

Molecular Characterization and Genotype Distribution of Enteroviruses in Pediatric Patients with Acute Gastroenteritis in Chiang Mai, Thailand, 2015-2016

การตรวจหาคุณลักษณะในระดับโมเลกุลและการกระจายตัวของสายพันธุ์ของเชื้อไวรัสเอนเทอโรในผู้ป่วยเด็กที่มีอาการกระเพาะอาหารและลำไส้อักเสบเฉียบพลันในจังหวัดเชียงใหม่ ประเทศไทย, ปี ค.ศ. 2015-2016

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ABSTRACT

Enterovirus (EV) has been reported to associate with acute gastroenteritis. This study aimed to investigate the prevalence and genotype diversity of enterovirus detected in children admitted to the hospitals with acute gastroenteritis. A total of 844 fecal specimens were collected from children hospitalized with acute gastroenteritis in Chiang Mai, Thailand during 2015 to 2016. All specimens were tested for the presence of enterovirus by reverse-transcription polymerase chain reaction (RT-PCR). The genotypes of EV detected were identified by nucleotide sequencing and phylogenetic analysis of viral protein 1 (VP1) gene. The enterovirus was detected in 70 of 844 fecal specimens (8.3%) and the virus was detected throughout the year with a peak of 20% in July and of 23.3% in April of 2015 and 2016, respectively. Twenty-one genotypes of enterovirus were identified and coxsackievirus-B5 was the most predominant genotype (9.4%). This study provides the information of epidemiology, seasonal distribution and genotype diversity of enterovirus circulating in children with acute gastroenteritis in Chiang Mai, Thailand.

บทคัดย่อ

มีรายงานการศึกษาว่าเชื้อไวรัสเอนเทอโรมีความสัมพันธ์กับการเกิดโรคกระเพาะอาหารและลำไส้อักเสบเฉียบพลัน การศึกษานี้มีวัตถุประสงค์เพื่อศึกษาความชุกและการกระจายตัวของสายพันธุ์ของเชื้อไวรัสเอนเทอโรในเด็กที่เข้ารับการรักษาในโรงพยาบาลด้วยอาการกระเพาะอาหารและลำไส้อักเสบเฉียบพลัน โดยทำการเก็บตัวอย่างอุจจาระจำนวน 844 ตัวอย่างจากเด็กที่เข้ารับการรักษาด้วยอาการอุจจาระร่วงในโรงพยาบาลในจังหวัดเชียงใหม่ ประเทศไทย ระหว่างปี ค.ศ. 2015-2016 แล้วนำมาตรวจหาเชื้อไวรัสเอนเทอโรด้วยวิธี reverse-transcription polymerase chain reaction (RT-PCR) ตัวอย่างที่ให้ผลบวกจะถูกนำไปหาลำดับนิวคลีโอไทด์บริเวณยีน viral protein 1 และนำลำดับนิวคลีโอไทด์ไปวิเคราะห์โดย phylogenetics ต่อไป ผลการศึกษาพบไวรัสเอนเทอโรใน 70 ตัวอย่างจากทั้งหมด 844 ตัวอย่าง (8.3%) โดยตรวจพบเชื้อได้ตลอดทั้งปีและพบมากที่สุดในเดือนกรกฎาคมของปี 2015 (20%) และ เมษายนของปี 2016 (23.3%) ซึ่งพบเชื้อทั้งหมด 24 สายพันธุ์ โดยตรวจพบสายพันธุ์ coxsackievirus-B5 มากที่สุด (9.4%) การศึกษานี้ทำให้ทราบข้อมูลทางด้านระบาดวิทยาของเชื้อไวรัสเอนเทอโร การกระจายตัวของเชื้อในแต่ละฤดูกาลและสายพันธุ์ที่ก่อให้เกิดโรคกระเพาะอาหารและลำไส้อักเสบเฉียบพลันในเด็ก ในจังหวัดเชียงใหม่ของประเทศไทย

Keywords: Enterovirus, Acute gastroenteritis, VP1 gene

คำสำคัญ: เชื้อไวรัสเอนเทอโร กระเพาะอาหารและลำไส้อักเสบเฉียบพลัน ยีน VP1

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Introduction

Acute gastroenteritis is one of the most common cause of morbidity and mortality in infants, children, and adults with the highest incidence in children at the age range of 6-24 months (Dennehy, 2011), particularly in low-resource settings. Each year, acute gastroenteritis accounts for 3-5 billion cases and nearly 2 million deaths of children globally (King et al., 2003). In developing countries, it is a common reason for admission to hospital. The cost of gastroenteritis in the community level is high and the disease remains an important health issue (Elliott, 2007).

Microbial infection is the cause of acute gastroenteritis, especially virus which accounts for more than 70% of diarrheic cases, followed by bacteria (10-20%) and protozoa (less than 10%) (Elliott, 2007). Viruses that are the common cause of acute gastroenteritis include rotaviruses, noroviruses, astrovirus, and adenovirus (Dennehy, 2011). However, about 30–40% of diarrheic cases are unknown etiology. Some previous epidemiological studies have reported the association of enterovirus (EV) with acute gastroenteritis (Rao et al., 2013; Patil et al., 2015).

Human EVs belong to genus *Enterovirus*, family *Picornaviridae*. The particle of EV is spheroidal, non-enveloped virion with 22–30 nm in diameter. The viral genome is a single-stranded RNA molecule with about 7,389 to 7,441 nucleotides long. EVs that infect human are currently classified by molecular method into 4 species including EV A, B, C, and D based on nucleotide sequence similarity and genome organization. Although most EV infection are commonly asymptomatic, but it is associated with a wide range of diseases, including febrile illness, rash, acute haemorrhagic conjunctivitis, hand-foot-and-mouth disease, herpangina, meningitis, encephalitis, paralysis or myelitis, myocarditis, respiratory diseases, and gastroenteritis (Pallansch et al., 2013). There are several reports of EV detection in fecal specimens from patients with gastroenteritis including Thailand, Italy, Japan, Vietnam, and India (Chaimongkol et al., 2012; Maslin et al. 2007; Patil et al., 2015; Phan et al., 2005; Rovida et al., 2013; Silva et al., 2008). The EV prevalences reported from these studies differ depending on geographical site ranged from 2.5 to 13.7%. In Thailand, the epidemiological data about enterovirus is very limited. So far, there are only 4 studies had been performed in Thailand which reported the prevalence of EV detected from patients with gastroenteritis ranging from 2.5 to 8.3% (Chaimongkol et al., 2012; Saikruang et al., 2014; Chansaenroj et al., 2017; Kumthip et al., 2017).

Objectives of the study

To investigate epidemiology and genotype diversity of EV detected in children with acute gastroenteritis in Chiang Mai, Thailand during 2015-2016.

Methodology

Specimen collection

A total of 844 fecal specimens were collected from children hospitalized with acute gastroenteritis in Maharaj Nakorn Chiang Mai hospital, Nakorn Ping hospital, and Sanpatong hospital, Chiang Mai Province, Thailand during January 2015 to December 2016. The ages of the patients ranged from neonate up to 5 years old. All fecal specimens were stored at -20°C until used.

Detection of enterovirus in fecal specimens

Viral genomic RNA was extracted from 200 μ l of supernatant of 10% fecal suspension using Geneaid Viral Genome Extraction Kit (Geneaid, Taipei, Taiwan) according to manufacturer's instruction. The viral RNA was then reverse transcribed by reverse transcriptase enzyme according to the manufacturer's protocol (Invitrogen, Carlsbad, USA). Polymerase chain reaction (PCR) amplification of 5' untranslated region (5' UTR) of EV genome was performed using EV-specific primers, sense primer F1 (5'-CAAGCACTTCTGTTTCCCCGG-3') and antisense primer R1 (5'-ATTGTCACCATAAGCAGCCA-3'). The PCR product size was 440 base pairs (Zoll et al., 1992). The amplification was performed under the following thermal cycling condition. First, 95°C for 3 minutes to initiate denaturation, 40 cycles of 94°C for 1 minute, followed by 50°C for 1 minute, 72°C for 1 minute and the final extension step at 72°C for 10 minutes. Then EV positive samples were further amplified for partial viral protein 1 (VP1) region using nested-PCR amplification with outer primers 224 (5'-GCIATGYTIGGIACICAYRT-3') and 222 (5'-CICCIIGIGGIAYRWACAT-3'), and inner primers AN89 (5'-CCAGCACTGACAGCAGYNGARAYNGG-3') and AN88 (5'-TACTGGACCACCTGGNGGNAYRWACAT-3') (Nix et al., 2006; Oberste et al., 2003). Thermal cycling condition of first round PCR were as followed, 95°C for 3 minutes to initiate denaturation, 35 cycles of 95°C for 1 minute, followed by 42°C for 45 seconds, 72°C for 1 minute and the final extension step at 72°C for 10 minutes. The second round PCR amplification was then performed using thermal cycling condition as followed, 95°C for 3 minutes to initiate denaturation, 40 cycles of 95°C for 1 minute, followed by 55°C for 45 seconds, 72°C for 40 seconds and the final extension step at 72°C for 10 minutes. The amplification site of the first and second PCR were 999 and 350 base pairs, respectively.

Nucleotide sequencing and phylogenetic analysis

The PCR products of VP1 gene of human EV were purified by Gel/PCR DNA Fragments Extraction Kit (Geneaid, Taipei, Taiwan) according to the manufacturer's protocol. Nucleotide sequencing of the purified PCR products was performed by fluorescence based cycle sequencing method using BigDye® Terminator Cycle Sequencing Kit (Applied Biosystem, Carlsbad, USA). The obtained nucleotide sequences of partial VP1 gene were assembled and analyzed manually by Mega (version X) software. Multiple sequence alignment was performed using MEGA (version X). The reference sequences were obtained from NCBI GenBank database by using BLAST server (<http://blast.ncbi.nlm.nih.gov/Blast.cgi>). Phylogenetic trees of the partial VP1 gene of EV were conducted by MEGA (version X) software with the neighbor-joining method.

Results

Prevalence and monthly distribution

The overall prevalence of EV detected between 2015 and 2016 was 8.3% (70 of 844). In the screening of samples collected in 2015 (335 samples) and 2016 (509 samples), 24 (7.2%) and 46 (9%) specimens were positive for human EVs, respectively. Monthly distribution of EV-positive cases revealed that enterovirus was detected throughout the whole year with peak in July (20%) and April (23.3%), of 2015 and 2016, respectively (Figure 1).

Genotypes distribution

The positive EVs were identified the virus genotypes and species by nucleotide sequence analysis of partial VP1 region. From 70 EV positive cases, 64 EV strains could be successfully amplified for VP1 gene. The result showed that human EV species A, B, and C (EV-A, EV-B, and EV-C) were found in this study. In 2015, EV-B was the most prevalence species (13 of 24; 54.2%) whereas EV-A and EV-C were detected at the prevalence of 20.8% (5 of 24), and 25% (6 of 24), respectively. In 2016, EV-B was also the most prevalence species (16 of 40; 40%), followed by EV-C (13 of 40; 32.5) and EV-A (11 of 40; 27.5%).

Overall, 21 EV genotypes were detected in this study. The most common genotype detected was coxsackievirus (CV)-B5 (9.4%), followed by CV-A4, echovirus 6 (E6), CV-A13, EV-C96 (each 7.8%), CV-A6, E5, CV-A24 (each 6.2%), CV-A10, E14, E18, PV2 (each 4.7%), CV-A5, CV-A8, CV-A9, E25, PV3 (each 3.1%), CV-B3, CV-B4, E1, and E13 (each 1.6%). Species A detected in this study included 5 different genotypes of CV-A4, CV-A5, CV-A6, CV-A8, and CV-A10. Species B comprised 11 different genotypes including E1, E5, E6, E13, E14, E18, E25, CV-A9, CV-B3, CV-B4, and CV-B5. Species C included 5 different genotypes of CV-A13, CV-A24, EV-C96, poliovirus 2 (PV2), and PV3.

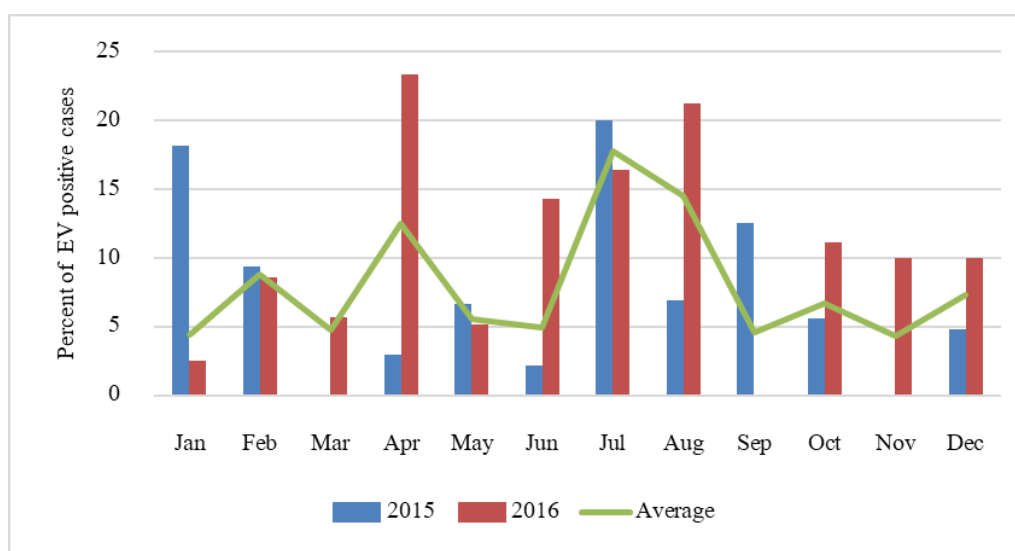


Figure 1 Monthly distribution of EV detected in this study during 2015-2016.

Phylogenetic analysis

Most of strains detected in this study were related to strains previously detected in China. In 2015, EV-A detected in this study were closely related to EV strains previously detected in China and Spain, sharing nucleotide sequence identities ranging from 80.6-98.2% and 96.9%, respectively. EV-B were closely related to EV strains previously reported from China with nucleotide sequence identity ranging from 90.9-100% and Thailand at 93.5%. Some strains of EV-B were closely related to strains previously detected in Japan and Netherlands sharing nucleotide sequence identity ranging from 90.5-97.8%. Some strains of EV-B were related to strains in Guinea, Australia, and

India with nucleotide sequence identity ranging from 85.8 to 91.8%. EV-C were related to EVs strains previously detected in China (sharing nucleotide sequence identity of 87.5%), Finland (77.6%), Greece (70.3-79.8%), Chad (75.5-77.2%), Madagascar (77.2-78.1%), Nigeria (76.8-78.5%), and Malawi (76.3-78.9%) (Figure 3). In 2016, EV-A detected in this study were closely related to strains previously detected in China and Japan with nucleotide sequence identities ranging from 95.5-99.5% and 89.7-98.2%, respectively. Some strains of EV-A were related to strains previously detected in Taiwan, India, Vietnam, Thailand, and Australia with nucleotide sequence identity of 89.2-97.7%, 90.6-95.5%, 98.2%, 89.2-98.6%, and 91.5-94.1%, respectively. EV-B were related to strains previously detected in China and India with nucleotide sequence identities ranging from 94.6-98.6% and 82.5-90.6%, respectively. Some strains of EV-B were closely related to strains detected in Australia, South Korea, Tunisia, Poland, Japan, Netherlands, and Thailand sharing nucleotide sequence identity from 80.3-84.8%, 8.3%, 95.5%, 95.9-98.6%, 96.8, 91.9-94.1%, and 93.3%, respectively. EV-C were related to strains previously detected in China with nucleotide sequence identity ranging from 77.2-100% and Greece at 99.1%. Some strains of EV-C were related to strains in Mexico at 99.5% and Russia at 98.6-99.5%. Some strains of EV-C were identical to EV strains in Australia, Netherlands, China, United Kingdom, and India with 100% nucleotide sequence identity. Some strains of EV-C were closely related to strains detected in Kenya, and Finland from 80.8-84.8%, 79.9% (Figure 4).

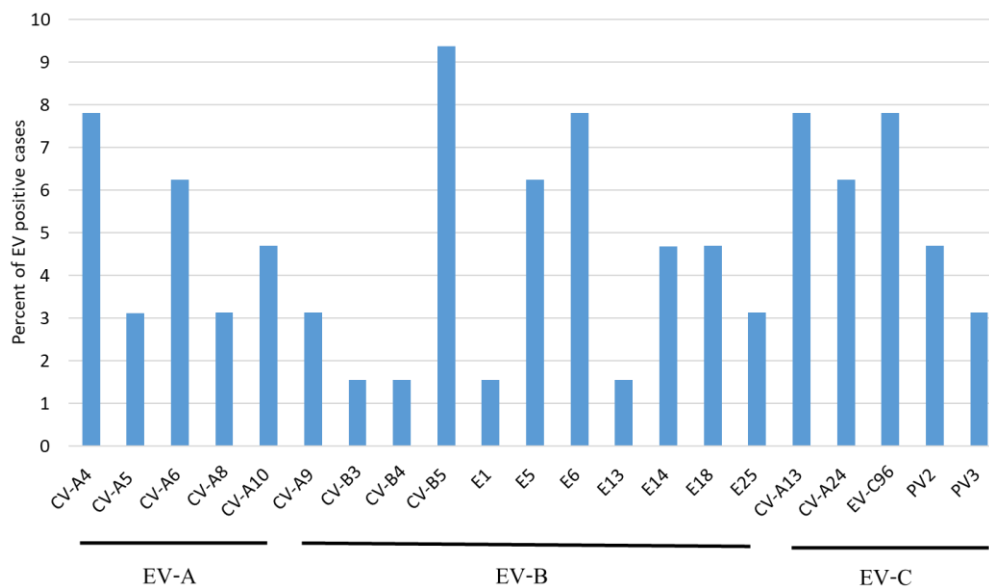


Figure 2 Genotypic distribution of enterovirus strains detected in this study.

Discussion and Conclusions

This study reported the epidemiology and molecular characteristics of EVs detected in pediatric patients hospitalized with acute gastroenteritis in Chiang Mai, Thailand between 2015 and 2016. The overall prevalence of EV detected in this study was 8.3%. The prevalences of human EVs in fecal specimens from patients with gastroenteritis have been reported worldwide varying from 2.5 to 13.7% (Chaimongkol et al., 2012; Maslin et al. 2007; Patil et al.,



2015; Phan et al., 2005; Rovida et al., 2013; Silva et al., 2008). In Thailand, previous studies reported the prevalences of EV in children with diarrhea varied from 2.5 to 6.2 % (Chaimongkol et al., 2012; Saikruang et al., 2014; Chansaenroj et al., 2017; Kumthip et al., 2017). The detection rate of EV reported in this study is slightly higher than those of previous studies in Thailand, but it is in accordance with the study from Vietnam in 2002 to 2003 which reported prevalence at 9.8% (Phan et al., 2005).

Of 64 EV isolates, 21 different EV genotypes were identified which CV-B5 was the most prevalence genotype detected in this study (9.4%) followed by CV-A4, echovirus 6 (E6), CV-A13, and EV-C96 (each 7.8%). The study in Ghana reported that CV-A24 was the most predominant genotype identified in children with diarrhea (Silva et al., 2008). In India, E11 and E30 were the most common genotypes detected in children with acute diarrhea (Rao et al., 2013). In Thailand, CV-A24 and EV-C96 were the most prevalent genotypes detected in diarrheic children (Kumthip et al., 2017). Another study in Thailand revealed that PV2 was the most common genotype detected in diarrheic children (Chansaenroj et al., 2017). The results in this study are in line with the previous study conducted in Thailand that the EV-C96 was the highly prevalent genotype detected in patients with acute gastroenteritis. All 5 strains of poliovirus detected in this study were the Sabin vaccine strains based on the partial VP1 sequence analysis. Nine human EV strains (CMHS191/15, CMHS176/16, CMHS177/16, CMHS245/16, CMHN54/16, CMHS165/16 CMHS185/16, CMHS199/16, and CMHS243/16) detected in the present study were highly similar to EV strains previously isolated in Thailand, suggesting these EV strains are circulating in this area. Several strains in this study were also closely related to strains previously detected in many countries around the world.

In conclusion, this study reports the prevalence, seasonal, and genotype distribution of human EV in children hospitalized with acute gastroenteritis in Chiang Mai, Thailand.

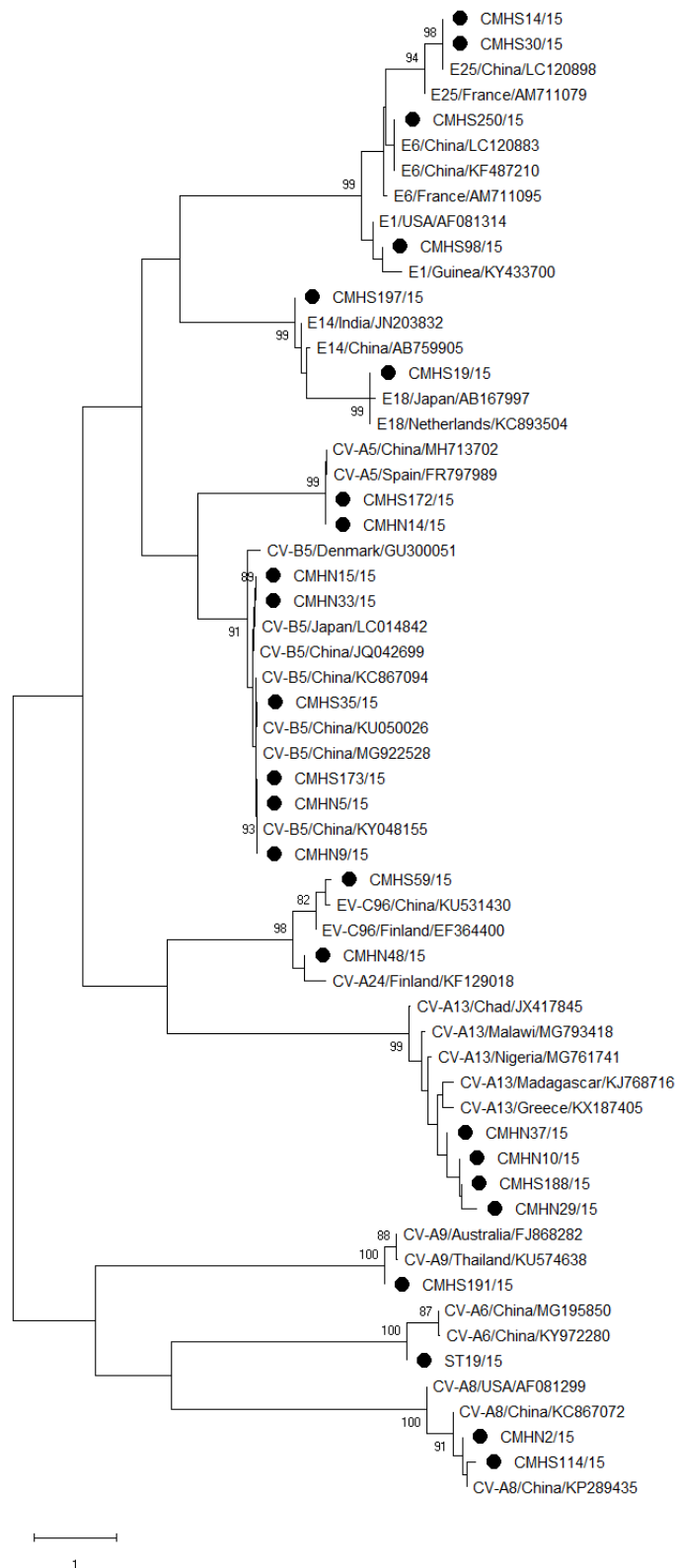


Figure 3 Phylogenetic analysis of the partial nucleotide sequence of VP1 region of EV strains detected in 2015. EV strains detected in this study are represented by circle marks. The tree was performed by neighbor-joining method. The number at each branch represents the bootstrap value.

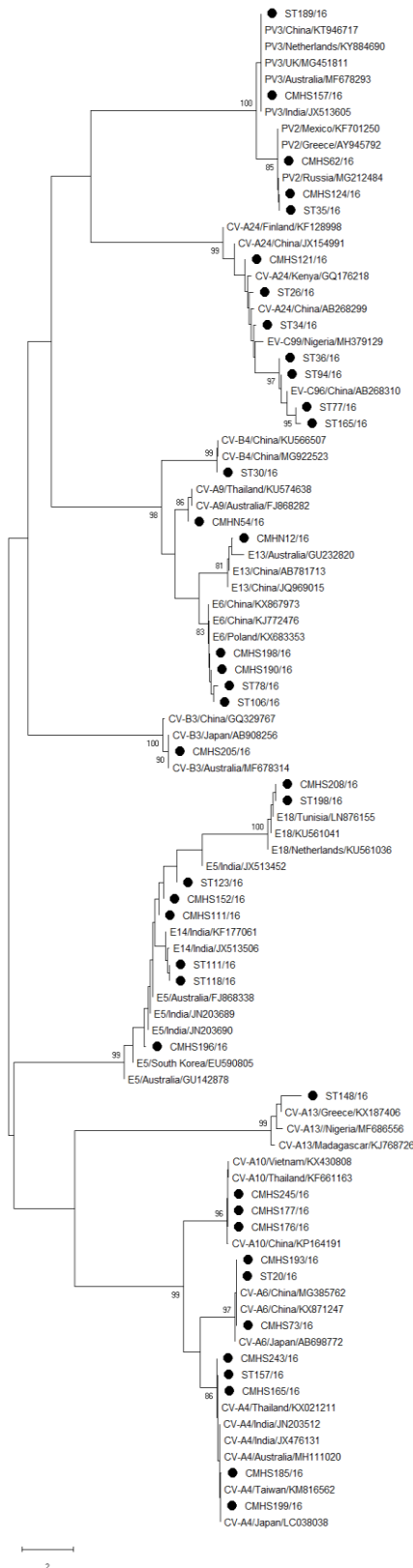


Figure 4 Phylogenetic analysis of the partial nucleotide sequence of VP1 region of EV strains detected in 2016. EV strains detected in this study are represented by circle marks. The tree was performed by neighbor-joining method. The number at each branch represents the bootstrap value.

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