Original Article

Comparison of the Detection Rates of RT-PCR and Virus Culture Using a Combination of Specimens from Multiple Sites for Enterovirus-Associated Encephalomyelitis during Enterovirus 71 Epidemic

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SUMMARY: Enterovirus (EV) 71 may cause severe neurological illness in the pediatric population. The present study aimed to compare the detection rates of reverse transcription-polymerase chain reaction (RT-PCR) with pan-EV/EV71 type-specific primers and virus culture (VC) for the identification of EV and EV71 using specimens from multiple sites. In total, specimens from throat/rectal swabs, cerebrospinal fluid (CSF), and blood from 66 patients diagnosed with EV encephalomyelitis were subjected to both RT-PCR and VC for detecting the presence of pan-EV and EV71. The results revealed that the positive RT-PCR rate was higher in throat swabs (60.6%) and rectum swabs (50.0%) than in CSF (16.7%) and blood (15.6%). The same trend was also observed in case of VC: throat swabs (22.7%), rectum swabs (10.6%), and blood (3.0%). The detection rate of EV encephalomyelitis by RT-PCR was 90.9% for all samples, 63.6% of which were subtyped as EV71. The detection rates of RT-PCR were superior to those of VC, and identification using specimens from throat/rectal swabs yielded higher positive results. These findings may help physicians to identify the etiologies at an early stage during EV71 epidemics and to make emergent medical decisions for minimizing patient morbidity and mortality.

INTRODUCTION

Enterovirus (EV) usually causes mild and self-limiting infections. However, it may lead to serious infections, including central nervous system (CNS) infections (1). EV71 infections often present as febrile illness and mucocutaneous manifestations with a benign course in the pediatric population (2). It may cause brain stem encephalitis and myelitis preceded by herpangina/hand-foot-mouth disease (HFMD); some affected children experienced complications with brain stem encephalitis or acute flaccid paralysis, leading to neurological pulmonary edema/hemorrhage (3,4). The brain stem dysfunction frequently presents as cranial nerve dysfunction, tachycardia/hypertension, apnea, and myoclonic jerks with preserved consciousness (5,6). The substantial neurological comorbidity and mortality has caused large psychological burdens to parents since the epidemic outbreak in Taiwan and pan-Asia since 1998 (7,8).

To assess the neurological complications caused by EV71 infections, the diagnosis is based on clinical

manifestations, pleocytosis in cerebrospinal fluid (CSF), and brain stem lesions on neurological images and is confirmed by positive virus cultures (VC) or serology tests (3). VC can be attempted using various clinical samples, including throat/rectal swabs, serum, CSF, vesicles, and ulcers (9,10). Although VC is the standard method for confirming a viral infection, laboratory assays by VC are not always positive in the acute stage of the illness. Reverse transcription-polymerase chain reaction (RT-PCR) has been used to detect EV RNA for a rapid diagnosis of clinical EV infections (1,10,11). In addition, it is useful for the rapid detection of EV71 with neurological involvement (12,13). The rapid establishment of EV etiology enhances the accuracy of diagnosing EV71 infection complicated with neurological involvement and leads to better patient management (14,15). Early diagnosis can also help limit the spread of the disease in the community during an outbreak (1,16).

The aims of this study were to compare the detection rates using different methodologies and specimens from multiple sites to identify EV-infected patients with encephalomyelitis. Early identification of cases of EV71 during epidemics enables physicians to make better and more timely medical decisions for patients and to minimize potential morbidity and mortality.

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MATERIALS AND METHODS

Patients diagnosed with herpangina or HFMD complicated with encephalomyelitis during EV71 epidemics were enrolled in the present study. All the patients were admitted to medical centers in Taichung, Taiwan. Diagnoses of EV-associated neurological involvement during the EV71 epidemic were made by pediatricians on the basis of laboratory tests and neurological images.

EV-associated encephalomyelitis was defined as follows: 1, a clinical diagnosis of herpangina/HFMD; 2, positive neurological signs; and 3, positive pleocytosis. Positive neurological signs were defined as neurological signs of ataxia, myoclonic jerks, cranial nerve dysfunction, or evidence of brain stem involvement from magnetic resonance imaging according to the definition of the Centers for Disease Control (CDC), Taiwan (<http://www.cdc.gov.tw/>). Positive pleocytosis is defined as more than 5 white cells per cubic millimeter and the absence of bacteria in laboratory tests. Cases who met these criteria were enrolled into the study, and all provided specimens for both RT-PCR and VC from 4 sites, i.e., of throat/rectal swabs, CSF, and blood samples, for detecting pan-EV and EV71 (Figure 1).

We investigated the applicability of RT-PCR using pan-EV and type-specific primers for the direct detection of pan-EV and EV71 using different specimens (Mission Biotech, Taiwan). Extracted viral RNA was reverse transcribed to cDNA and amplified by RT-PCR. The appropriate primers of target genes were designed for the human-EV71 isolate 4643-TW98 polyprotein gene (GenBank no. JN544418.1). The 2 primer pairs used for the 5'-UTR-1 region of the pan-EV genome were sense 5'-ACCTTTGTGCGCCTGTTT-3'/antisense 5'-GGATGGCCAATCCAATAGC-3' and 5'-UTR-1-1 of the sense 5'-AAGCACTTCTGTTTCC-3'/antisense 5'-CATTTCAGGGGCCGAGGA-3'. Two other primer pairs used for EV71 in the VP1 region were sense 5'-TGGCAGATGTGATTGAGAGTT-3'/antisense 5'-TGAACAGCTCCACCTTTC-3' and VP1-1 of the sense 5'-AGTGATGAGAGTATGATTGA-3'/antisense 5'-TTATGTCTATGTCCCAGTT-3'. All samples were amplified through 35 cycles of denaturation, annealing and extension in a thermal cycler. Identity of the causative pathogen was based on the PCR product size resolved on 2% agarose gel. Samples with successful amplification of 574 and 297 base-pair products from the 5'-UTR region of the viral genome were defined as being positive for pan-EV (Figure 2A). Subsequently, samples with 2 positive bands of 368 and 180 base pairs amplified from the VP1 region were defined as being positive for EV71 (Figure 2B). A positive and a negative control were amplified in parallel and run on the same gel for comparison.

Specimens of VC were collected in transport medium and inoculated on monolayer A549 cells, RD cells, green monkey kidney cells, HEp-2 cells, and VERO cells within 24 h. When the EV cytopathic effect involved more than 50% of the cell layer, the cells were scraped and indirect fluorescent-antibody staining (Millipore, Bedford, MA, USA) with pan-EV antibody was performed to identify EV (Figure 3). These isolates were subsequently identified as EV71 by immunofluorescence with EV71 monoclonal antibody. The Institu-

Fig. 1. Flow chart of identify Pan-EV and EV71 by RT-PCR and VC.

tional Review Board of the Chung Shan Medical Hospital and China Medical University Hospital approved the study, and the parents of all the participants provided informed consent.

Statistical methods: Nonparametric data were assessed using the Mann-Whitney U test and expressed as medians and ranges. Differences between RT-PCR and VC methods were assessed using the chi-square test. Unconditional logistic regression was used to estimate the odds ratios (ORs) and 95% confidence intervals (95% CIs) for the risk of neurological complications between HDMD and herpangina. All statistical analyses were performed using SAS software version 9.1 (SAS Institute Inc., Carey, NC, USA), and the significance level was set at 0.05 in 2-tailed tests.

RESULTS

Sixty-six patients (male:female = 40:26, HFMD: herpangina = 55:11) met the criteria of EV encephalomyelitis during the EV71 epidemic and were enrolled into this study (Table 1). By RT-PCR, 60 (90.9%) were found to be positive for pan-EV and 42 (63.6%) were subsequently found to be positive for EV71. The remaining specimens from 6 (9.1%) patients were all negative. VC showed a similar trend, with 24 (36.4%) patients found to be positive for pan-EV and 18 (27.3%) found to be positive for EV71. Therefore, the result revealed a higher yield by RT-PCR than by VC for detecting EV ($p < 0.001$), EV71 ($p < 0.01$), and non-EV71 ($p < 0.001$) (Table 2). Overall, RT-PCR takes 4 days (range: 0–16) and VC takes 25.5 days (range: 3–322). By comparison, results of RT-PCR are significantly rapid than those of VC ($p < 0.001$).

With regard to the detection of EV71 or non-71EV in-

Fig. 2. Visualization of nested RT-PCR products of pan-enterovirus and EV71 by gel electrophoresis. Figure 2A. RT-PCR products of pan-enterovirus: DNA ladder molecular mass marker was included in lane 1 and 5. Lanes 2 and 6 represent positive control. Lanes 3 and 7 represent negative control. Lanes 4 and 8 represent RT-PCR-amplified products is 574 (First RT-PCR; Using of the 5'-UTR1-1 S/AS primer) and 297 b.p. (Nested PCR; Using of the 5'-UTR1-1 S/AS primer), respectively. Figure 2B. RT-PCR products of enterovirus 71: Lane 1. DNA ladder molecular mass marker was included in lane 1. Lanes 2 and 6 represent positive control. Lanes 3 and 7 represent negative control. Lanes 4 and 8 represent RT-PCR-amplified 368 (First RT-PCR; Using of the VP-1 S/AS primer) and 180 b.p. (Nested PCR; Using of the VP-1 S/AS primer).

Fig. 3. (Color online) Enterovirus detected by indirect fluorescent antibody. RD cells were infected and stained with antibody to enterovirus. Positive reaction appears as green fluorescence (arrow).

fection from specimens from individual sites, identifications of EV from throat swabs and rectal swabs by RT-PCR were predominantly higher than those from CSF and blood. As shown in Figure 4, the detection rates of EV71 by RT-PCR and VC were 40 (60.6%) and 15 (22.7%), respectively, from throat swabs ($p < 0.001$); 33 (50.0%) and 7 (10.6%), respectively, from rectal swabs ($p < 0.001$); 11 (16.7%) and 0, respectively, from CSF ($p = 0.001$); and 10 (15.2%) and 2 (3.0%), respectively, from blood ($p = 0.008$). In comparison with the use of throat swabs ($n = 40$, 60.6%) for RT-PCR, the use of rectal swabs ($n = 33$, 50.0%) did not show a significant difference ($p = 0.946$); however, the differences were significant when CSF ($n = 11$, 16.7%,

Table 1. Demographic and clinical manifestations of EV71 encephalomyelitis ($n = 66$)

Characteristic	No. (%)	Mean (SD)
Male/female	40/26	
Age (mo)		
Male		23.9 (15.4)
Female		24.2 (15.9)
HFMD/Herpangina	55/11	
Symptom and sign		
Pyretic illness	60 (90.9)	
Myoclonic jerks	47 (71.2)	
Vomiting	19 (28.8)	
Lethargy	19 (28.8)	
Ataxia	15 (22.7)	
Seizure	8 (12.1)	
Tachycardia	55 (83.3)	
Hypertension	43 (65.2)	

Table 2. Results of RT-PCR and VC for EV, and EV subtyping ($n = 66$)

EV species	RT-PCR (%)	VC (%)	p -value
EV71	42 (63.6)	18 (27.3)	< 0.01
Non-71EV	18 (27.3)	6 (9.1)	< 0.001
Total	60 (90.9)	24 (36.4)	< 0.001
Time (day)	4.0 (0–16.0)	25.5 (3–322.0)	< 0.001

EV71, enterovirus 71; Non-71EV, enterovirus species other than EV71; RT-PCR, reverse transcription-polymerase chain reaction; VC, virus culture.

Time: duration between specimens sampling and results obtained. Data are shown as number (percentage) or medians (ranges).

Fig. 4. Results of EV identification with combination of specimens from multiple sites of throat swabs, rectal swabs, CSF, and blood ($n = 66$). CSF, cerebrospinal fluid; Non-71 EV, enterovirus species other than EV71.

Table 3. Compare the results of EV71 by RT-PCR and VC from different sites of specimens ($n = 66$)

Specimen	Result	VC + (%)	VC - (%)	p-value ¹⁾
Throat	RT-PCR +	13 (19.7)	27 (40.9)	<0.001
	RT-PCR -	2 (3.0)	24 (36.4)	
Rectal	RT-PCR +	5 (7.6)	28 (42.4)	<0.001
	RT-PCR -	2 (3.1)	31 (47.0)	
CSF	RT-PCR +	0 (0)	11 (16.7)	N/A
	RT-PCR -	0 (0)	55 (83.3)	
Blood	RT-PCR +	1 (1.5)	9 (13.6)	<0.05
	RT-PCR -	1 (1.5)	55 (83.3)	

¹⁾: McNemar test.

CSF, cerebrospinal fluid; +, positive of EV71; -, negative of EV71; N/A, not available.

$p < 0.001$) and blood ($n = 10$, 15.2%, $p < 0.001$) were used. In comparison with the use of throat swabs ($n = 15$, 22.7%) for VC, the use of rectal swabs ($n = 7$, 10.6%) did not show a significant difference ($p = 0.062$); however, the differences were significant when CSF ($n = 0$, $p < 0.001$) and blood ($n = 2$, 3.0%, $p = 0.001$) were used. In case of EV71, as shown in Table 3, RT-PCR was more sensitive than VC using throat swabs ($p < 0.001$), rectal swabs ($p < 0.001$), and blood ($p < 0.05$). Figure 5 shows the identification of EV encephalomyelitis by RT-PCR and VC from multiple sites. With regard to the numbers of positive results, the positive rates of RT-PCR versus VC were 13.6% versus 22.7% with 1 positive result, 51.5% versus 12.1% with 2 positive results, 15.5% versus 1.5% with 3 positive results, and 10.6% versus 0 with 4 positive results from different sites ($p = 0.018$, $p < 0.001$, $p < 0.001$, and

$p < 0.001$, respectively). Based on cumulative results, 90.9% of cases of EV infections were identified by RT-PCR and 36.4% were identified by VC ($p < 0.001$).

DISCUSSION

The neurological signs with regard to cortical function as a result of EV71 encephalomyelitis are usually subtle, except for brain stem dysfunction. The missing signs of EV71 encephalomyelitis resulted in a large comorbidity and a consequent large psychological burden to parents (17). Pediatricians in Taiwan experienced the consequent impacts on patient management during the EV71 epidemic (5-7). The Taiwan CDC had established a virological surveillance system for EV and concluded that EV71 was one of the most common serotypes in Taiwan during the epidemics and was associated with severe and fatal cases in young children (18,19). The clinical and laboratory-based system continues to aid clinical physicians and enable the earlier implementation of disease control for limiting the spread of EV71 (<http://www.cdc.gov.tw/>).

The strength of the present study depends on routine testing by RT-PCR and using combination of specimens from all 4 sites during EV71 epidemics from each patient. The paired primers for pan-EV and EV71 may minimize false-positive results and enhance specificity in the identification of epidemic EV71. EV71 plays a major role in causing severe neurological complications. In addition, other non-71EV viruses such as coxsackie or echo virus may have had a similar critical neurological involvement during the EV71 epidemic in the present study (18,19). With regard to clinical manifestations, EV71 cases are more likely to present with HFMD

Fig. 5. Numbers of positive results by RT-PCR and VC in identification of EV with specimens from multiple sites ($n = 66$).

than herpangina. We evaluated the patients with herpangina and HFMD from pediatric clinics, and found a significant risk of neurological complications in patients with HFMD (20). Although the common benign forms of EV diseases are well recognized by clinicians, the early identification of herpangina and HFMD can help predict and prevent morbidities from EV71 infections (21,22). In addition, early recognition of the pathogen during EV71 epidemics using different methods and specimens collected from different sites helps the clinical physicians to get more information for making decisions.

Throat swabs were proposed to be more a recognizable site than rectal swabs for the identification of EV71 infection, suggesting that the oral-oral route may be a more important route of transmission than the fecal-oral route (18,22,23). In contrast, the present study compared the identification of specimens from multiple sites and showed that throat and rectal swabs are more sensitive than CFS and blood. This may imply that both the oral-oral route and the fecal-oral route are the main transmission routes for acute EV71 illness. Moreover, RT-PCR is more sensitive in identifying cases of EV71 than VC using specimens from each site, which may imply the practical utility in the identification of acute EV71 illness (21).

The laboratory diagnosis of EV infection is primarily based on VC or molecular detection of the viral nucleic acid using specimens from various sites. Traditionally, VC is the gold standard to confirm the diagnosis because the procedure is highly specific and reliable for EV typing (1). Various cell lines can be used to identify cytopathic effects. However, typing by immunofluores-

cence is laborious, and the results are reliable only during the acute phase of the illness, which results in low positivity even when combining specimens collected from different sites (21). Once a virus is detected from a patient's specimen, there is no question regarding whether the result is truly positive. However, when a virus is not detected from a specimen, there are always questions about the techniques used in obtaining that negative result and whether the results is truly negative. It is known that culture depends on the presence and viability of the virus in the specimen.

RT-PCR is considered to be significantly better than VC in diagnosing viral infections. PCR was developed as a technique to amplify nucleic acid, and it only requires small amounts of clinical specimens. The procedure is rapid and sensitive and is well suited to identify infectious pathogens (24). RT-PCR has been applied to detect EV RNA with direct and rapid diagnosis of epidemic EV71 infections since the 1998 outbreak in Taiwan (25). In EV studies, the confirmation of EV associated with neurological diseases is typically performed by RT-PCR using CSF specimens because it is more sensitive and more rapid than VC. For the diagnosis of EV complicated with neurological diseases, the sensitivity and specificity of EV PCR have been reported to be 100% and 83%, respectively, in CSF (9). However, CSF specimens may not be the only optimal choice for the detection of EV71 by PCR or VC (26). Hosoya et al. (27) detected EV using CSF samples from patients with CNS infections by PCR and VC and demonstrated a higher sensitivity by PCR, with 39.1% EV infections being detected by PCR and only 8.7% by VC. Singh et al. (28) detected EV71 by RT-PCR and

found sensitivity rates of 71% and 53% for pan-EV and EV71, respectively.

A combination of specimens from multiple sites should be used for laboratory investigations according to the disease manifestations. Studies of EV infections complicated with CNS infections show that the yields are frequently lower in CSF and blood (9,21). Possible explanations include the virus being transiently present in CSF and blood and a lower amount of virus being present in CSF and blood, making detection difficult. The identification of epidemic EV71 infections relies on throat and rectal swabs because they are more useful specimens in detecting EV by either PCR or VC (15,21). In the present study, the detection rate was 51.5% in 2 specimens by RT-PCR and 22.7% in 1 specimen by VC; this reflects the differences in sensitivity between the methods, and using specimens from multiple sites helps identify the pathogen.

Molecular testing does not depend on the viability of a virus to be detected; therefore, less emphasis is placed on transport and the timing between specimen collection and processing. However, more emphasis is placed on the method itself. It becomes very important to describe the multiple positive and negative controls included in the testing to confirm that the system detects the specific virus desired and differentiates that virus from other viruses. It is important to include the results of known positive and negative specimens tested along with the results of the patient's specimens. The sensitivity and specificity of the 2 methods were not determined in the present study; the results of the 2 methods were discrepant. The problems associated with the discrepant results may be resolved by re-testing those specimens using a third method. Specimens that are positive using 2 of 3 methods are considered true positives.

The findings of our study may help in comparisons between the different methods, encourage the clinical physicians to diagnose the disease at an early stage, and consequently support early decisions for the management of cases.

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Conflict of interest None to declare.

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