients

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who had a clinical diagnosis of acute gastroenteritis aged from 2 months to 15 years in six prefectures including Hokkaido, Tokyo, Shizuoka, Kyoto, Osaka, and Saga during 2-year period from July 2014 to June 2016, 63 samples (6.5%) determined to be positive for EV by multiplex RT-PCR with a primer pair amplified the 5'UTR of EV genome were subjected for genotyping based on the partial VP1 region amplified by CODEHOP method (Nix et al., 2006; Thongprachum et al., 2017; Zoll et al., 1992). Of these, 36 samples (36/543, 6.6%) were collected from July 2014 to June 2015 and the remaining 27 samples (27/429, 6.3%) were from July 2015 to June 2016. In addition, the samples were also tested for other enteric viruses including rotavirus, norovirus, adenovirus, human astrovirus, sapovirus, Aichi virus, human parechovirus, cosavirus, bocavirus, and Saffold virus by multiplex RT-PCR (Thongprachum et al., 2017). The study was approved by the Ethics Committee of the Nihon University School of Medicine, Tokyo, Japan (No. 22–15 and No. 25-13-0).

2.2. Enterovirus genotyping and sequence analysis

EV genotyping was done by amplifying the VP1 region using the CODEHOP method and sequence analysis. In brief, after RNA extraction, synthesis of cDNA was conducted as previously described (Nix et al., 2006). First round PCR was performed using the primer pair 224 and 222 followed by a second PCR with the nested primer pair AN89 and AN88. After sequencing, the amplicon sequences were compared with the VP1 sequences of EV reference strains. For one sample negative with CODEHOP method, it was investigated by sequencing of the 5'UTR. The sequence data and phylogenesis were analyzed using BioEdit v7.0.5. A parsimony analysis was conducted using MEGA version 7 to determine the evolutionary relationship among studied sequences (Kumar et al., 2016). The nucleotide sequences of the EV strains described in this study have been deposited in GenBank under accession numbers: MF589244-MF589304.

3. Results and discussion

All the patients whose stool specimens showed positive for EV were infants and children aged from 3 to 101 months with the mean and median ages of 22.1 and 15 months respectively. More than a half of the patients (43 of 63; 68.3%) were from 6 to 24 months of age with the highest prevalence (28 of 63; 44%) in children aged from 12 to 24 months. These findings are slightly variable than the previous studies (Patil et al., 2015; Phan et al., 2005; Rao et al., 2013) where most of the EV infections were less than 12 months of age. This variation might be due to difference in the studied participants in which outpatient cohorts were targeted in this study, whereas the previous studies were conducted in hospitalized patients.

The majority of the EV-positive cases (41 of 63; 65%) were from July to December with the peak occurring in September (Fig. 1). This seasonal pattern was similar to other studies which noted that the highest prevalence of EV infection was in summer (Moritsugu et al., 1970; Patil et al., 2015; Rao et al., 2013).

The percentage of EV mono-infection was found to be 31.7% (20 out of 63) whereas EV co-infection with other enteric viruses including rotavirus, norovirus, adenovirus, human astrovirus, sapovirus, and human parechovirus was observed in 68.3% (43 of 63). The double infection and triple infection were seen in 54% (34 of 63) and 14.3% (9 of 63), respectively. Among these, norovirus was the most predominant virus that was co-infected with EV (Table 1).

For clinical characterization, besides diarrhea, other symptoms such as abdominal pain, nausea, vomiting or fever were present in most of the patients. However, no severe cases were noted in this study, except one patient infected with EV-D68 had been transferred to a hospital because the patient was 3 month old with fever more than 38 °C (Pham et al., 2017).

In this study, EV types were determined by sequencing of the partial

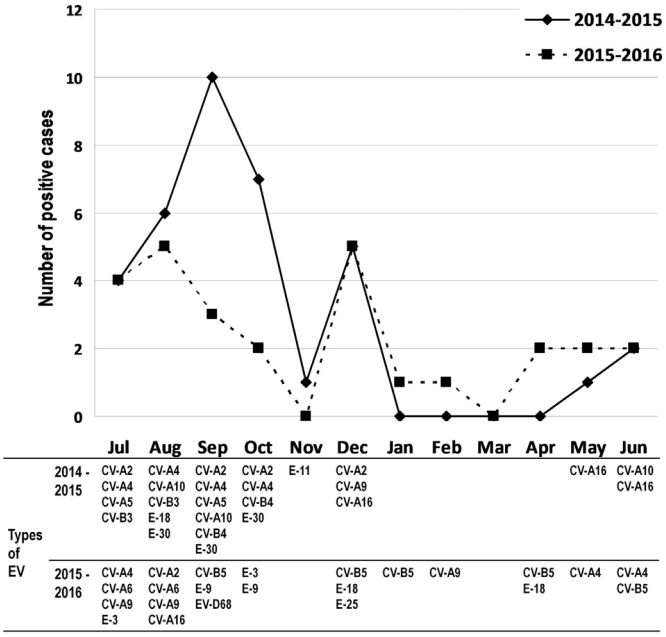

Fig. 1. Seasonal pattern of enterovirus infection in Japanese children with diarrhea during 2-year period 2014–2016; and monthly distribution of enterovirus types identified in this study. EV: enterovirus.

Table 1
Enterovirus mono-infection and co-infection with other enteric viruses. RV: rotavirus; NoV: norovirus; AdV: adenovirus; HAstV: human astrovirus; SV: sapovirus; HPeV: human parechovirus.

Mono- or Co-infection of EV	Number of cases (n = 63)	Percent (%) (100%)
Mono-infection of EV	**20**	**31.7**
Double infection:	**(34)**	**(54)**
EV + RV	8	12.7
EV + NoV	21	33.3
EV + AstV	2	3.2
EV + HPeV	2	3.2
EV + SV	1	1.6
Triple infection:	**(9)**	**(14.3)**
EV + RV + NoV	4	6.3
EV + NoV + AdV	2	3.2
EV + NoV + SV	2	3.2
EV + RV + SV	1	1.6

The numbers in boldface type (with or without bracket) are the total numbers and their percentages of mono-infection, double infection, and triple infection of EV-positive cases in this study.

VP1 region. Of the 63 EV strains subjected to genotyping, 62 strains were succeeded in amplifying and sequencing of the 327- nucleotide sequences of the VP1 gene. Fig. 2 shows a phylogenetic tree constructed based on partial VP1 gene sequences of reference EV strains and the 62 strains found in this study. Overall, 17 different EV types belonging to 3 EV species, EV- A (6 types, 30/62 samples; 48.4%), EV- B (10 types, 31/62; 50%), and EV- D (1 type, 1/62; 1.6%) were identified. None of the studied strains belonged to EV- C species. The most commonly detected types were CV-A4 (n = 8) followed by CV-B5 (n = 7), CV-A5 (n = 6) and CV-A16 (n = 5) (Fig. 2). The other types consisted of CV-A2 (n = 4), CV-A9 (n = 4), CV-A10 (n = 4), CV-B4 (n = 5), E-30 (n = 4), CV-A6 (n = 3), E-18 (n = 3), CV-B3 (n = 2), E-3 (n = 2), E-9 (n = 2), E-11 (n = 1), E-25 (n = 1), and EV-D68 (n = 1) and mixed genotype infections were not noted in this study (Fig. 2).

The predominant EV types found in this study were CV-A5 (6/35; 17.1%) followed by CV-A4 (4/35; 11.4%) during 2014–2015. In 2015–2016, CV-B5 (7/27; 26%) and CV-A4 (4/27; 14.8%) were found