An echovirus 18-associated outbreak of aseptic meningitis in Taiwan: epidemiology and diagnostic and genetic aspects

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In 2006, an outbreak of aseptic meningitis was noted in Taiwan. From January to October 2006, a total of 3283 specimens collected from patients with viral infection, including 173 cerebrospinal fluid (CSF) samples, were examined for virus isolation and identification. Overall, 339 enterovirus (EV)-positive cases were identified by virus culture: echovirus 18 (E18) formed the majority (27.4 %, 93 cases), followed by coxsackievirus B2 (13.8 %, 47 cases) and coxsackievirus A2 (10.8 %, 37 cases). The manifestations of the 93 E18 cases were aseptic meningitis (44.1 %), viral exanthema (23.6 %), acute tonsillitis (15.1 %), acute pharyngitis (14.0 %), acute gastritis (11.8 %), herpangina (7.5 %) and bronchopneumonia (5.3 %). Of 107 E18 isolates identified, 100, 62.5 and 19 % were obtained following culture in RD, MRC-5 and A549 cells, respectively. E18 was identified most frequently from throat swabs (67.2 %) and less frequently from stool samples (15.9 %) and CSF (16.8 %). The detection rate of E18 was 78.2 % from CSF, 50 % from stool samples and 22.9 % from throat swabs. Phylogenetic relationships among the E18 strains were examined. Analysis of the partial VP1 gene showed 3.7–23.8 % variation in sequence compared with sequences from GenBank and, notably, the amino acid change V152S was detected in a protruding loop within the VP1 protein. These results indicate that a genetic variant of E18 was circulating and caused an outbreak of aseptic meningitis in Taiwan in 2006.

INTRODUCTION

Millions of human enterovirus (EV) infections occur worldwide every year. HEV is a small positive-strand RNA virus belonging to the family Picornaviridae. The genus Enterovirus contains more than 60 human EV serotypes, which are divided into four clusters, A–D (Pöyry et al., 1996). Although most human EV infections are asymptomatic, some infections have clinical manifestations, such as hand, foot and mouth disease, the common cold, acute haemorrhagic conjunctivitis, myocarditis, aseptic meningitis and poliomylitis (Modlin, 1996). Outbreaks of aseptic meningitis caused by a major serotype have frequently been reported (Cabreroiz et al., 2008; Chambon et al., 2001; dos Santos et al., 2006; Mistchenko et al., 2006; Richter et al., 2006; Thoelen et al., 2003; Wang et al., 2002). Between 1970 and 2005, two peaks of human echovirus 18 (E18) activity

Abbreviations: CSF, cerebrospinal fluid; E, echovirus; EV, enterovirus; NCKUMC, National Cheng Kung University Medical Center.

The GenBank/EMBL/DBJ accession numbers for the sequences determined in this study are GQ205563–GQ205579.
were observed in the USA (1986–1987 and 1995–2005). In 2001, the activity of E18 reached its highest level, accounting for 22 % of EV isolations and resulting in an outbreak of E18 aseptic meningitis at a children’s summer camp in Alaska (McLaughlin et al., 2004). The mortality rate was around 1.8 % from 1983 to 2005 in the USA (CDC, 2002; Khetsuriani et al., 2006).

Traditional laboratory identification of EV infections is based on immunofluorescent staining, using commercially available mAbs and/or neutralization tests with polyclonal antibodies. However, antigenic variants frequently caused failure of identification (Muir et al., 1998). RT-PCR and DNA sequencing of the EV genome are often used for epidemiological analysis and classification. Because the VP1 protein contains several key neutralization sites (Mateu, 1995), the VP1-encoding region of the EV genome is amenable to sequence analysis to determine genetic variations.

An outbreak of aseptic meningitis occurred in Taiwan from April to August 2006. The majority of EV isolates were found to be E18 (27.4 %) and the case fatality rate among children was 4 % (Wang et al., 2011). The current study analysed the epidemiology, laboratory diagnosis and genetic variations of the virus in this E18 outbreak.

**METHODS**

**Virus isolation and identification.** Throat swab or stool specimens from patients with EV-like symptoms were collected by physicians of sentinel clinics and the National Cheng Kung University Medical Center (NCKUMC) in southern Taiwan. Cerebrospinal fluid (CSF) specimens were collected directly from inpatients with suspected EV infections. Swabs were placed immediately in 2 ml viral transport medium containing 0.5 % gelatin, Earle’s minimum essential medium (Gibco), 200 U penicillin/streptomycin ml\(^{-1}\), 0.05 mg gentamicin ml\(^{-1}\) and 1.25 μg amphotericin B (Fungizone) ml\(^{-1}\). Refrigerated specimens were inoculated into different tubes containing RD, A549 and MRC-5 cells within 24 h. Infected cells with cytopathic effect were confirmed by a neutralization test and/or immunofluorescent staining. Immunofluorescent staining was performed using mAbs with an EV screening kit (Chemicon). A Lim and Benyesh-Melnick antiserum pool and polyclonal antibodies were used with a low concentration of chloroform to disperse the virus particles prior to the neutralization test (Kapsenberg et al., 1979).

**RNA extraction and RT-PCR.** Viral RNA was extracted from 140 μl infected tissue culture fluid using a QIaAmp Viral RNA Mini kit. Five microliters of eluted RNA was used for amplification of the partial VP1 gene using a OneStep RT-PCR kit (Qiagen). The primers used for RT-PCR and sequencing were sense primer 012 (5’-ATGTAYGTICCCCGGIGGIG-3’, nt 2875–2894) and antisense primer 011 (5’-GICICGAYGTTGIGGCRRA-3’, nt 3311–3292) (Oberste et al., 1999a). Cycling conditions for RT-PCR amplification were: 30 min at 50 °C for reverse transcription, and one cycle for 5 min at 95 °C, followed by 35 cycles of 30 s at 95 °C, 45 s at 45 °C and 45 s at 72 °C, with extension for 10 min at 72 °C and subsequent holding at 4 °C.

**Sequencing and phylogenetic analysis.** PCR products were purified using a QIAquick PCR Purification kit (Qiagen) and the purified DNA was sequenced using an ABI 3130xl DNA sequencer (Applied Biosystems). Sequence data were analysed using version 10.1 of the sequencing analysis software package GCG of the Wisconsin Genetics Computer Group (Butler, 1998). Bootstrapping of 1000 replicates was performed. A phylogenetic tree was generated using maximum-parsimony criteria via the PAUP program (Page, 2002; Posada, 2003). Pairwise nucleotide and amino acid sequence comparisons were performed by the Distance program in DAMBE (Xia & Xie, 2001).

**Three-dimensional structure.** The VP1 amino acid changes of the Taiwan E18 isolates were projected onto the three-dimensional structure of the prototype E18 Metcalf strain VP1 region (GenBank accession no. AF081331), using the (PS)\(^2\) program (Protein Structure Prediction Server, http://ps2.life.nctu.edu.tw/) on the GMBD bioinformation website (Bioinformatics Core for Genomic Medicine and Biotechnology Development, http://www.tbi.org.tw/about/index.htm). Amino acids were numbered according to E18 Metcalf.

**RESULTS**

**Investigation of circulating EVs**

Between January and October 2006, a total of 3283 specimens, including 173 CSF samples, suspected of viral infections were received at the Virology Laboratory of NCKUMC in southern Taiwan for viral isolation. From 339 EV-positive cases, a total of 372 EVs were isolated: 315 (84.6 %) from throat swabs, 34 (9.1 %) from stool samples and 23 (6.1 %) from CSF. E18 (27.4 %, 93/339 EV-positive cases), coxsackievirus B2 (13.9 %, 47 cases) and coxsackievirus A2 (10.9 %, 37 cases) were identified most frequently (Table 1). Of 23 EVs isolated from CSF specimens, E18 accounted for 78.3 % (18 cases), whilst the others comprised coxsackievirus A9 (two cases), coxsackievirus B2 (one case) and untyped EVs (two cases).

**E18 outbreak description**

During the summer of 2006, most of the cases of aseptic meningitis were caused by E18. The clinical manifestations of the 93 E18-positive cases included aseptic meningitis (44.1 %), viral exanthema (23.6 %), acute tonsillitis (15.1 %), acute pharyngitis (14.0 %), acute gastritis (11.8 %), herpangina (7.5 %) and bronchopneumonia (5.3 %). One patient died of meningitis complications. The peak activity of E18 infection was identified in June–July 2006 (Table 1). The age of the E18-infected patients ranged from 1 month to 67 years, but most were pre-school and elementary school children. The age distribution was: <1 year, 16.1 % (15 patients); 1–3 years, 17.2 % (16 patients); 3–6 years, 28.0 % (26 patients); 7–12 years, 33.3 % (31 patients); and >12 years old, 5.4 % (five patients). Note that patients with positive CSF specimens were mostly aged 3–12 years (88.2 %, 15/18 patients). The ratio of males to females was 49:44.

**Detection of E18 in cell culture**

MRC-5, A549 and RD cells are used for the routine isolation of EVs in our laboratory. Of 107 E18 isolates that
were identified, the detection rates of primary isolation from RD, MRC-5 and A549 cells were 100, 62.5 and 19% with mean numbers of days to detection of 6.4 (range 2–11), 6.8 (range 3–10) and 7.1 (range 2–10), respectively. Among the 107 E18 isolates, the virus was isolated most frequently from throat swab specimens (67.3 %, 72 E18-positive throat swab specimens/107 total E18 positive specimens) and less frequently from stool specimens (15.9 %, 17/107) and CSF specimens (16.8 %, 18/107). A significantly higher detection rate of E18 was found in CSF (78.2 %, 18/23) than in stool samples (50 %, 17/34) or throat swabs (22.9 %, 72/315).

**Identification of E18**

Of the 107 E18 isolates, 44 and 63 isolates were initially identified as E4 and E18 by immunofluorescence staining, respectively. According to the results of the neutralization test and sequencing alignment of the partial VP1 gene, these isolates were identified and confirmed as E18.

**Genetic analysis of the partial VP1 gene**

Phylogenetic analysis of the partial VP1 gene was conducted. Sixteen E18 Taiwan strains were obtained from cases with different clinical manifestations and from various specimens (Table 2). Reference sequences from GenBank (12 E18 strains and three E4 strains) were included for comparison. A phylogenetic tree of these sequences was generated with E30 as the outgroup using PAUP. Phylogenetic analysis of the VP1 gene of the isolates in this outbreak and the sequences from GenBank showed that they clustered with the TR178027-05 strain from France (Mirand et al., 2006); by contrast, all 16 E18 Taiwan strains grouped in

### Table 1. Monthly distribution of EV serotypes in 2006

<table>
<thead>
<tr>
<th>Virus type</th>
<th>Jan</th>
<th>Feb</th>
<th>Mar</th>
<th>Apr</th>
<th>May</th>
<th>Jun</th>
<th>Jul</th>
<th>Aug</th>
<th>Sep</th>
<th>Oct</th>
<th>Total</th>
<th>%</th>
</tr>
</thead>
<tbody>
<tr>
<td>Coxsackievirus A2</td>
<td>0</td>
<td>2</td>
<td>0</td>
<td>4</td>
<td>11</td>
<td>10</td>
<td>6</td>
<td>2</td>
<td>2</td>
<td>0</td>
<td>37</td>
<td>10.9</td>
</tr>
<tr>
<td>Coxsackievirus A4</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>2</td>
<td>7</td>
<td>4</td>
<td>1</td>
<td>1</td>
<td>2</td>
<td>1</td>
<td>18</td>
<td>5.3</td>
</tr>
<tr>
<td>Coxsackievirus A5</td>
<td>3</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>4</td>
<td>1</td>
<td>0</td>
<td>6</td>
<td>2</td>
<td>1</td>
<td>17</td>
<td>5</td>
</tr>
<tr>
<td>Coxsackievirus A6</td>
<td>0</td>
<td>0</td>
<td>1</td>
<td>0</td>
<td>4</td>
<td>3</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>8</td>
<td>2.3</td>
</tr>
<tr>
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<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>2</td>
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<td>0</td>
<td>0</td>
<td>0</td>
<td>10</td>
<td>2.9</td>
</tr>
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<td>0</td>
<td>0</td>
<td>0</td>
<td>2</td>
<td>2</td>
<td>2</td>
<td>1</td>
<td>1</td>
<td>0</td>
<td>7</td>
<td>2.1</td>
</tr>
<tr>
<td>Coxsackievirus A16</td>
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<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>1</td>
<td>0</td>
<td>0</td>
<td>1</td>
<td>0</td>
<td>3</td>
<td>1</td>
</tr>
<tr>
<td>Coxsackievirus B2</td>
<td>7</td>
<td>4</td>
<td>2</td>
<td>7</td>
<td>2</td>
<td>10</td>
<td>6</td>
<td>8</td>
<td>1</td>
<td>0</td>
<td>47</td>
<td>13.9</td>
</tr>
<tr>
<td>E9</td>
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<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>1</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>1</td>
<td>0.3</td>
</tr>
<tr>
<td>E18</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>3</td>
<td>11</td>
<td>37</td>
<td>30</td>
<td>10</td>
<td>2</td>
<td>0</td>
<td>93</td>
<td>27.4</td>
</tr>
<tr>
<td>Poliovirus Sabin</td>
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<td>0</td>
<td>0</td>
<td>1</td>
<td>5</td>
<td>2</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>8</td>
<td>2.4</td>
</tr>
<tr>
<td>Untyped EV</td>
<td>1</td>
<td>2</td>
<td>3</td>
<td>7</td>
<td>11</td>
<td>22</td>
<td>15</td>
<td>9</td>
<td>16</td>
<td>4</td>
<td>90</td>
<td>26.5</td>
</tr>
<tr>
<td>Total</td>
<td>12</td>
<td>8</td>
<td>6</td>
<td>23</td>
<td>53</td>
<td>102</td>
<td>64</td>
<td>38</td>
<td>27</td>
<td>6</td>
<td>339</td>
<td>100</td>
</tr>
</tbody>
</table>

### Table 2. Clinical characteristics and manifestations of the E18 infection cases shown in the phylogenetic tree in this study

<table>
<thead>
<tr>
<th>Case no.</th>
<th>Date specimen received</th>
<th>Age of patient (years)</th>
<th>Specimen</th>
<th>Clinical diagnosis</th>
</tr>
</thead>
<tbody>
<tr>
<td>H97-TW-06</td>
<td>15 June 2006</td>
<td>8</td>
<td>CSF</td>
<td>Aseptic meningitis</td>
</tr>
<tr>
<td>N1447-TW-06</td>
<td>8 May 2006</td>
<td>5</td>
<td>Throat swab</td>
<td>Aseptic meningitis</td>
</tr>
<tr>
<td>N1719-TW-06</td>
<td>29 May 2006</td>
<td>6</td>
<td>Rectal swab</td>
<td>Aseptic meningitis</td>
</tr>
<tr>
<td>N1757-TW-06</td>
<td>1 June 2006</td>
<td>6</td>
<td>CSF</td>
<td>Aseptic meningitis</td>
</tr>
<tr>
<td>N1900-TW-06</td>
<td>12 June 2006</td>
<td>2</td>
<td>Throat swab</td>
<td>Fever</td>
</tr>
<tr>
<td>N1958-TW-06</td>
<td>15 June 2006</td>
<td>5</td>
<td>Throat swab</td>
<td>Acute pharyngitis</td>
</tr>
<tr>
<td>N2098-TW-06</td>
<td>26 June 2006</td>
<td>8</td>
<td>CSF</td>
<td>Aseptic meningitis</td>
</tr>
<tr>
<td>N2144-TW-06</td>
<td>27 June 2006</td>
<td>10</td>
<td>CSF</td>
<td>Aseptic meningitis</td>
</tr>
<tr>
<td>N2157-TW-06</td>
<td>28 June 2006</td>
<td>9</td>
<td>Throat swab</td>
<td>Aseptic meningitis</td>
</tr>
<tr>
<td>N2209-TW-06</td>
<td>3 July 2006</td>
<td>6</td>
<td>Throat swab</td>
<td>Aseptic meningitis</td>
</tr>
<tr>
<td>N2237-TW-06</td>
<td>4 July 2006</td>
<td>3</td>
<td>Rectal swab</td>
<td>Death, pneumonia, aseptic meningitis</td>
</tr>
<tr>
<td>N2243-TW-06</td>
<td>4 July 2006</td>
<td>1</td>
<td>Throat swab</td>
<td>Acute pharyngitis</td>
</tr>
<tr>
<td>N2273-TW-06</td>
<td>6 July 2006</td>
<td>2</td>
<td>Throat swab</td>
<td>Viral exanthema, vomiting</td>
</tr>
<tr>
<td>N2306-TW-06</td>
<td>10 July 2006</td>
<td>7</td>
<td>Throat swab</td>
<td>Aseptic meningitis</td>
</tr>
<tr>
<td>N2546-TW-06</td>
<td>28 July 2006</td>
<td>4</td>
<td>Throat swab</td>
<td>Aseptic meningitis</td>
</tr>
<tr>
<td>N2665-TW-06</td>
<td>7 August 2006</td>
<td>4</td>
<td>Throat swab</td>
<td>Aseptic meningitis</td>
</tr>
</tbody>
</table>
another clade (Fig. 1). Pairwise comparisons of the nucleotide sequences of the 16 Taiwan E18 strains showed them to be closely related to each other (0–3.2 % difference in 420 nt sequences); they differed from the E18 and E4 sequences obtained from GenBank by 3.7–23.8 % and 33.1–36.4 %, respectively. The VP1 regions of the Taiwan E18 strains had 98.3–100 % aa similarity; their amino acid sequences varied from the E18 sequences in GenBank by 1.7–6.7 % (data not shown). Within the VP1 140 aa region that we sequenced, a frequent amino acid substitution site was identified as V152S (13/16 strains; position relative to E18 prototype Metcalf strain).

**Structure of the VP1 protein of the E18 isolates**

Three-dimensional structure analysis located the V152S amino acid change in the DE loop structure of the VP1 protein (Fig. 2), which is located on the surface of the virion (Hogle et al., 1985). The genetic variation of the Taiwan E18 isolates hinted at antigenic drift in this outbreak. The results indicated that a genetic variant E18 strain was associated with the 2006 outbreak in Taiwan.

**DISCUSSION**

Human E18 infections may present various degrees of illness, from asymptomatic to aseptic meningitis; the virus can also cause sporadic cases, outbreaks and epidemic infections. This study reported an outbreak of aseptic meningitis in the summer of 2006 in Taiwan. Compared with an E30 outbreak in 2001 in Taiwan (Wang et al., 2002), the rate of hospitalization for E18 infections was lower (38.7 %, 36/93) than that for the E30 infections (73.9 %, 139/188), although the
age groups of severely infected patients were similar. Children of school age were more prone to echovirus infections. Our molecular analysis found that a variant strain E18 with a mutation in the VP1 region was involved in this outbreak.

E18 can be identified from different kinds of specimens. In healthy children, E18 can be found in stool samples, and was found to account for 8.9% of EV isolations in Norway (Witsø et al., 2006). In the 2000–2001 outbreak of E18 in the USA, it became the predominant serotype, accounting for 22.0% of 1863 specimens. Our study found that the detection rate was higher in CSF (78.2%) than in stool samples (50%) and throat swabs (22.9%).

Generally, E18 has been classified as untyped EV, as no commercial mAb was available that recognized E18, except for an EV blend (Chemicon) used for immunofluorescent staining (Oberste et al., 2000). However, false virus identification was noted initially when using the mouse anti-E4 mAb, indicating a cross-reaction of this antibody with E18 strains. Antigenic drift has been shown in E18 resulting in failure to identify current E18 strains by the Lim and Benyesh-Melnick antiserum pool (Bendig & Earl, 2005; Künkel & Schreier, 2000; Oberste et al., 1999b; Wang et al., 2002). Antigenic sites have been reported for other EVs, such as site A, B, and C antigenic variations in the VP1 protein detected in Sabin poliovirus 1, 2 and 3 in 1986 (Minor et al., 1986). Five neutralization epitope sites (Ia, Ib, II, III and IV) were found in swine vesicular disease virus, located in VP1–3 (Borrego et al., 2002; Nijhar et al., 1999). Neutralization sites of E18 have not been reported. Genetic variation of E18 in Taiwan is suggested to have caused antigenic drift in this strain.

The complete nucleotide sequence for the prototype E18 Metcalf strain was determined by Andersson et al. (2002).

Different regions in the EV genome have been evaluated for molecular characterization, including the 5'-untranslated, VP4, VP2 and VP3 regions. However, there was no correlation between genotype and serotype (Andersson et al., 2002; Oberste et al., 1998). By contrast, the VP1 region shows a good correlation between genotype and serotype. We reviewed various EV outbreaks and molecular typing methods (Chambon et al., 2001; Oberste et al., 1999a) used to identify E18 serotypes in recent years (Table 3). In our review of the literature from 1998 to 2006, in addition to the E18 epidemic in the USA during 2000–2001, sporadic cases of E18 infection have been reported in several EV outbreaks associated with aseptic meningitis: in France (one E18 case/33 EVs in 1998–2000; 4/41 in 2005), Brazil (5/162 in 1998–2003), Argentina (2/60 in 1998–2003), Belgium (2/122 in 2000) and Spain (4/116 in 2006). Sequencing of the 5'-end of VP1 and the VP1–2A junction of the partial VP1 gene are the most popular methods in typing of E18 strains; genomic sequencing of EVs can also help identify unknown serotypes if reagents are not readily available for immunofluorescent staining or neutralization tests.

In conclusion, a genetic variant E18 strain was found to be the cause of an aseptic meningitis outbreak in Taiwan in 2006. Molecular typing of the VP1 sequence is a rapid and precise method to identify EV serotypes during epidemics.

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