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## Echovirus serotypes circulating in Malaysia from 2002 to 2013

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## ABSTRACT

**Objective:** To identify the circulating serotypes of human echovirus in Malaysia from 2002 to 2013.**Methods:** A total of 31 retrospective samples from non-polio acute flaccid paralysis, hand-food-and-mouth disease, viral meningitis and enterovirus cases were subjected to amplification of partial VP1 gene by RT-PCR.**Results:** Sequencing and phylogenetic analysis of the partial sequences identified presence of human echovirus and human coxsackie viruses. It was found that echovirus 11 was the commonly circulating serotype followed by echovirus 6, echovirus 7, echovirus 3, echovirus 9, echovirus 30 and echovirus 1 in decreasing order. Additionally two types of human coxsackie virus isolates were detected which were coxsackie A24 and B3.**Conclusions:** From the findings, there is a possibility that echovirus 11 is the predominant serotype among Malaysian patients with echovirus infection. However, a larger sample size will yield a more confident result to support this evidence.

## 1. Introduction

The echovirus, being the largest subgroup of Enteroviruses in the *Picornaviridae* family, consists of 32 serotypes. Echovirus is accounted for causing a huge spectrum of human diseases from asymptomatic or acute febrile illness in infants and young children to fatal encephalitis, aplastic anemia and pulmonary hypertension [1]. It is also reported to be the most common cause of aseptic meningitis [2].

Laboratory diagnosis for echovirus was initially focused on classical method which required viral isolation from cell culture of clinical specimens. However, this method has its limitation whereby not all serotypes were able to grow in cell cultures. To date, molecular assays particularly RT-PCR are being widely used [3–5] as it provided a more sensitive, rapid and accurate platform for characterization and serotyping of echovirus.

The VP1 gene was often targeted for serotyping of non-polio enteroviruses as it codes for the major antigenic sites and most type-specific neutralization determinants [6]. Partial sequence analysis of VP1 gene served as tool of identification of

echovirus serotypes, thus discriminating it from other non-polio enteroviruses. Therefore, using this method, we report the molecular epidemiology of echovirus serotypes circulating in Malaysia from 2002 to 2013.

## 2. Materials and methods

## 2.1. Samples

A total of 31 patient samples were obtained from the Virology Unit, Institute for Medical Research, Kuala Lumpur, Malaysia over a period of 12 years (2002–2013). Samples were selected from non-polio acute flaccid paralysis cases (60.0%), hand-food-and-mouth disease cases (3.3%), viral meningitis (26.7%) and enterovirus cases (13.3%) The samples were previously confirmed as echovirus-positive by culture (86.7%) and pan-enterovirus positive (13.3%) by PCR. The sources of sample procurement include cerebrospinal fluid, stool, throat swab, rectal swab and lung fluid.

## 2.2. RNA isolation

Viral RNA isolation from virus-infected culture supernatant was performed using QIAamp Viral RNA Mini Kit (Qiagen, Germany) according to the manufacturer's instructions. The isolation procedure was based on spin-column method. The final

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elution volume of 50 µL containing viral RNA from each sample was stored at -20 °C for long-term usage.

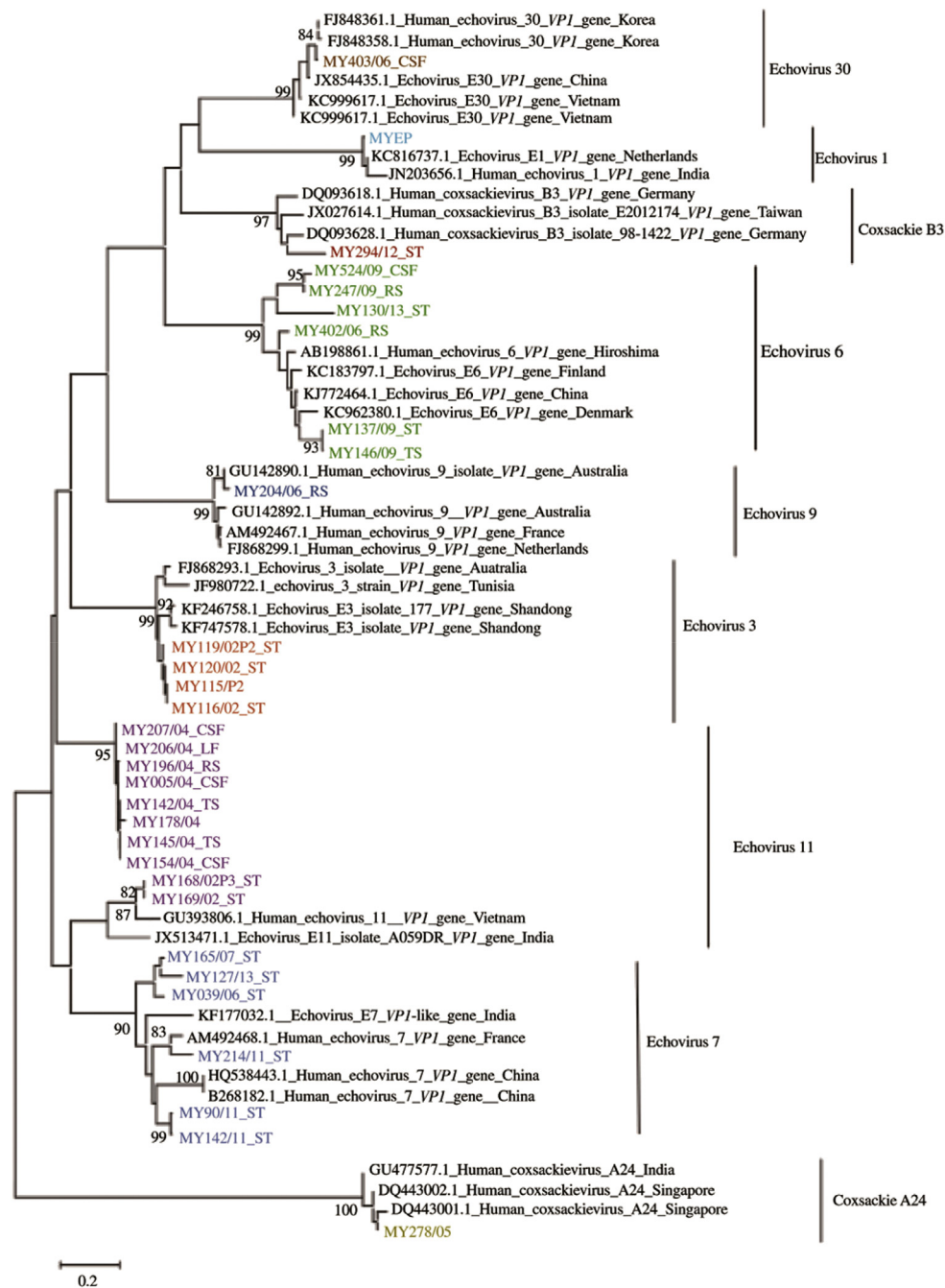
### 2.3. VP1 gene partial amplification

Isolated RNA's were subjected to RT-PCR amplification of partial VP1 gene by using published primers [7]. All amplification reactions were carried out in a 96-well Thermal Cycler (Bio Rad, USA). PCR was undertaken at 50 °C for 30 min, 94 °C for 3 min followed by thermocycling for 35 cycles at 94 °C, 30 s; 42 °C, 30 s and 60 °C, 30 s and a final incubation at 72 °C for 5 min. PCR reaction was composed of 12.5 µL of 2× MyFi RT-PCR Mix (Bioline, USA), 1.0 µL of each oligonucleotides (10 µM), 1.0 µL of Rnase Inhibitor,

0.5 µL of RT enzyme, 5 µL of extracted RNA and 4.0 µL of sterile distilled water.

### 2.4. Post PCR purification and sequencing

A 15 µL aliquot of each PCR reaction was analyzed on 2% agarose by gel electrophoresis and viewed under UV illumination. The agarose was pre-stained with Red Safe Dye (Intron Biotech, Korea). The corresponding amplicons were extracted from the agarose gel and purified using Gel Extraction Kit (Qiagen, USA) according to the manufacturer's instruction. Final elution contained 35 µL of purified PCR amplicons from which 5 µL was reanalyzed on 2% agarose gel to confirm that the purification step was performed precisely. All purified PCR were



**Figure 1.** Phylogram of partial VP1 sequences of human echovirus and human coxsackievirus isolates.

Malaysian isolates were labeled as MY and color coded. RS = rectal swab; ST = stool; CSF = cerebrospinal fluid; TS = throat swab; LF = lung fluid.

subjected to sequencing in 3730 genetic analyzer (Applied Biosystem, USA).

### 2.5. Data analysis

Overlapping sense and antisense sequences derived from sequencing were aligned to produce partial *VP1* gene sequence using CLUSTAL Omega (<http://www.ebi.ac.uk/Tools/msa/clustalo/>) bioinformatics tool. Subsequently, the serotype of each isolates was determined by BLAST search of derived *VP1* gene sequences against the GenBank NCBI database and further verified by construction of phylogenetic tree by Mega 6.06, neighbor joining method (Tamura–Nei model, bootstrap replication 1000×).

## 3. Results

### 3.1. *VP1* gene amplification and serotyping

*VP1* partial gene amplification was observed in all 31 isolates. Alignment of forward and reverse sequences produced *VP1* amplicon of 348 bp. The sequences were deposited in GenBank (Accession numbers: KP204987–KF205039). BLAST search revealed the serotype similarity of Malaysian echovirus isolates with the existing echovirus database in GenBank. It was found that of 31 isolates sequenced, 32.3% (10/31) were echovirus 11 (E11), 19.4% (6/31) were echovirus 6 (E6) and echovirus 7 (E7) respectively 12.9% (4/31) were echovirus 3 (E3) and 3.2% (1/31) were echovirus 1 (E1), echovirus 9 (E9), echovirus 30 (E30), Coxsackie A24 and Coxsackie B3 respectively.

### 3.2. Phylogenetic analysis

The constructed phylogram revealed a 100% concordance with serotyping result by BLAST search. The phylogram grouped Malaysian isolates into 9 clades comprising of E1, E3, E6, E7, E9, E11, E30, coxsackievirus B3 and coxsackievirus A24 (Figure 1). Majority of the Malaysian echovirus isolates were shown to be closely related to the echovirus isolates from Asian countries such as India, Vietnam, China & Korea.

## 4. Discussion

Sequencing and phylogenetic analysis of partial *VP1* gene of Echovirus revealed E11 as the most common serotype circulating in Malaysia from 2002 to 2013. E11 has been associated with a number of outbreaks in neonatal nurseries [8,9]. In an enterovirus surveillance conducted in United States from 1970 to 2005, E11 was reported to be the second most common serotypes [10]. However, no recent reporting of E11 outbreak was found. Few researches in the Asian region demonstrated E30 as the predominant serotypes [11–13]. Recent outbreaks of E30 were seen in other region of the world such as the United States [14] and Europe [15]. Enteroviruses were known to circulate in variable patterns in different years and regions even inside a country, therefore, inconsistency in circulating serotypes and epidemiological reports are well anticipated [16–18].

In the current study, two samples (MY278/05 & MY294/12 ST) which were previously identified as echovirus by culture

appeared to be coxsackieviruses (Coxsackie A24 & B3) as revealed by sequencing and phylogenetic analysis. The misdiagnose could have been due to the fact that some enteroviruses particularly Coxsackie A are not readily detectable in cell culture, therefore a more extensive method such as suckling mice is required for isolation [19]. However, the latter method is no longer commonly used.

The current investigation also found high sequence similarities between different types of samples obtained from the same patient or different passage of the same type of sample (data not shown). Sequence similarities ranged from 97% to 100% of which none have induced amino acid changes.

This study finding showed that E11 was the most common echovirus serotype circulating in Malaysia from 2003 to 2013. Detection and analysis by RT-PCR, sequencing and phylogenetic analysis further identified presence of other serotypes such as E6, E7, E3, E9, E30 and E1, coxsackie A24 and coxsackie B3.

### Conflict of interest statement

We declare that we have no conflict of interest.

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