Identification of enterovirus 71 isolates from an outbreak of hand, foot and mouth disease (HFMD) with fatal cases of encephalomyelitis in Malaysia

Sazaly AbuBakar a,*, Hui-Yee Chee a, Muhannad F. Al-Kobaisi b, Jiang Xiaoshan a, Kaw Bing Chua a, Sai Kit Lam a

a Department of Medical Microbiology, University Malaya Medical Center, 50603 Kuala Lumpur, Malaysia
b Institute of Postgraduate Studies and Research, University Malaya, 50603 Kuala Lumpur, Malaysia

Received 4 October 1998; accepted 23 February 1999

Abstract

Thirteen enterovirus 71 (EV71) isolates were obtained from both fatal and non-fatal infections of patients seen in Peninsula Malaysia and in Sarawak during an outbreak of hand, foot and mouth disease (HFMD) in Malaysia in 1997, with incidences of fatal brainstem encephalomyelitis. The isolates were identified using immunofluorescence staining, neutralization assays, and partial sequencing of the 5’ untranslated regions (UTR). Assessment of the potential genetic relationships of the isolates using the partial 5’UTR sequences suggested clustering of the isolates into at least two main clusters. Isolates from Peninsula Malaysia were found in both clusters whereas Sarawak-derived isolates clustered only in cluster II. Isolates derived from fatal infections, however, occurred in both clusters and no distinctive nucleotide sequences could be attributed to the fatal isolates. Examination of the nucleotide sequences revealed at least 13 nucleotide positions in all the isolates which differ completely from the previously reported EV71 5’UTR sequences. In addition, at least 11 nucleotide position differences within the 5’UTR were noted which differentiated cluster I from cluster II. Predicted secondary RNA structures drawn using the nucleotide sequences also suggested differences between isolates from the two clusters. These findings suggest the presence of at least two potentially virulent EV71 co-circulating in Malaysia during the 1997 HFMD outbreak. © 1999 Elsevier Science B.V. All rights reserved.

Keywords: Encephalomyelitis; Enterovirus; Hand, foot and mouth disease; Malaysia; Sarawak; 5’ untranslated regions

Enterovirus 71 (EV71), a positive single stranded RNA virus belonging to the family of Picornaviridae is known to cause hand, foot and mouth disease (HFMD) in young children (Hagiwara et al., 1978; Miwa et al., 1980; Tagaya et al., 1981). The virus has also been implicated to cause severe neurological manifestations including encephalitis, meningitis, polio-like paresis (Ishimaru...
Table 1
Identification of EV71 isolates of HFMD patients and those who succumbed to a HFMD-associated brainstem encephalomyelitis

<table>
<thead>
<tr>
<th>EV71 Isolate</th>
<th>Location</th>
<th>Infection Site of isolation</th>
<th>Passage in Vero cell culture</th>
<th>IF&lt;br&gt;</th>
<th>NT&lt;br&gt;</th>
<th>PCR&lt;br&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>EV71/7/97/UH1</td>
<td>PM</td>
<td>Fatal Cerebrum</td>
<td>6</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>EV71/7/97/UH1SB</td>
<td>PM</td>
<td>HFMD Stool</td>
<td>3</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>EV71/8/97/UH2</td>
<td>PM</td>
<td>Fatal Brain stem</td>
<td>3</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>EV71/8/97/UH2S</td>
<td>PM</td>
<td>HFMD Stool</td>
<td>2</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>EV71/9/97/UH3</td>
<td>PM</td>
<td>Fatal Brain stem</td>
<td>2</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>EV71/10/97/UH4</td>
<td>PM</td>
<td>Fatal Brain stem</td>
<td>2</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>EV71/8/97/MH1</td>
<td>PM</td>
<td>Fatal Cerebellum</td>
<td>3</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>EV71/PM/SHA51</td>
<td>PM</td>
<td>HFMD Vesicle swab</td>
<td>2</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>EV71/PM/SHA61</td>
<td>PM</td>
<td>HFMD Oral swab</td>
<td>2</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>EV71/SAR/C3</td>
<td>SAR</td>
<td>Fatal* Stool</td>
<td>4</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>EV71/SAR/UM3</td>
<td>SAR</td>
<td>Fatal* Serum</td>
<td>4</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>EV71/SAR/SHA63</td>
<td>SAR</td>
<td>HFMD Rectal swab</td>
<td>2</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>EV71/SAR/SHA66</td>
<td>SAR</td>
<td>HFMD Stool</td>
<td>2</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
</tbody>
</table>

a PM, Peninsula Malaysia; SAR, Sarawak; Fatal, fatal brainstem encephalomyelitis; Fatal*, cause of death not identified; HFMD, suspected hand, foot and mouth disease; +, positive for the test performed.

b EV71 specific monoclonal antibody (Chemicon, USA) was used.

c Neutralization tests performed using EV71 specific antiserum.

d The PCR was performed using pan-enterovirus primers and parameters as described in text.

e Isolate from the surviving younger brother of the deceased child from which the isolate EV71/7/97/UH1 was derived.

f Isolate from the surviving younger brother of the deceased child from which the isolate EV71/8/97/UH2 was derived.

et al., 1980; Nagy et al., 1982; Gilbert et al., 1988; Da Silva et al., 1990; Alexander et al., 1994) and isolated outbreaks of fatal meningoencephalitis (Chumakov et al., 1979). Similar to other enteroviruses, EV71 is thought to spread by contact with fecal contaminated materials. Infection by the virus is often asymptomatic or may manifest as mild self-limiting illness which is often characterized by the presence of characteristic lesions on the palms, soles and oral mucosa. It is not presently understood, however, how the infection could progress to involve the brain resulting in neurological disorders and in some cases death notably in young children. Zheng et al., (1995) based on findings of the poliovirus and Venezuelan equine encephalitis, postulated that changes in the 5% untranslated region (UTR) sequences perhaps lead to the increased virulence of the otherwise innocuous virus.

In 1997 a number of unexplained sudden childhood deaths occurred in Malaysia in the midst of an outbreak of HFMD. Thirty one children succumbed to the infection in Sarawak, a state in Malaysia located on the Borneo island, within hours of admission to the hospitals. Subsequently, at least four children presenting with almost similar clinical features of high fever and rapid deterioration of vital functions died within 12 h of onset of cardiopulmonary symptoms in the Peninsula Malaysia. From these later cases, EV71 was isolated from the CNS tissues of all four patients (Lum et al., 1998) and two isolates from non-neuronal tissues were obtained from the Sarawak fatal cases. We report here isolation, identification and partial sequencing results of the 5'UTR of these EV71 isolates and seven additional isolates obtained from suspected HFMD patients and one from an unreported fatal case from Peninsula Malaysia within the same outbreak period.

Patients’ specimens including stool, serum, and rectal, throat and vesicle swabs were processed for virus isolation following the routine laboratory procedures. Briefly, sera, stool, and cerebrospinal fluid were clarified by centrifugation and the supernatants were filtered through a 0.22 µm filter. Swabs samples, on the other hand, were first vortexed gently in transport medium and then centrifuged and treated as above. Post-mortem tissues whenever available, were ground with pestle and mortar to dissociate the cells. The cell
suspension was freeze–thawed twice, centrifuged to sediment cells and debris, and filtered through a 0.22 µm filter as above. All the filtrates were then used as inocula to infect freshly prepared Vero cell cultures. The cytopathic effects were monitored daily and the presence of EV71 antigens was detected using the pan-enterovirus antibodies and EV71 specific monoclonal antibody (Chemicon, USA). Neutralization assays to confirm for the presence of the EV71 were performed at the Singapore General Hospital using antiserum provided by Dr Margery Kennett (Regional Polio Reference Laboratory, VIDRL, Australia). The neutralizing antiserum developed using the JS strain EV71 has also been shown to neutralize the prototype EV71BrCr strain (Kennett et al., 1974). The presence of enteroviruses genomic sequences in the infected Vero cells, on the other hand, was initially examined by the RT-PCR performed using the pan-enterovirus primers and parameters as described in Abebe et al. (1992). A total of 68 specimens from 51 patients; 29 from Peninsula Malaysia and 22 from Sarawak which included five and nine fatal cases, respectively, were examined for the presence of enteroviruses genomic sequences. From these patients, enteroviruses 5'UTR sequences were noted in 26 patients, including 21 from Peninsula Malaysia and the remaining from Sarawak. The presence of EV71 was serologically confirmed in only 13 of these samples (Table 1). Seven of these positive samples were from fatal cases which included the five cases from Peninsula Malaysia. The six additional EV71 positive samples were
Fig. 2. Partial 5'UTR nucleotide sequences of EV71 isolates of patients seen in Malaysia in an outbreak of HFMD in 1997. The sequences were aligned and numbered with respect to the EV71MS (Accession # U22522) genomic sequence (Brown and Pallansch, 1995) using Clustal X (Thompson et al., 1994) and displayed using GeneDoc 2.3 (Nicholas and Nicholas, 1997). Nucleotide positions which differentiated the EV71 isolates into at least two major clusters are shaded. Gaps and homologous nucleotides are indicated by (−) and (●), respectively.

from suspected HFMD patients which included two from Sarawak and two from contacts of the fatal cases from Peninsula Malaysia. To examine the potential genetic relationships of the EV71 isolates, partial length 5'UTR sequences were amplified by the RT-PCR using the enterovirus primers described in Arola et al. (1995) and the following parameters: reverse transcription at 37°C for 1 h; denaturation at 95°C for 2 min; and 40 cycles of denaturation at 95°C for 1 min, annealing at 55°C for 1 min, and extension at 72°C for 1 min with the last extension at 72°C for 5 min. The primer was chosen to enable amplification of longer 5'UTR sequences of approximately 470 bp. The resulting cDNA fragments were sequenced at ACGT (USA) and the results were compared using FASTA (Pearson and Lipman, 1988) to EV71MS/7423/87 (ETU22522) and EV71BrCr (ETU22521) strain sequences (Brown and Pallansch, 1995) previously reported in the GenBank nucleotide sequence database. Nucleotide sequences were then aligned using Clustal X (Thompson et al., 1994) and phylogenetic trees were constructed using the neighbor joining
method (Saitou and Nei, 1987). Bootstrap values which indicated how well supported the interior branches of the phylogenetic tree were determined using 1000 random samples of the multiple alignments. The dendrograms were displayed using TreeView 1.5 (Page, 1996).

Results from the search of the GenBank DNA sequence database showed that the 13 enterovirus isolates shared high (~88%) sequence identity to EV71. Based on the aligned sequences, a dendrogram indicating the potential relationships of the 13 EV71 isolates was drawn using the prototype EV71BrCr strain as the outward group, since pairwise comparisons of the sequences suggested that the strain was the least similar to the isolates (Fig. 1). Two well bootstrap supported (>97%) clusters of the isolates were obtained. Cluster I comprised of five isolates of patients from Peninsula Malaysia and cluster II consisted of the remaining isolates which included all four isolates from Sarawak. Isolates in cluster I were differentiated from cluster II in at least 11 nucleotide positions (Fig. 2). At least eight nucleotide substitutions within the 11 nucleotide positions would be needed to place all the 13 isolates into a cluster. Approximately 60% (3/5) of the isolates from cluster I and 50% (4/8) from cluster II were from fatal cases. Analyses of the aligned sequences, however, did not indicate the presence of any significant nucleotide differences between the EV71 isolates derived from fatal and non-fatal HFMD-associated cases. This is especially true
when sequences of the isolates of the fatal cases were compared to the non-fatal infection of the deceased siblings. Differences in a few nucleotides noted in EV71/8/97/UH2S (non-fatal) sequences in comparison to the sibling’s EV71/8/97/UH2 (fatal) were not significant since similar nucleotides were also noted in other isolates derived from the fatal cases. However, enough nucleotide differences between EV71/8/97/UH2 and EV71/8/97/UH2S occurred to separate them into the two different clusters suggesting that perhaps both the EV71 strains were co-circulating in Peninsula Malaysia within the same period. Nucleotide sequence comparisons made to examine the potential differences between EV71 isolates from Peninsula Malaysia and Sarawak also did not reveal any characteristic nucleotide sequences specific to either in the 5’UTR. Comparisons of the nucleotide sequences of isolates EV71/SAR/UM3 and EV71/SAR/C3 derived from the Sarawak fatal cases with that of the non-fatal isolates from Sarawak also did not indicate the presence of any significant nucleotide differences. Although we cannot draw any definite conclusion from the limited number of samples, it is suggested that since all (4/4) isolates from Sarawak were found only in cluster II, perhaps only a single EV71 strain was circulating in Sarawak during the period.

Even though the sequencing results obtained in this study suggested that sufficient nucleotide differences were present at the 5’UTR to enable
differentiation of the isolates into two clusters, significant differences were not noted that would differentiate between the virulent EV71 which could contribute to the fatal infections and the non-virulent EV71 which caused only mild HFMD. On the other hand, sequencing results of the thirteen isolates showed the presence of characteristic nucleotides: A, A, C, C, A, G, C, T, G, A, G, C, and C at positions 120, 125, 136, 139, 144, 147, 156, 159, 229, 260, 298, 369, and 389, respectively, which were completely different from either of the established EV71 strains, EV71MS and EV71BrCr. Since the enterovirus genomic 5' UTR is known to form secondary RNA structures important in virus replication, it is possible that the presence of these nucleotides within the isolates 5' UTR would affect the RNA structure formation. This possibility was examined by determining the predicted RNA structures of two representative isolates; EV71UH1 and EV71UM3 for cluster I and II respectively and comparing them to that of EV71MS and EV71BrCr. Using RNAstructure 2.52 (Software distributed by Mathews DH and Burkard ME at http://128.151.176.70/RNAstructure.html), it was revealed that at least three major identifiable RNA stem-loop structures or domains (I, II, III) comparable to that previously reported as domains II, IV and V, respectively for polioviruses (Zell and Stelzner, 1997) were present within the amplified 5' UTR sequences. Domains I (nucleotides 129–169) and III (nucleotides 479–537) of both the
isolates shared almost similar predicted secondary RNA structures to that of the EV71BrCr and EV71MS, respectively. Domain II of EV71MS and EV71BrCr strains in our study on the other hand, consisted of nucleotides from positions 243–446 and 241–444, respectively. Whereas, for EV71UM3 and EV71UH1 strains, the predicted stable secondary RNA structure which made-up domain II included nucleotides at positions 201–472 and 197–436, respectively. These nucleotide sequences included the reported domain III (nucleotide 186–236) of the enteroviruses (Zell and Stelzner, 1997). The RNA secondary structures between nucleotide positions 269–421 within domain II for all the EV71 isolates and the reference strains were similar suggesting the potential importance of the structure. A longer stem structure and an additional stem at nucleotide positions 225–240 for EV71UM3 were predicted. A similar stem was also predicted at nucleotides 222–241 for EV71MS. In contrast, an extended additional stem-loop structure at nucleotides 212–266 was predicted for EV71UH1 (Fig. 3). Whether differences in the predicted RNA secondary structures of domain II for the EV71 isolates in this study have any real significance will require further investigation. Nevertheless, findings presented in this study suggested that there were at least two potentially virulent EV71 strains co-circulating within Malaysia during the outbreak of HFMD in 1997.
Fig. 3. Predicted RNA secondary structure of EV71 isolates partial 5′UTR. The figure is a schematic representation of the RNA structures of domain II predicted based on the lowest free energy using Zuker algorithm and displayed using RNA-Draw 1.1b2 (Matzura and Wennborg, 1996). Domain II of isolates EV71UM3 (A) and EV71UH1 (B) are shown from nucleotides 243–446 and 197–436, respectively in 5′→3′ direction (left to right). Arrow head indicate nucleotide at position 269. Figures are drawn not according to scale.

References


