Current Status of Dengue Diagnosis at the Center for Disease Control, Taiwan

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Abstract

A national-level diagnostic laboratory has been set up in Taiwan for routine diagnosis of reported cases of dengue fever (DF)/dengue haemorrhagic fever (DHF), Japanese encephalitis (JE) and yellow fever (YF). The facilities include serological diagnosis, virus isolation by cell culture, molecular diagnosis and molecular tools for epidemiological investigations. To detect and differentiate dengue, JE and YF virus infections, a differential diagnostic system has been developed. For acute-phase sera, virus isolation by cell culture and real-time one-step reverse transcription-polymerase chain reaction (RT-PCR) has been established. For all of the serum samples reported, serological diagnosis of specific antibodies based on envelope and membrane (E/M)-specific capture IgM and IgG enzyme-linked immunosorbent assay (ELISA) are performed. In this report, a case study from Taiwan has been presented with the analysis of 959 serum samples (including some paired sera) collected between day 1-30 of illness from 799 confirmed dengue cases reported in 2002. The results demonstrated that 94.5% of acute-phase serum samples of confirmed dengue cases could be identified as positive or probable with the combined use of real-time one-step RT-PCR and E/M-specific capture IgM and IgG ELISA. Furthermore, a nonstructural protein NS1 serotype-specific indirect IgG ELISA has been developed and used to analyse dengue NS1-specific IgG antibodies. Both E/M-specific capture IgM and IgG ELISA and the NS1 serotype-specific indirect IgG ELISA have been used to detect and differentiate primary and secondary dengue virus infections. In addition, the NS1 serotype-specific indirect IgG ELISA has the potential of replacing the plaque-reduction neutralization test (PRNT) and is being used for a large-scale seroepidemiological study.

Keywords: Dengue virus, virus isolation, real-time one-step RT-PCR, E/M-specific capture IgM and IgG ELISA, NS1 serotype-specific indirect IgG ELISA.

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Introduction

The dengue viruses cause a broad spectrum of illness ranging from inapparent infection, mild undifferentiated fever and classic dengue hemorrhagic fever/dengue shock syndrome (DHF/DSS), resulting in high morbidity and mortality. The diagnosis of dengue virus infection based on clinical syndromes is not reliable, and a confirmation of the infection should rely on laboratory diagnosis with the detection of the specific virus, viral antigen, genomic sequence and/or antibodies.

A rapid, simple, sensitive and specific assay system to detect the virus in the acute-phase serum is essential to improve the clinical treatment, etiological investigation and disease control of dengue virus infection. Among the various assays for virus detection, virus isolation by cell culture and dengue virus antigen detection by ELISA suffer from some disadvantages - while the former needs a longer time, the latter has low sensitivity. However, recent advances in molecular diagnosis have demonstrated that various RT-PCR protocols can be reliably used to detect the viral genomic sequence with high sensitivity and specificity. More recently, several investigators have reported real-time RT-PCR assays for the detection of dengue virus in acute-phase serum samples. The real-time RT-PCR assay has many advantages over the conventional RT-PCR methods, which include rapidity, quantitative measurement, lower contamination rate, higher sensitivity, higher specificity and easy standardization.

For convalescent sera, detection of specific IgM and IgG antibodies based on haemagglutination inhibition (HI) test and E/M-specific capture IgM and IgG ELISA are the two most commonly used serological techniques for the routine diagnosis of flavivirus infection. The serodiagnosis of flavivirus is rather complicated due to the high cross-reactivity of IgG antibodies to homologous and heterologous viruses. We have attempted to set up an ELISA system that can be easily and reliably used to detect and differentiate various flavivirus infections. To accomplish this goal, three different forms of ELISA were developed including: (i) E/M-specific capture IgM and IgG ELISA; (ii) E/M-specific antigen-coated indirect IgM and IgG ELISA; and (iii) NS1 serotype-specific indirect IgG ELISA.

Case study of 2002 outbreak of DEN-2 in southern Taiwan

We present here a case study of the analysis of biological material obtained during a DEN-2 outbreak in 2002 in southern Taiwan, by utilizing the facilities available in the national diagnostic laboratory.

A major DEN-2 epidemic occurred in southern Taiwan, affecting Kaohsiung city, Kaohsiung county and Pingtung county between October 2001 and December 2002, with more than 5,000 confirmed cases. Among these, 227 cases were classified as of DHF with 21 deaths. This outbreak was a repeat of the 1987-1988 DEN-1 epidemic in many aspects. In this report, we present the results of a total of 959 acute- and convalescent-phase sera collected from 799 confirmed dengue patients reported to the Kun-Yang office of the Center for Disease Control (CDC), Taiwan, 2002.
Materials and methods

Human serum samples
The serum samples used in this study were collected from the confirmed cases of dengue patients reported to the Arbovirus Laboratory in the Kun-Yang office, CDC, Department of Health, 2002. A total of 959 acute- and convalescent-phase sera collected from 799 confirmed dengue patients were analysed. Most of these serum samples were from the major DEN-2 outbreak, together with a few serum samples from imported cases contracted during travel to the neighbouring South-East Asian countries.

Case definitions
A confirmed case of dengue virus infection was defined as febrile illness associated with: (i) the isolation of dengue virus; (ii) positive test of real-time one-step RT-PCR; (iii) positive seroconversion or ≥ four-fold increase in dengue-specific IgM or IgG antibody from appropriately timed paired serum; or (iv) high-titer dengue-specific IgM and IgG antibody in a single serum specimen where cross-reaction to Japanese encephalitis (JE) had been excluded. Sera collected during day 1-7 after the onset of symptoms are referred to as acute-phase sera. Early and late convalescent sera refer to the specimens collected during day 8-13 and day 14-30, respectively.

Virus isolation by cell culture and virus antigen preparation
The isolation of dengue virus by cell culture and virus antigen preparation from culture supernatants of DEN-1, DEN-2, DEN-3, DEN-4 or JE-virus infected Vero cells were performed as previously described. The culture supernatants were used as the source of E/M and NS1 antigens for ELISA. The control antigen was prepared by the same procedure from Vero cells culture without viral infection.

One-Step SYBR Green I Real-Time RT-PCR
One-step SYBR Green I real-time RT-PCR for dengue virus was performed in the Mx4000™ quantitative PCR system (Stratagene) as recently described. Briefly, a set of flavivirus- (in the NS5 gene region), dengue- and serotype-specific primer pairs (in the core gene region) was selected and used for analysis. To assure the specificity of amplicons produced from SYBR Green I real-time RT-PCR in daily routine screening, both flavivirus- and dengue-specific primer pairs were used for each of the serum samples tested. Serum samples found positive for initial screening were then tested for serotype by each of the four serotype-specific primer pairs.

ELISA
E/M-specific capture IgM and IgG ELISA
A modified E/M-specific capture IgM and IgG ELISA was performed to measure the dengue-specific IgM and IgG antibodies as recently described. Briefly, each microtiter 8 wells strip was coated with 5 µg/ml, 100 µl/well of affinity purified goat anti-human IgM (µ-specific) or IgG (γ-specific) antibodies, followed by incubation with 1:100 diluted serum, incubation with cocktail contained 1:3 diluted pooled virus antigens from culture supernatants of DEN-1, DEN-2, DEN-3 or DEN-4 infected Vero cells.
and 1 µg/ml mAb D56.3, incubation with 1:1,000 diluted alkaline phosphatase-conjugated goat anti-mouse IgG (γ-specific). The enzyme activity was developed with the substrate p-nitrophenyl-phosphate and optical density (OD) was taken 30 minutes later. For routine screening, culture supernatant from JE virus-infected Vero cells was used as negative control antigen due to the limited cross-reactivity between dengue- and JE-specific IgM antibodies measured by E/M-specific capture IgM ELISA. This was in contrast to the high cross-reactivity of dengue- and JE-specific IgG antibodies among dengue patients with secondary infection.

E/M-specific antigen-coated indirect IgM and IgG ELISA

E/M-specific antigen-coated indirect IgM and IgG ELISA were performed as previously described[10]. Two-fold serial dilutions of appropriately timed paired sera diluted from 1:100 to 1:12,800 were analysed to determine whether ≥ four fold increase of dengue-specific IgM or IgG antibody could be found. This assay has the advantage of better sensitivity in the detection of IgM and IgG antibody increase than E/M-specific capture IgM and IgG ELISA.

NS1 serotype-specific indirect IgG ELISA

NS1 serotype-specific indirect IgG ELISA was performed as previously described[9,13]. Briefly, each microtiter 8 wells strip was coated with 5 µg/ml, 100 µl/well of mAbs D2/8-1, followed by incubation with 1:3 diluted NS1-containing culture supernatants of DEN-1, DEN-2, DEN-3, DEN-4 or JE viruses-infected Vero cells, incubation of serum samples at a 1:50 dilution, incubation with goat anti-human IgG conjugated to alkaline phosphatase. The enzyme activity was developed and OD was taken 30 minutes later.

Data analysis

For E/M-specific capture IgM and IgG ELISA, primary dengue virus infection was defined if the IgM:IgG OD ratio was ≥1.2, or secondary if the OD ratio was <1.2. For those sera with positive NS1-specific IgG antibody response, NS1 serotyping was calculated by the ratio of the highest OD value and the second highest OD value read from the four dengue serotypes. Positive serotype-specificity is defined if the OD ratio is ≥1.2 and negative serotype-specificity is defined if the OD ratio is <1.2. Based on NS1 serotype-specific indirect IgG ELISA, primary dengue virus infection was defined if: (i) negative NS1-specific IgG antibody response was found for sera collected between day 1 and 14 of illness, or (ii) positive serotype-specificity for sera collected ≥9 days of illness. Secondary dengue virus infection was defined if: (i) positive NS1-specific IgG antibody response was found for sera collected between day 1 and 8 of illness, or (ii) positive NS1-specific IgG antibody response and negative serotype-specificity was found any time after the onset of infection.

Results

Dengue surveillance system and laboratory diagnosis in Taiwan

Taiwan has an integrated programme for dengue surveillance and control. The dengue prevention and control centre is a
mission-oriented structure jointly sponsored by the Department of Health and the Environment Protection Administration responsible for the planning and execution of dengue control. To assure the effectiveness of dengue surveillance, three report systems are currently associated with the dengue surveillance programme including: (i) hospital-based passive report system; (ii) syndrome report system (under the classification of viral haemorrhagic fever); and (iii) active surveillance system. The Arbovirus Laboratory in Kun-Yang office, Taipei City, CDC, Department of Health, is responsible for the diagnosis of various flavivirus. In addition, a second dengue diagnostic laboratory was set up in the Fourth Branch, Kaohsiung City, CDC, in July 2002 to provide prompt service to Kaohsiung city, Kaohsiung county and Pingtung county due to the large samples generated by the DEN-2 outbreak. For routine diagnosis, serum samples from the reported cases were sent to the laboratory on a daily basis and tested according to the flow chart shown in Figure 1. The periods of time required to complete these tests were 7 days, 6 hours and 4 hours for virus isolation, real-time one-step RT-PCR and E/M-specific capture IgM and IgG ELISA, respectively. The results were reported as positive, negative or probable cases. The probable case was referred to ELISA result with only IgM or IgG antibody positive. For negative and probable cases, the convalescent serum samples collected after day 14 of the illness were demanded and tested for the presence of or increase in IgM and/or IgG antibodies.

Figure 1. Flow chart of laboratory diagnosis of dengue virus infection

```
Serum
1-7 days
Virus isolation (+) Result
1-30 days
Real-Time RT-PCR
(+ Result
E/M-specific Capture IgM/IgG ELISA 1:100 dilution
Current dengue infection
Probable or (-) Result
>=14 days serum
Probable or (- Result
E/M-specific Capture IgM/IgG ELISA 1:100 dilution
Final Result
```
Representative results of routine diagnosis measured by virus isolation, real-time one-step RT-PCR, and E/M-specific capture IgM and IgG ELISA

Table 1 shows the representative results of serum samples analysed by virus isolation, real-time one-step RT-PCR and E/M-specific capture IgM and IgG ELISA. The results provided a good example of the dynamic change of dengue virus and specific IgM and IgG antibodies in the acute- and convalescence-phase sera from patients with primary or secondary dengue virus infection covering all four serotypes. As shown in Figure 1, all of the three assays were performed for acute-phase sera, whereas only E/M-specific capture IgM and IgG ELISA was tested for convalescence-phase sera.

Table 1. Representative results of routine diagnosis of serum samples from reported dengue cases

<table>
<thead>
<tr>
<th>Dengue infection</th>
<th>Dengue serotype</th>
<th>Onset days</th>
<th>Virus isolation</th>
<th>Real-time one-step RT-PCR Ct value</th>
<th>E/M-specific capture IgM and IgG ELISA OD 405 nm</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Dengue- specific</td>
<td>Dengue- specific</td>
</tr>
<tr>
<td>Primary infection</td>
<td>DEN-1</td>
<td>3 16</td>
<td>+</td>
<td>28 25 25</td>
<td>0.526 2.791</td>
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<tr>
<td></td>
<td>DEN-2</td>
<td>2 15</td>
<td>+</td>
<td>26 33 29</td>
<td>0.209 2.484</td>
</tr>
<tr>
<td></td>
<td>DEN-3</td>
<td>3 16</td>
<td>+</td>
<td>25 31 32</td>
<td>0.478 3.484</td>
</tr>
<tr>
<td></td>
<td>DEN-4</td>
<td>3 15</td>
<td>-</td>
<td>26 35 35</td>
<td>0.094 2.225</td>
</tr>
<tr>
<td>Secondary infection</td>
<td>DEN-1</td>
<td>1 16</td>
<td>+</td>
<td>27 28 24</td>
<td>0.166 1.949</td>
</tr>
<tr>
<td></td>
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<td>2 18</td>
<td>+</td>
<td>17 18 20</td>
<td>0.267 0.496</td>
</tr>
<tr>
<td></td>
<td>DEN-3</td>
<td>1 7</td>
<td>+</td>
<td>18 23 24</td>
<td>0.175 3.631</td>
</tr>
<tr>
<td></td>
<td>DEN-4</td>
<td>7 12</td>
<td>+</td>
<td>20 28 29</td>
<td>1.298 1.468</td>
</tr>
</tbody>
</table>

+ = positive
- = negative

Due to the long time needed to isolate virus using the cell culture method, it has limited value in rapid diagnosis. The isolated virus, however, is the key material for the later studies of molecular epidemiology and pathogenesis. The real-time one-step SYBR Green I RT-PCR we developed is a simple, reliable and universal RT-PCR protocol that can be used to systemically detect and differentiate various flavivirus. To assure the specificity of amplicons produced from SYBR Green I real-time RT-PCR in routine screening, both flavivirus- and dengue-specific primer pairs were run (Table 1).
Those serum samples positive for initial screening were then tested for serotype by each of the four serotype-specific primer pairs. The analysis of acute-phase serum samples demonstrated that the one-step SYBR Green RT-PCR was more sensitive to the virus isolation method and could detect two-times more the acute-phase sera with positive dengue-specific IgM and/or IgG antibodies.

The E/M-specific capture IgM and IgG ELISA has several advantages in the detection of dengue-specific IgM and IgG antibodies including: (i) high sensitivity; (ii) high specificity (only for IgM antibody); (iii) analysis of isotype-specific antibody responses; (iv) easy automation to test large amount of serum samples; and (v) differentiation of primary and secondary dengue infections. The results shown in Table 1 demonstrated the low cross-reactivity between dengue- and JE-specific IgM antibody and inverse pattern of IgM:IgG OD ratio of primary and secondary infection.

Therefore, a positive dengue-specific IgM and IgG antibody response can be easily used to detect and differentiate primary and secondary dengue virus infections.

**E/M-specific antigen-coated indirect IgM and IgG ELISA for the detection of dengue virus infection**

Occasionally, there were acute-phase sera which tested positive with E/M-specific capture IgM or IgG antibody response, but did not show an apparent increase in antibody titers in convalescent sera. Due to the higher sensitivity of E/M-specific indirect IgM and IgG ELISA (especially for IgG antibody), it can be reliably used to determine whether ≥ four-fold increase of dengue-specific IgM or IgG antibody were presented. Figure 2 shows an example where significant dengue-specific IgG antibody increase in a convalescent serum.
Statistical analysis of results of serum samples from confirmed dengue cases reported in 2002

Table 2 shows the comprehensive analysis of the results of serum samples from confirmed dengue cases reported to Kun-Yang office in 2002. The results of virus isolation, real-time RT-PCR and E/M-specific capture IgM and IgG ELISA were analysed separately or in combination from the sera samples collected on 1-30 day of illness. The positive rate for real-time RT-PCR was 74.7%, 69.5%, 72.3%, 76.6%, 57.7%, 36.3% and 22.2% for day 1-7 of illness, respectively. The positive rate for E/M-specific capture IgM and/or IgG ELISA was 31.6%, 32.6%, 30%, 39%, 52.6%, 87.5% and 80% for day 1-7 of illness, respectively. Thus, the combined results of real-time RT-PCR and E/M-specific capture IgM and IgG ELISA (IgM and/or IgG positive) could detect an average 94.5% (89.7% to 97.5%) of acute-phase serum samples of confirmed dengue cases. The results also showed that the real-time RT-PCR was more sensitive than virus isolation although very few sera, which were virus-isolation positive, were missed by real-time RT-PCR.

Table 2. Statistical analysis of results of serum samples from confirmed dengue cases reported in 2002

<table>
<thead>
<tr>
<th>Assays</th>
<th>Days after onset</th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>4</th>
<th>5</th>
<th>6</th>
<th>7</th>
<th>8</th>
<th>9</th>
<th>10</th>
<th>11</th>
<th>12</th>
<th>13</th>
<th>14-30</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total serum no. tested</td>
<td></td>
<td>95</td>
<td>95</td>
<td>130</td>
<td>128</td>
<td>97</td>
<td>80</td>
<td>45</td>
<td>58</td>
<td>27</td>
<td>24</td>
<td>16</td>
<td>7</td>
<td>7</td>
<td>148</td>
<td>959</td>
</tr>
<tr>
<td>V.I. * (Virus isolation)</td>
<td></td>
<td>53</td>
<td>52</td>
<td>64</td>
<td>58</td>
<td>32</td>
<td>9</td>
<td>2</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>270</td>
<td></td>
</tr>
<tr>
<td>% of V.I. *</td>
<td></td>
<td>55.8</td>
<td>54.7</td>
<td>49.2</td>
<td>45.3</td>
<td>33.0</td>
<td>11.3</td>
<td>4.4</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td>RT-PCR* and V.I. *</td>
<td></td>
<td>52</td>
<td>50</td>
<td>60</td>
<td>57</td>
<td>31</td>
<td>8</td>
<td>2</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>260</td>
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<td>RT-PCR* or V.I. *</td>
<td></td>
<td>72</td>
<td>68</td>
<td>98</td>
<td>99</td>
<td>57</td>
<td>30</td>
<td>10</td>
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<td>-</td>
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<td>-</td>
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<td>RT-PCR* (Real-time)</td>
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<td>71</td>
<td>66</td>
<td>94</td>
<td>98</td>
<td>56</td>
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<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
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<td>% of RT-PCR*</td>
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<td>74.7</td>
<td>69.5</td>
<td>72.3</td>
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<td>57.7</td>
<td>36.3</td>
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<td>-</td>
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<td></td>
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<tr>
<td>RT-PCR* (Real-time)</td>
<td></td>
<td>24</td>
<td>29</td>
<td>36</td>
<td>30</td>
<td>41</td>
<td>51</td>
<td>35</td>
<td>-</td>
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<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>246</td>
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<tr>
<td>ELISA+ (E/M-specific capture IgM* or IgG*)</td>
<td></td>
<td>20</td>
<td>25</td>
<td>21</td>
<td>27</td>
<td>29</td>
<td>41</td>
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<td>8</td>
<td>12</td>
<td>7</td>
<td>140</td>
<td>444</td>
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<tr>
<td>Probable (ELISA IgM* or IgG*)</td>
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<td>10</td>
<td>6</td>
<td>18</td>
<td>23</td>
<td>22</td>
<td>29</td>
<td>9</td>
<td>9</td>
<td>8</td>
<td>1</td>
<td>1</td>
<td>3</td>
<td>0</td>
<td>8</td>
<td>147</td>
</tr>
<tr>
<td>% of IgM* and/or IgG</td>
<td></td>
<td>31.6</td>
<td>32.6</td>
<td>30.0</td>
<td>39.1</td>
<td>52.6</td>
<td>87.5</td>
<td>80.0</td>
<td>93.1</td>
<td>100</td>
<td>100</td>
<td>100</td>
<td>93.8</td>
<td>100</td>
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<tr>
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<td></td>
<td>3</td>
<td>3</td>
<td>5</td>
<td>3</td>
<td>9</td>
<td>1</td>
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<td>4</td>
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<td>1</td>
<td>0</td>
<td>0</td>
<td>32</td>
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### NS1 serotype-specific indirect IgG ELISA in the differentiation of JE, primary and secondary dengue virus infections and for the DEN serotyping of primary infection

More recently, we have developed a NS1 serotype-specific indirect IgG ELISA in the detection and differentiation of primary and secondary infections. Comparisons of E/M-
specific capture IgM and IgG ELISA and NS1 serotype-specific indirect IgG ELISA showed good correlation with 95.90% agreement[13]. Most importantly, retrospective seroepidemiological studies on serum samples collected from Liuchiu Hsiang, Pingtung county and Tainan city in southern Taiwan, demonstrated that NS1 serotype-specific indirect IgG ELISA could replace plaque-reduction neutralization test (PRNT) for seroepidemiological study to differentiate JE.
primary and secondary dengue virus infections and for the DEN serotyping of primary infection\textsuperscript{[11]}.

**Discussion**

Recent advances in molecular and serological assays have revolutionized the laboratory diagnosis of flavivirus infection\textsuperscript{[7,13,14]}. Rapid diagnosis of dengue virus infection in the acute-phase sera, which is important for disease control measures and potential treatment, will require very sensitive and specific assays. With the maturation of real-time RT-PCR technique, its routine application to clinical and laboratory diagnosis has now become a reality. For serodiagnosis, E/M-specific capture IgM and IgG ELISA has become the new standard assay for the detection and differentiation of flavivirus infection.

The large DEN-2 epidemic in southern Taiwan, was uncontrolled despite vigorous attempts to contain it by the central and local health governments during October 2001 – December 2002. Although insecticide-resistance was blamed as an important factor for this disaster, other elements including, political, social, environmental, community and human factors were also responsible for this setback. This epidemic was a strong warning to us and suggested that more effective measures should be sought and applied. There is an urgent need to improve the surveillance system and laboratory diagnosis which would help to identify confirmed cases in the acute-phase sera and respond promptly and effectively to control the transmission chain.

Along with the progress of the DEN-2 outbreak in 2002, we have developed and evaluated the real-time RT-PCR method for rapid detection of dengue virus in the acute-phase sera. In this report, we have presented a detailed analysis of a total of 959 acute- and convalescent-phase sera collected from 799 confirmed dengue patients reported to the Kun-Yang office of CDC, Taiwan, in 2002. The results demonstrated that 94.5% of acute-phase serum samples of confirmed dengue cases could be identified as positive or probable with the combined use of real-time one-step RT-PCR and E/M-specific capture IgM and IgG ELISA. The results are very encouraging and suggest that these two assays are well-suited for routine tests for the early diagnosis of dengue virus infection.

**Conclusion**

The real-time RT-PCR assay has many advantages over conventional RT-PCR methods, which include rapidity, quantitative measurement, lower contamination rate, higher sensitivity, higher specificity and easy standardization. Therefore, real-time quantitative assay might eventually replace virus isolation and conventional RT-PCR as the new gold standard for the rapid diagnosis of virus infection in the acute-phase serum samples.

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