Viral Load in Blood Is Correlated with Disease Severity of Neonatal Coxsackievirus B3 Infection: Early Diagnosis and Predicting Disease Severity Is Possible in Severe Neonatal Enterovirus Infection

Meng-Hsiu Yen,1 Kuo-Chien Tsao,2,3 Yhu-Chering Huang,1,3 Chung-Guei Huang,2 Ya-Ling Huang,2 Reyin Lin,1,3 Mei-Ling Chang,1 Chi-Chia Huang,2 Dah-Chin Yan,1,3 and Tzou-Yien Lin1,3

1Department of Pediatrics, Chang Gung Children’s Hospital, 2Department of Clinical Pathology, Chang Gung Memorial Hospital, and 3Chang Gung University School of Medicine, Taoyuan, Taiwan

We conducted a study during an outbreak of coxsackievirus B3 infection in 2005 and found that viral RNA could be detected in patients’ blood specimens soon after the onset of fever, and the level of viral RNA was positively correlated with disease severity. Timely diagnosis is possible in severe neonatal enterovirus infection.

Manifestations of enterovirus infection in neonates range from unapparent infection to overwhelming illness and even death. Virus serotype, mode of transmission, and presence of passively acquired, serotype-specific maternal antibody correlate with the severity and outcome of perinatally acquired enterovirus infection [1, 2]. Treatment of neonatal enteroviral infection is primarily supportive. Intravenous (IV) IgG is advocated for treatment of neonatal enterovirus infection, but its benefits are still debated [3–5]. The antiviral agent pleconaril is a potential treatment for enterovirus infection in neonates or infants, but the experience is still limited [6, 7].

Viremia plays an important role in the pathophysiology of enteroviral infection. Investigation of the role of viremia in the clinical manifestations of enterovirus infection is not easy to perform using the traditional method of virus isolation. Because of the transient character of viremia and the large amount of blood needed for virus isolation, the use of viremia in diagnosis of enterovirus infection is limited. Development of molecular techniques made the detection of viral RNA in blood or CSF practical. Use of RT-PCR during the first few days of illness offers potential benefits for early diagnosis, timely treatment, and cost savings for infants with mild enterovirus infection [3, 8]. Nonetheless, the correlation between disease severity and blood viral load by real-time RT-PCR had not been assessed in neonates with enteroviral infection. During May–August 2005, an outbreak of coxsackievirus B3 (CVB3) infection occurred in Taiwan, offering an opportunity to conduct this study to assess the correlation between disease severity and viral load with the help of modern molecular techniques.

Patients and methods. A total of 97 infants and children with culture-proven CVB3 infection were treated at Chang Gung Children’s Hospital (Taiwan) during May–August 2005. A total of 24 neonatal patients were noted, and among them, 11 were <7 days of age. Among all of the neonatal patients, 11 (10 of whom were aged <7 days) were classified as having severe infection. Severe infection was defined as the presence of hepatitis (serum aspartate aminotransferase and alanine aminotransferase levels of >3 times the upper limits of normal for the age) and coagulopathy (platelet count, <100,000 platelets/μL, plus an abnormal coagulation profile) [1, 9]. On the other hand, the remaining 13 neonates (only 1 of whom was aged <7 days) were classified as having mild infection, which included nonspecific febrile illness or viral meningitis.

During the study period, all of the parents of the patients admitted to the newborn care center (where infants aged <3 months are treated) were contacted if enterovirus infection was confirmed by culture or was highly suspected (e.g., if there was a family history of enterovirus infection or if maternal illness around the time of delivery was noted). All together, 10 patients, including 6 neonates and 4 infants aged 1–2 months, were included in this study, because written consent was available. Blood samples (1.5 mL in an EDTA tube) obtained for measurement of other parameters during hospitalization and outpatient follow-up were also used for viral load measurement. Follow-up throat and rectal swabs for virus isolation and identification were obtained during each visit to the outpatient clinic until enterovirus disappeared. Treatment of each patient was independently directed by the attending neonatologists.

Isolation of enterovirus and identification of CVB3 was performed using standard procedures for respiratory and enteric pathogens. The protocol for real-time RT-PCR was modified...
from that used by Watkins-Riedel et al. [10]. Briefly, viral RNA was extracted from samples of whole blood using the QIAamp viral RNA Mini kit (Qiagen). A 1-step RT-PCR kit (Qiagen) was used, and the position of the primer was the conserved region of the 5′-noncoding region of the enterovirus. The primer and fluorescent probe sequences were (1) forward primer, 5′-CCCCTGAATGCGCTAATCC-3′; (2) reverse primer, 5′-CAATTGTCAAGGATAACGCGGCC-3′; and (3) probe sequence, 5′-FAM-CAGGACACCCAAAATGCTCGTTCC-TAMRA. Real-time RT-PCR was performed using the Bio-Rad iCycler (Bio-Rad). Thermal cycling conditions consisted of 30 min at 48°C for reverse transcription and 10 min at 95°C for activation of Taq polymerase, followed by 40 cycles of 15 s at 95°C and 1 min at 58°C. The amplified PCR product size was 157 bp. For quantitative enteroviral RNA determination, cDNA of a CVB3 standard was cloned by the Escherichia coli system and was performed in parallel with specimens for real-time RT-PCR. The detection limit of CVB3 was 20 copies/μL by real-time RT-PCR.

Mann-Whitney tests (Wilcoxon rank sum tests) were used for testing the difference in viral loads between severe and mild enterovirus infections. Kaplan-Meier estimate (log rank test) was used to test the difference of persistence of viremia between severe and mild infections.

Results. Demographic data, laboratory data, blood viral RNA level, and methods used for treatment of the 10 patients are shown in table 1. Among the 6 neonates included in this study, 5 had severe disease (patients 1–5), and 1 had mild disease (patient 6). The other 4 infants (patients 7–10) were 1–2 months of age, and all had mild disease. Fever was noted in all 10 patients, and empirical antibiotic therapy was given to all of the patients at admission to the hospital. All 5 patients with severe disease (patients 1–5) received blood component transfusions, including platelets, packed RBCs, fresh frozen plasma, and IV IgG infusions. The patients with mild disease (patients 6–10) received supportive care only. All 10 patients were discharged in stable condition, and no complications were noted at their 6-month follow-up visits.

Because the duration of the period between disease onset and hospitalization varied, the timing of blood sampling for viral RNA determination was inconsistent in each case (table 1). Nonetheless, we noted that viremia was detected soon after disease onset. In all patients, the highest viral RNA load was detected in the first blood specimen, and then the viral RNA load decreased gradually. For patients aged <7 days (patients 1–4, all with severe disease), the highest viral RNA load detected was >20,000 copies/μL. For patients aged 1–2 months (patients 7–10, all with mild disease), the highest viral load detected was <2000 copies/μL. The highest viral RNA load in patients with severe disease (patients 1–5) was significantly higher than that in patients with mild disease (patients 6–10) (P = .009, by the Mann-Whitney test). Two patients (patients 3 and 4) had blood specimens available for viral load determination before and after the administration of IV IgG. The blood viral RNA load decreased rapidly after the administration of IV IgG in both cases. We also noted that a higher viral RNA load corresponded with a higher peak serum aspartate aminotransferase level in these 10 infants (table 1). The duration of detectable viral RNA in blood specimens was significantly longer in patients with severe disease (P = .0331, by log rank test, Kaplan-Meier analysis). In patients 2 and 4, the viral RNA remained detectable in the blood 2 months after disease onset.

The duration of viral shedding in the stool and throat was <4 weeks in 7 patients who received regular follow-up visits at the outpatient clinic, and no significant difference was noted between the patients with severe and mild disease.

Discussion. To our knowledge, this is the first study to investigate the correlation of blood viral load with disease severity in neonatal enterovirus infection. A strong association was noted among higher viral load, younger age, and greater disease severity; this finding is compatible with that proposed by Dagan et al. [11]. The association of higher viral load and younger age may be a result of either mode of transmission (perinatal transmission) or of the immaturity of the neonatal immune system, and it is possible that higher viral load is responsible for higher disease severity in such patients. Nonetheless, this hypothesis needs to be further clarified because of the lack of control subjects (patients aged <7 days with mild disease). On the basis of the study data, higher viral load, younger age, and greater disease severity could not be separated.

We believe that the data show that blood viral RNA load can be used as a tool for early diagnosis and prediction of disease severity, in addition to age at onset, virus serotype, and mode of transmission (whether vertical transmission occurred). Further investigation is required to determine a clear cutoff level for blood viral RNA load to predict severe disease. Additionally, the duration of detectable viral RNA in blood was significantly longer in patients with severe disease, despite the cessation of viral shedding from the rectum (as noted in patients 2 and 4). Because these patients were clinically healthy, the persistence of viral RNA load 2 months after disease onset may indicate free nucleic acid, instead of infectious particles, in the blood. The difference in persistence of viral load between severely and mildly infected patients needs further clarification using standardized and more frequent follow-up sampling.

IV IgG contains different level of antibodies to several types of enteroviruses, and more rapid cessation of viremia and viuria is seen in patients receiving IV IgG containing a high titer to their own viral isolates [5, 12]. We found a rapid decrease in blood viral RNA load after the administration of IV IgG in 2 neonates who received it (patients 3 and 4). It is possible that IV IgG administration may be beneficial for reducing blood
Table 1. Demographic data, clinical manifestations, and use of intravenous (IV) IgG for study patients.

<table>
<thead>
<tr>
<th>Status of infection, patient</th>
<th>Age at onset, days</th>
<th>GA/BBW, weeks/g</th>
<th>Site where virus was isolated</th>
<th>Blood RNA load, copies/μL (time&lt;sup&gt;a&lt;/sup&gt;)</th>
<th>Highest AST level, IU/L (time)</th>
<th>Nadir of platelet count, platelets/μL (time)</th>
<th>Timing of IV IgG administration</th>
<th>Blood products transfusion</th>
</tr>
</thead>
<tbody>
<tr>
<td>Severe infection</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Patient 1</td>
<td>4</td>
<td>39/2998</td>
<td>T, R, CSF</td>
<td>395,000 (D16) ... ... ... ... ... ...</td>
<td>2889 (D3)</td>
<td>15,000 (D2)</td>
<td>D 3–6</td>
<td>IV IgG, Plt, PRBCs, FFP</td>
</tr>
<tr>
<td>Patient 2</td>
<td>4</td>
<td>39/3296</td>
<td>T, R, CSF</td>
<td>31,200 (D12) 8100 (D25) 3280 (D68) UD (D128) ... ...</td>
<td>189 (D0)</td>
<td>42,000 (D0)</td>
<td>D 0–3</td>
<td>IV IgG, Plt, PRBC, FFP</td>
</tr>
<tr>
<td>Patient 3</td>
<td>5</td>
<td>39/3060</td>
<td>T, R, CSF</td>
<td>23,400 (D2) 3270 (D6) 2970 (D7) 1480 (D12) 720 (D20)</td>
<td>141 (D1)</td>
<td>20,000 (D2)</td>
<td>D 2–5</td>
<td>IV IgG, Plt, PRBCs, FFP</td>
</tr>
<tr>
<td>Patient 4</td>
<td>3</td>
<td>36/3035</td>
<td>T, R, B</td>
<td>320,000 (D1) 121,000 (D2) 12,500 (D5) 6150 (D50) 1210 (D73)</td>
<td>745 (D1)</td>
<td>1000 (D1)</td>
<td>D 1–5</td>
<td>IV IgG, Plt, PRBCs, FFP</td>
</tr>
<tr>
<td>Patient 5</td>
<td>19</td>
<td>37/2800</td>
<td>T, R, CSF</td>
<td>3562 (D6) ... ... ... ... ... ... ...</td>
<td>202 (D2)</td>
<td>60,000 (D4)</td>
<td>D 4–5</td>
<td>IV IgG, Plt, PRBCs, FFP</td>
</tr>
<tr>
<td>Mild infection</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Patient 6</td>
<td>18</td>
<td>39/3305</td>
<td>T, R</td>
<td>UD (D1) UD (D11) ... ... ... ... ... ...</td>
<td>28 (D1)</td>
<td>339,000 (D4)</td>
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<td>None</td>
</tr>
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<td>Patient 7</td>
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<td>41/3746</td>
<td>T, R, CSF</td>
<td>1350 (D0) 209 (D3) UD (D7) ... ... ... ...</td>
<td>41 (D2)</td>
<td>253,000 (D0)</td>
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<td>None</td>
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<td>Patient 8</td>
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<td>T, R, CSF</td>
<td>225 (D6) ... ... ... ... ... ... ... ...</td>
<td>31 (D2)</td>
<td>289,000 (D2)</td>
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<td>None</td>
</tr>
<tr>
<td>Patient 9</td>
<td>49</td>
<td>38/3000</td>
<td>T, R, CSF</td>
<td>1860 (D7) 982 (D10) UD (D48) ... ... ...</td>
<td>Not checked</td>
<td>429,000 (D0)</td>
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<td>None</td>
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<tr>
<td>Patient 10</td>
<td>58</td>
<td>39/3168</td>
<td>T, R, CSF</td>
<td>906 (D6) 828 (D8) ... ... ... ... ... ...</td>
<td>98 (D0)</td>
<td>181,000 (D3)</td>
<td>None</td>
<td>None</td>
</tr>
</tbody>
</table>

**NOTE.** AST, aspartate aminotransferase; B, blood; D, day; FFP, fresh frozen plasma; GA/BBW, gestational age/birth body weight; Plt, platelets; PRBCs, packed RBCs; R, rectum; T, throat; UD, undetectable.

* No. of days after fever onset, with day 0 defined as fever onset.
viral load, but additional studies are required to elucidate this issue because of the lack of standardization of IV IgG administration and the schedule of viral load determination in this study.

Our study revealed the possibility of using blood viral load as determined by real-time RT-PCR for early diagnosis and for predicting disease severity in neonatal enterovirus infection—much earlier than from results from virus isolation. Earlier diagnosis leads to more prompt and definitive treatment of neonates with severe enterovirus infection, especially during an outbreak of enterovirus.

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Potential conflict of interest. All authors: no conflicts.

References