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ORIGINAL ARTICLE

Molecular diagnosis and clinical presentations of enteroviral infections in Taipei during the 2008 epidemic

Min-Hsiung Lee [a], Li Min Huang [b], Wing-Wai Wong [c], Tsung-Zu Wu [d], Ting-Fang Chiu [e], Luan-Yin Chang [b,*]

[a] *Department of Pediatrics, Cathay General Hospital, Taipei, Taiwan*
[b] *Department of Pediatrics, National Taiwan University Hospital, Taipei, Taiwan*
[c] *Department of Internal Medicine, Kunming Branch, Taipei City Hospital, Taipei, Taiwan*
[d] *Department of Pediatrics, Branch for Women and Children, Taipei City Hospital, Taipei, Taiwan*
[e] *Department of Pediatrics, Zhongxiao Branch, Taipei City Hospital, Taipei, Taiwan*

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Background: In 2008, an epidemic of enterovirus (EV) infection caused hand, foot, and mouth disease (HFMD) and herpangina in children in Taiwan, and some of them died. To establish an early detection and effective management for children with EV71 infection, sensitive molecular diagnostic methods were applied from May to July 2008.
Methods: We used virus isolation, EV71 real-time reverse-transcription polymerase chain reaction (RT-PCR), and viral protein 1 (VP1) RT-PCR followed by direct sequencing to detect EV71 and the other EVs in the infected outpatient or inpatient children. Clinical presentations of children infected with EV71 and other EVs were compared.
Results: From May 2008 to July 2008, 255 swabs were tested by both PCR diagnostic methods. Based on the viral isolation results, the sensitivities of EV71 real-time RT-PCR and VP1 RT-PCR followed by direct sequencing were 71% and 86%, respectively. Among the 221 children who were enrolled for clinical analysis, 73% (161 of 221) had herpangina, and 27% (60 of 221) had HFMD. Coxsackievirus A2 (CA2) was the most prevalent among the identifiable viruses (65%, 104 of 160), followed by EV71 (28%, 45 of 160). EV71 was the most commonly detected virus among the HFMD cases (63%, 38 of 60), whereas herpangina was mainly caused by CA2 (61%, 98 of 161). Of the CA2 cases, 94% (98 of 104) had herpangina, and the most common manifestation of EV71 infection was HFMD with or without complications (84%, 38 of 45). Phylogenetic study revealed that the genotype of EV71 cases during this epidemic was of B5 lineage.

* Corresponding author. Department of Pediatrics, National Taiwan University Hospital, No. 8, Chung-Shan South Road, Taipei 10016, Taiwan.
E-mail address: ly7077@tpts6.seed.net.tw (L.-Y. Chang).

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Conclusion: During the 2008 EV epidemic, most of the HFMD was caused by EV71, whereas herpangina was mainly caused by CA2. Real-time RT-PCR for EV71 is a time-saving and sensitive diagnostic tool.

Introduction

Enteroviruses (EVs) are the main etiological agents of illness in children. They are RNA viruses containing about 7,400 base pairs. The genome encodes 11 proteins, including VP1 to VP4, 2A to 2C, and 3A to 3D. Among these sequences, VP1 is the one that can induce human neutralizing antibody response and correlate well with serotypes.[1] Compared with other EV serotypes, EV71 causes more serious complications of the central nervous system, such as encephalitis, meningitis, and poliomyelitis syndrome. Several large outbreaks of EV71 occurred around the world, including Bulgaria in 1975, Hungary in 1978, Malaysia in 1997, and Taiwan in 1998.[2–5]

Early diagnosis of EV71 infection makes early management and better outcome possible. The traditional diagnostic method for EVs is isolation in cell culture followed by indirect immunofluorescence assay with type-specific monoclonal antibody to verify the serotype. However, it was time consuming. Previous studies of children suggested that the use of the polymerase chain reaction (PCR) assay for EVs has a clinical benefit by reducing antibiotic therapy and avoiding ancillary tests, and also, it reduces hospital-related costs.[6,7] Another report by Tsao et al.[8] revealed that reverse-transcription (RT)-PCR is a good method for identifying EV71 with high sensitivity and specificity.

In 2008, an epidemic of EV infection caused hand, foot, and mouth disease (HFMD) and herpangina in children in Taiwan. Three hundred and seventy cases were confirmed by Centers for Disease Control (CDC) Taiwan as EV infection with severe complications. Three hundred and forty-four (93%) cases were infected with EV71, and 14 cases were fatal. Besides, the most common serotype of EV isolated from contract laboratories was coxsackievirus A2 (CA2), followed by EV71.[9]

To understand the dominant serotype and establish a method for early detection and effective management for children with EV71 infection, we applied molecular diagnostic methods during 2008 EV epidemic and evaluated their accuracies.

Methods

Patients

We enrolled children with the clinical diagnosis of herpangina or HFMD and those with possible EV infections in Taipei, Taiwan, from May 17 to July 23, 2008. This study population was composed of patients in outpatient departments and wards of National Taiwan University Hospital (NTUH), a medical center in northern Taiwan, and three branches of Taipei City Hospital. Throat swabs were collected immediately or within 24 hours after hospitalization by experienced physicians in outpatient departments. These samples were sent to our laboratory for further molecular diagnosis. In the meantime, virus isolation was done for cases from NTUH for comparison. Clinical presentations and ages of children infected with EV71 and other EV serotypes were compared.

Laboratory methods

The swabs were placed in viral transport media. We then drew 200-μL viral transport media for the extraction of RNA. The extraction was performed by using MagNA Pure LC Total Nucleic Acid Isolation Kit (Roche Inc., Mannheim, Germany). We used EV71 real-time RT-PCR and VP1 RT-PCR followed by direct sequencing to detect EV71 and the other serotypes of EV infection in the infected people. One set of primers was used in EV71 real-time RT-PCR, 5'-CAG CRC TYC AAG CYG CTG ARA TTG-3' and 5'-CGC ATT TGY GCG TAA CCW GTT AT-3'. For VP1 RT-PCR followed by direct sequencing, three genogroup-specific degenerate oligonucleotide primers flanking VP1 region were made, including EntAF TNCARGCWGCNGAR ACNGG, EntAR outer ANGGRTTNGTNGMWGTYTGCCA, EntAR inner GGNGGNACRWACATRTAYTG, EntBF GCNGYNGARAC NGGNCACAC, EntBR outer CTNGGRTTNGTNGANGWYTGCC, EntBR inner CCNCCNGGBGGNAYRTACAT, EntCF TNACNG CNGTNGANACHGG, EntCR outer TGCCANGTRTANTCRTCCC, and EntCR inner GCNCCWGGDGGNAYRTACAT. Amplicons of PCR with three sets of VP1-specific primers were purified using the Gel/PCR DNA Fragments Extraction Kit (Geneaid, Taipei County, Taiwan) before sequencing, and direct sequencing was performed with the previous genogroup-specific primers on 377 PE/ABI automatic sequencer (Perkin-Elmer Cetus, Norwalk, CA, USA) with ABI Prism BigDye Termination Cycle Sequencing Ready Reaction Kit (Applied Biosystems, Perkin-Elmer, Foster City, California, USA). The serotype of EVs was inferred by comparison of the partial VP1 sequence with those in the public gene database containing VP1 sequences for the strains of all the 67 human EV serotypes.

Phylogenetic analysis

Phylogenetic and molecular evolutionary analyses were conducted using Molecular Evolutionary Genetics Analysis (MEGA) Version 4.1.[10] The alignment of VP1 sequences from present cases and the reference sequences was performed using the Clustal W method with MEGA Version 3.1. Reference sequences for EV71 prototype BrCr strain of genotype A (accession number U22521); the genotype B strains (U22522, 2289-MAA-97, SHA63VP1, SHA66VP1, 2609-AUA-74, 7673-CT-87, 5865-SINCP1, 5666-SINVP1, SHA89VP1, 5511-SIN-00, 2933-Yamagata-03, S110031-SAR-03); the genotype C strains (S1105-SAR-98, SHA52VP1, SNA71-VP1, 03907-MAA-97, 1245aVP1, KOR-EV71-05, SHZH98VP1, F1-CHN-00, SHZH03-VP1); CA16 prototype G10 strain (U05876); and Poliovirus 1

(V01150), Poliovirus 2 (X00595), and Poliovirus 3 (X00596) were obtained from the GenBank and used in our phylogenetic analysis.

The tree was outgroup rooted with the strain of CA16 prototype G10 strain (U50876), and it was built by using the Neighbor-joining method and bootstrap analysis with 1,000 bootstrap replications, and p-distance substitution model was used to evaluate the strength of the topologies by MEGA Version 4.1. The genetic distance was calculated by a pairwise estimation of percent divergence among the sequences. The genotypes of EV71 followed the classification of Brown et al.'s study[11] and the subgenogroups of EV71 followed the grouping of Cardosa et al. and Huang et al.'s studies.[12,13]

Statistics

Comparisons of selected parameters were performed by Chi-square test with Yates' correction for categorical variables and by Student *t* test for the continuous variables. A *p* value less than 0.05 was considered statistically significant.

Results

Demography and diagnosis

From May 17, 2008, to July 23, 2008, a total of 255 swab specimens from 235 patients were tested by EV71 real-time RT-PCR and VP1 RT-PCR followed by direct sequencing. The case number for PCR diagnosis in our laboratory peaked in June with average number of 4.3 cases per day. Because only 14 cases of NTUH had positive EV71 results of virus culture, we can only calculate the sensitivity of these 14 cases. Based on these viral isolation results, the sensitivities of real-time EV71 RT-PCR and VP1 PCR followed by direct sequencing were 71% (10 of 14) and 86% (12 of 14), respectively. The specificity of real-time EV71 PCR was 96% (115 of 120) according to the results among cases infected with viruses other than EV71.

We exclude seven cases whose age was greater than 18 years. The diagnosis of pediatric population is shown in Fig. 1. The number of cases without HFMD or herpangina is far less than that with HFMD or herpangina. Among these, seven cases had neither herpangina nor HFMD; therefore, we investigated the clinical data of 221 patients.

The age distribution is shown in Fig. 2 except for nine children who lacked the data for age. The mean age (±standard deviation) of the 212 patients with the diagnosis of herpangina or HFMD was 3.69 ± 2.37 years. The median (range) age at the time of infection diagnosis was 3.45 years (from 65 days to 11.79 years). Most cases (87%) occurred in children who were aged 6 years or younger.

Children with EV71 were slightly younger relative to children with CA2. However, the difference was not significant ($p > 0.05$) (Table 1). The age distribution also has no significant difference between the herpangina group and the HFMD group (Table 2).

Serotype distribution

Among these EVs identified by VP1 molecular serotyping, CA2 was the most prevalent (104 of 160, 65%), followed by EV71 (45 of 160, 28%); CA16 (3 of 160, 2%); CA10 (3 of 160, 2%); CB4 (3 of 160, 2%); CA4 (1 case); and CA6 (1 case).

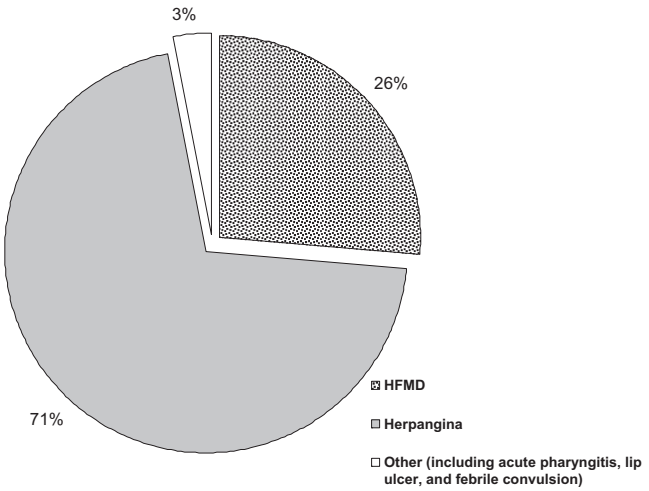

Figure 1. Distribution of diagnosis ($n = 228$). The HFMD cases with neurological complications ($n = 9$) were included in the sector of HFMD. Other cases included acute pharyngitis ($n = 4$), lip ulcer ($n = 1$), and febrile convulsion ($n = 2$). HFMD = hand, foot, and mouth disease.

Among these 221 children, 72.9% (161 of 221) had herpangina, and 27.1% (60 of 221) had HFMD. Distribution of EV serotypes that was identified among the HFMD and herpangina cases is shown in Fig. 3. No virus was detected in 59 children. During this epidemic, 63% of HFMD was caused by EV71, 10% by CA2, 5% by CA16, 2% by CA6, and 2% by CA10; 61% of herpangina was caused by CA2, 4% by EV71, 2% by CB4, 1% by CA4, and 1% by CA10. Therefore, EV71 was the most commonly detectable virus among HFMD cases (38 out of 60, 63%), and herpangina was mainly caused by CA2 (98 of 161, 61%).

Of all the CA2 cases, 94% (98 of 104) had herpangina, and the major manifestations of EV71 infection were HFMD with or without complication (38 of 45, 84%). The difference is statistically significant ($p < 0.01$).

Complications

Nine children with HFMD, eight of whom were infected by EV71, suffered from central nervous system involvement. All the eight EV71-infected cases developed myoclonic jerk. Of them, three cases were presented as HFMD and

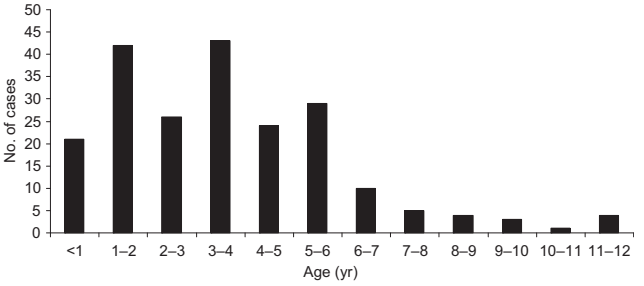

Figure 2. Age distribution of patients.

Table 1 Age distribution of CA2 and EV71

	CA2 ($n = 104$)	EV71 ($n = 43$)	p
Age, yr	3.80 ± 2.23	3.38 ± 2.20	0.11

CA2 = coxsackievirus A2; EV71 = enterovirus 71; SD = standard deviation.

Table 2 Age distribution of herpangina and HFMD

	Herpangina ($n = 153$)	HFMD ($n = 59$)	p
Age, yr	3.74 ± 2.48	3.54 ± 2.05	0.38

Data were expressed as mean ± SD and analyzed with Student t test.
CA2 = coxsackievirus A2; HFMD = hand, foot, and mouth disease; SD = standard deviation.

myoclonic jerks and treated with supportive care only. Another one case had headache, fever, and vomiting. The Cerebrospinal fluid (CSF) pleocytosis was found. He was diagnosed of HFMD with aseptic meningitis and discharged home without sequelae.

The remaining four children had brainstem encephalitis plus cardiopulmonary failure, and all received mechanical ventilation, intravenous immunoglobulin, and milrinone according to the guidelines of CDC Taiwan. The duration of hospitalization ranged from 17 days to 65 days. One child recovered completely, and two cases had sequelae of limb weakness and central hypoventilation needing racheostomy and ventilator support. One child died of subsequent sepsis after coma for 5 weeks.

Phylogenetic study revealed that the genotype of EV71 cases during this epidemic was of B5 lineage (Fig. 4). All the other cases did not have complications.

Discussion

During the epidemic of EV infection in 2008, 370 cases were reported to CDC Taiwan as EV infection with severe complications. However, few publications described the epidemiological data of 2008 epidemic in Taiwan, except Chen et al.[14] Furthermore, the number of cases of complicated EV infection in 2008 reached its peak in June, which was covered by our study period. Our study described the characteristics of this epidemic in northern Taiwan. At the same time, because EV71 can cause severe complications, we established a rapid and reliable method to detect EV71, which may be helpful for the prevention and control in the future.

In contrast with previous years, the most common serotype in 2008 was CA2, although our samples were mainly collected from children in northern Taiwan. Even the CA2 is the dominant serotype; no CNS complication was reported in children infected by CA2.

Because only data of age and diagnosis were available in our study, we can only describe the primitive characteristics of CA2 infection. There were 107 children infected with CA2, including 98 who presented with herpangina (92%), six suffered from HFMD (6%), two had febrile convulsions, and one child suffered from pharyngitis. The age distribution ranged from 2 months to 11 years, and the mean of age was 3.80 ± 2.23 years in our study. Chen et al.[13] reported the comparison of clinical features between CA2 and EV71 in 2008. Among 183 CA2-infected cases, most also presented with herpangina (83.6%), and the mean age was 2.1 years in their study population, which was younger than that of our cases infected with CA2.

Although CA16 has a main role in causing HFMD, only three children with HFMD had a positive result for CA16 infection in our population in 2008. Our analyses provided a snapshot of the relationship between EV serotypes and predominant clinical presentations, but detailed prospective surveillance would be needed to clarify these associations.

Previous data suggest that the prevalence of human EV infections has been underestimated in epidemiological studies based on virus culture.[15] The sensitivity and specificity of PCR were affected by many factors, including primer–probe set, extraction method, instrument, and skill of the performers. One hundred percent sensitivity of PCR had been reported by using clinical isolates from virus laboratory.[8] Most of the literatures used CSF as the clinical specimen, and a few studies used throat swabs. One study revealed that the sensitivity and specificity were 95% and 81%, respectively, when compared with viral culture and using throat swabs as the clinical specimen.[16]

However, compared with PCR in CSF and/or serum specimens, detection of EV RNA in throat swabs showed a sensitivity of 62.5% and a specificity of 75.6%.[17]

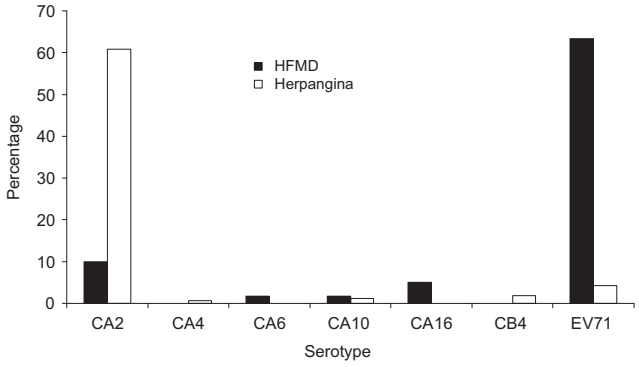

Figure 3. Serotype distribution between HFMD and herpangina. CA2 = coxsackievirus A2; CA4 = coxsackievirus A4; CA6 = coxsackievirus A6; CA10 = coxsackievirus A10; CA16 = coxsackievirus A16; CB4 = coxsackievirus B4; HFMD = hand, foot, and mouth disease.

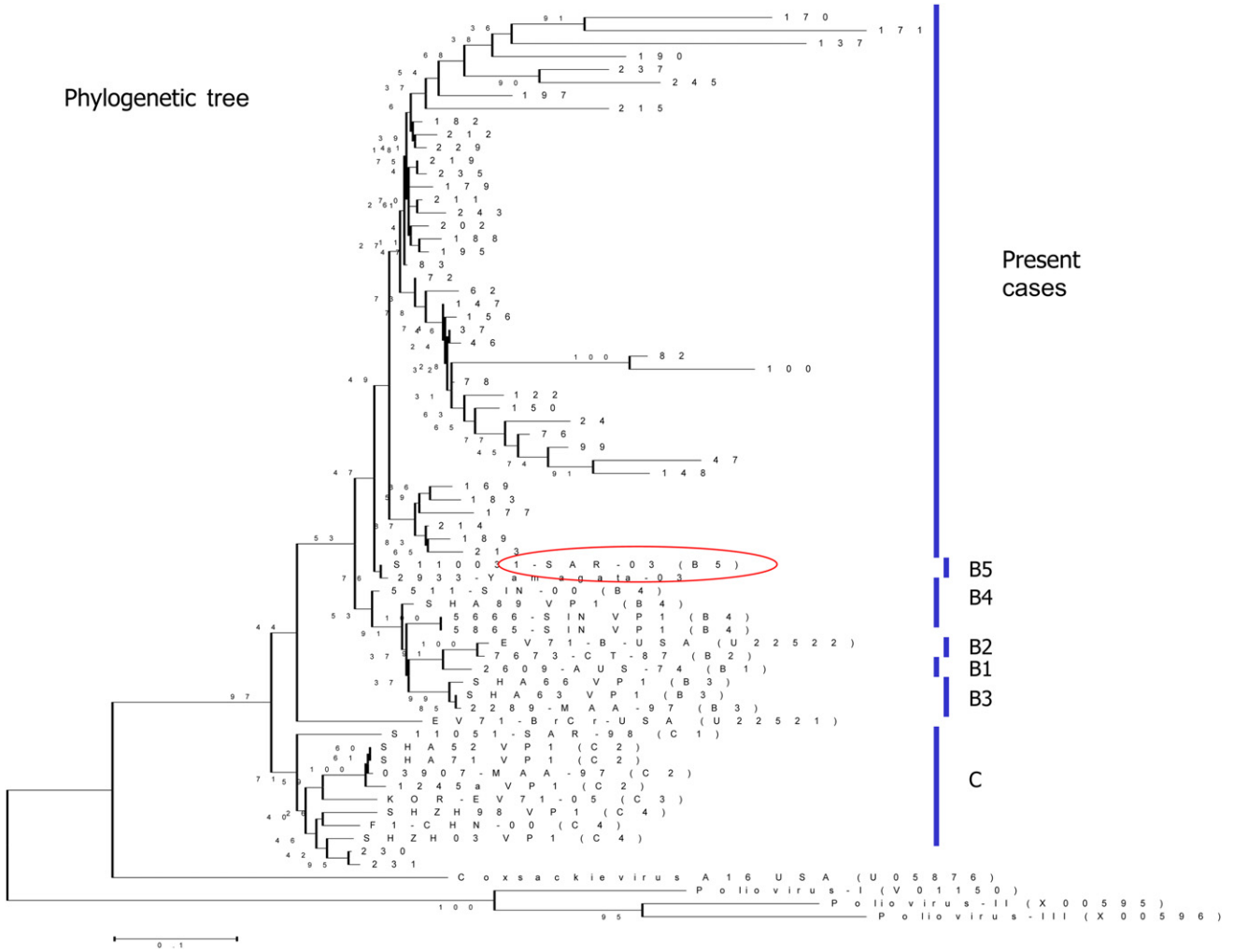

Figure 4. Phylogenetic tree of enterovirus EV71 of the present cases and some reference strains.

The possible explanation for our sensitivity (71%) is that, sometimes, swabs for PCR do not catch virus adequately, whereas swabs for culture do. Certainly, the extracted clinical specimens could contain impurities that inhibit nucleic acid amplification steps. Our sensitivity could be better by means of obtaining multiple samplings in each case. Although PCR is a rapid and sensitive tool, it was also limited by the lack of standardization and inability to detect coinfection with two or more virus species.

Earlier data showed that most (93%) patients with HFMD/herpangina were very young children (<4 years).[18] In our data, only 62% cases were younger than 4 years, but 87% cases were younger than 6 years.

Conventional cell culture was the traditional method for the diagnosis of EV infection. However, it is time consuming as the observation of characteristic cytopathic effect takes 2—7 days or longer. In addition, certain EV serotypes do not replicate in common cell culture. RT-PCR-based testing for EV in throat or rectal swabs offers a solution to this problem by allowing rapid accurate diagnosis of children with EV infection.

We had applied PCR methods in CSF specimen in three cases. Two HFMD cases caused by EV71 infection had pleocytosis in CSF but both CSF virus culture and CSF PCR revealed negative results. Another one case of herpangina with myoclonic jerk had normal CSF cell count, and both CSF virus culture and CSF PCR revealed negative results. Although we have not evaluated the sensitivity and specificity for CSF yet, this PCR method can theoretically be used in CSF specimens.

In conclusion, the major circulation serotypes during 2008 epidemic in Taiwan were CA2 and EV71. CA2 caused most of herpangina, whereas EV71 caused most of HFMD. We developed a real-time RT-PCR to detect EV71, and we hope EV71 transmission will be limited through this rapid diagnosis of real-time RT-PCR and the preventive measures.

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