Factors Influencing Dengue Virus Isolation by C6/36 Cell Culture and Mosquito Inoculation of Nested PCR-Positive Clinical Samples


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Abstract. Dengue viral isolation is necessary for definitive diagnosis, pathogenesis and evolutionary research, vaccine candidates, and diagnostic materials. Using standardized techniques, we analyzed isolation rates of 1,544 randomly selected polymerase chain reaction (PCR)-positive samples, representing all four dengue serotypes, from patients with serologically confirmed dengue infections and evaluated whether clinical and laboratory results could be predictive of isolation using standard and mosquito isolation techniques. Viruses were isolated from 62.5% of the samples by direct application to C6/36 cells and increased to 79.4% when amplifying C6/36 negative samples by intrathoracic inoculation in Toxorhynchites splendens mosquitoes. High viremia, measured by reverse transcriptase (RT)-PCR, was a strong predictor for viral isolation by either method. Isolation was most successful in samples collected early in the disease, had low antibody levels, temperatures greater than 38°C, and had a final clinical diagnosis of dengue fever. Dengue serotypes also played a role in the success of viral isolation.

INTRODUCTION

Dengue is an endemic disease affecting tropical and subtropical regions worldwide. Dengue virus (DENV) is transmitted to humans primarily by Aedes aegypti and Aedes albopictus mosquitoes. Currently, the incidence of the disease has been estimated to be 50–100 million cases per year; this incidence is increasing. Currently, the incidence of the disease has been increasing. There are four closely related dengue serotypes, DENV 1–4 and infection by a given serotype induces a lifelong protective immunity against the homologous serotype, but only a transient and partial protection against the three other serotypes. Secondary infection with another serotype is considered to be a major risk factor for developing dengue hemorrhagic fever (DHF) and dengue shock syndrome.

Routine laboratory testing has classically involved either virus isolation or culture followed by fluorescent staining or detection of anti-dengue immunoglobulin M (IgM)/IgG antibodies by enzyme-linked immunosorbent assay (ELISA). However, virus isolation is time-consuming, requiring greater than 7 days to obtain results, and serology is often inaccurate because of cross-reactivity among flaviviruses. Molecular biology techniques have become the primary methods to detect dengue virus RNA in the plasma or serum of patients. These molecular techniques have the advantage of allowing more rapid diagnosis of acute dengue infection, which can then guide the clinical management of these patients. Viral isolation continues to be a highly useful tool, however, allowing detection of dengue virus but also providing valuable reagents for the study of longitudinally collected specimens to evaluate virus evolution and epidemiology, molecular markers of virulence or attenuation, virus-antibody interactions, and other factors that may be implicated in disease pathogenesis and/or protection from disease.

Before the availability of molecular approaches, our laboratory used direct C6/36 cell culture and Toxorhynchites splendens amplification followed by C6/36 cell culture for dengue virus isolations. Currently, polymerase chain reaction (PCR) is the method of choice for rapid and early virological diagnosis of dengue infections, but viral isolation remains a key diagnostic tool. We routinely perform PCR on all acute phase serum/plasma samples when screening for dengue viremia. If virus isolation is desired, PCR-positive samples are inoculated onto C6/36 cell culture. Those samples that are not isolated in C6/36 cell culture are injected into T. splendens followed by C6/36 cell culture of infected mosquito homogenates. In this study, we analyzed isolation rates of 1,544 PCR-positive samples, representing all four dengue serotypes, from patients with serologically confirmed dengue infections and evaluated whether clinical and laboratory results could be predictive of isolation using standard and mosquito isolation techniques. We believe this is the first study to use standardized laboratory and clinical results, obtained from a single laboratory, using a large randomized selection of dengue-positive clinical samples from both primary and secondary infections, consisting of all four serotypes, from patients experiencing dengue fever (DF) and DHF to determine how these results contribute to viral isolation.

MATERIALS AND METHODS

Specimens. Samples were randomly selected among positive nested PCR serum/plasma specimens from service testing performed on patients admitted to Queen Sirikit National Institute of Child Health (QSNICH) between 2000 and 2002. Acute specimens were collected from patients with a history of fever and meeting at least one of the following additional criteria: positive tourniquet test, leukopenia, or bleeding manifestation. Each sample was aliquoted when delivered to the laboratory and stored at −70°C and previously unthawed were used for PCR and viral isolation. All patients were serologically confirmed as acute primary or secondary dengue infections. Of these samples there were 644 DENV-1, 499 DENV-2, 302 DENV-3, and 79 DENV-4 (1,544 total), representing 488 DF and 959 DHF according to World Health Organization (WHO) established criteria.

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**Detection of virus genome by reverse transcriptase (RT)-PCR.** The RT-PCR was performed according to the protocol of Lanciotti and others with modifications as described by Kungthong and others.  

**Virus isolation in C6/36 cells and identification of serotypes.** The PCR-positive serum specimens were used to infect C6/36 cell cultures. Original serum or plasma (0.3 mL) was blindly passaged three times on C6/36 cell culture with a 7-day incubation period for each passage. Following the third passage, the culture fluid was tested against a panel of monoclonal antibodies against each of the four dengue virus serotypes.  

**Mosquito amplification.** All samples that could not be recovered by C6/36 cell culture were intrathoracically inoculated with 0.34 μL of the clinical sample into 15–20 live T. splendens mosquitoes. After 14 days, ~10–15 surviving mosquitoes were triturated and passaged once in C6/36 cell culture as described previously. The virus present in culture fluid was then serotyped as above.  

**Dengue/Japanese encephalitis (JE) IgM/IgG enzyme immunoassay (EIA).** All serum/plasma were tested for dengue and JE IgM and IgG by Armed Forces Research Institute of Medical Sciences (AFRIMS) antibody capture EIA to serologically confirm the diagnosis and to differentiate primary and JE IgM and IgG by Armed Forces Research Institute of Medical Sciences (AFRIMS) antibody capture EIA to serologically confirm the diagnosis and to differentiate primary and secondary dengue infection. For specimens, 40 units (U) of anti-dengue IgM (with anti-dengue IgM greater than anti-Japanese encephalitis virus [JEV] IgM) were considered evidence of acute dengue infection. From paired sera (acute and convalescent interval of ≥ 7 days), a dengue IgM-to-IgG ratio ≥ 1.8 defined a primary dengue virus infection. A ratio < 1.8 defined a secondary dengue virus infection. With serial specimens, a 2-fold increase in IgG to dengue with an absolute value of ≥ 100 U indicated a secondary infection in the absence of anti-dengue IgM of ≥ 40 U.  

**Statistical analysis.** Data were entered and manipulated using FoxPro for Windows software (Microsoft, Redmond, WA) and analyzed using SPSS for Windows version 12.0 (SPSS Inc., Chicago, IL) and SAS analytic software, version 9.1 (SAS Institute, Inc., Cary, NC). The χ² analysis was done for contingency tables. Logistic regression was used for multivariate analysis. All variables that were significantly associated with isolation positivity by bivariate analysis were initially entered as predictors in the multivariate regression model. The best model was selected by the method of backward elimination, in which the variable with the highest P value greater than a chosen cut-off (we selected P = 0.10) is removed at each step until all remaining variables in the model have P values less than the cut-off.  

**RESULTS**

We selected 1,544 PCR-positive clinical samples to determine the isolation rates using standard C6/36 culture and mosquito inoculation and to apply clinical and laboratory values to predict a successful isolation. Serum directly applied to C6/36 cells yielded dengue virus recovery in 966 samples (62.5%). An additional 260 (45%) of the 578 that failed to amplify in C6/36 cells were recovered after mosquito amplification and subsequent plating of infected mosquito homogenate on C6/36 cells. The combination of both methods resulted in viral isolation of 1,226 of 1,544 (79.4%) as shown in Table 1.  

**Laboratory predictors.** *RT-PCR positivity.* Viral load was assessed, qualitatively, by considering that samples that were positive in the first RT-PCR round contained a higher viral load than those samples that were only positive in the second round nested PCR. Of the 1,544 PCR-positive samples 907 (58.7%) were positive in the first round (Table 2). Eighty-three percent (753/907) of the positive RT-PCR samples were isolated with mosquito amplification for a total isolation rate of 96% (872/912) for first round RT-PCR-positive samples (data not shown). Thirty-four percent of RT-PCR-negative (nested PCR-positive) samples were isolated using direct application on C6/36 cells and 75% (119/158) of those that were negative for direct C6/36 isolation samples were isolated with mosquito amplification for a total isolation rate of 96% (872/912) for first round RT-PCR-positive samples (data not shown). Thirty-four percent of RT-PCR-negative (nested PCR-positive) samples were isolated using direct C6/36 inoculation; 33% of the RT-PCR-negative samples that were not isolatable by direct application to C6/36 cells were successfully isolated following mosquito inoculation (data not shown). This yielded a total isolation rate of 56% for first-round PCR-negative samples.

**Serotype.** All four serotypes were identified in the sample population (664 DENV-1; 499 DENV-2; 302 DENV-3; and 79 DENV-4) as shown in Table 2. In bivariate analysis of RT-PCR positivity, DENV-3 was significantly more likely to be RT-PCR positive (67.5%) and DENV-4 was the least (45.6%) (P = 0.001). Combining both methods of viral isolation, DENV-3 was the least likely to be isolated (71.5%), and DENV-1 the most likely (84.2%) (P < 0.001).

**Levels of anti-dengue IgM and IgG.** Using diagnostic cutoff levels, samples that contained less than 40 U of IgM were more likely to have virus isolated by either method (947/1055, or 89.8%, for those with IgM < 40 versus 300/489, or 62.9% for those with IgM ≥ 40). Those samples with IgM less than 40 U were also less likely to be RT-PCR positive (300/489, or 61.3%, for those with IgM < 40 versus 279/489, or 57.2% for those with IgM ≥ 40). Similar trends were observed using anti-dengue IgG levels. Those samples containing IgG level less than 50 were more likely to be isolated by either method 1,050/1,248 (84%). Samples containing greater than or equal to 50 U were less likely to be RT-PCR positive and exhibited a reduction in the isolation rates (Table 2).  

**Clinical parameters.** *Day of illness.* Time of blood collection after the first appearance of symptoms was a strong indicator of viral isolation rates. One thousand eighty-five (70%) samples were collected 4 days or less after symptoms appeared. An 85.3% viral recovery rate was seen in the first 4 days of infection, which was reduced to 65.4% when samples were collected more than 4 days after the initial appearance of symptoms, as shown in Table 3.

**Severity of disease.** Severity of disease, in terms of final diagnosis as defined by the WHO, had a significant effect in the
Table 2

<table>
<thead>
<tr>
<th>Virus isolation</th>
<th>Isol neg</th>
<th>Isol pos</th>
<th>P value*</th>
<th>Level of viremia by RT-PCR</th>
<th>Low viremia</th>
<th>High viremia</th>
<th>P value*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Direct C6/36 inoculation</td>
<td>578 (37.4%)</td>
<td>966 (62.5%)</td>
<td>–</td>
<td>637 (41.3%)</td>
<td>907 (58.7%)</td>
<td>–</td>
<td></td>
</tr>
<tr>
<td>Mosquito followed by C6/36 inoculation (among direct C6/36 negatives)</td>
<td>318 (55.0%)</td>
<td>260 (44.9%)</td>
<td>–</td>
<td>158 (27.3%)</td>
<td>420 (72.7%)</td>
<td>P &lt; 0.001</td>
<td></td>
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<tr>
<td>Infecting Serotype</td>
<td></td>
<td></td>
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</tr>
<tr>
<td>DENV-1</td>
<td>105 (15.8%)</td>
<td>559 (84.2%)</td>
<td>P &lt; 0.001</td>
<td>290 (43.7%)</td>
<td>374 (56.3%)</td>
<td>P = 0.001</td>
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<tr>
<td>DENV-2</td>
<td>112 (22.4%)</td>
<td>387 (77.6%)</td>
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<td>206 (41.3%)</td>
<td>293 (58.7%)</td>
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<td></td>
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<tr>
<td>DENV-3</td>
<td>86 (28.5%)</td>
<td>216 (71.5%)</td>
<td></td>
<td>98 (32.5%)</td>
<td>204 (67.5%)</td>
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<tr>
<td>DENV-4</td>
<td>15 (19.0%)</td>
<td>64 (81.0%)</td>
<td></td>
<td>43 (54.4%)</td>
<td>36 (45.6%)</td>
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<td>IgM titer by ELISA</td>
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<tr>
<td>&lt; 40</td>
<td>108 (10.2%)</td>
<td>947 (89.8%)</td>
<td>P &lt; 0.001</td>
<td>337 (31.9%)</td>
<td>718 (68.1%)</td>
<td>P &lt; 0.001</td>
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<tr>
<td>&gt; 40</td>
<td>210 (42.9%)</td>
<td>279 (57.1%)</td>
<td></td>
<td>300 (61.3%)</td>
<td>189 (38.7%)</td>
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<tr>
<td>IgG titer by ELISA</td>
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<tr>
<td>&lt; 50</td>
<td>198 (15.9%)</td>
<td>1050 (84.1%)</td>
<td>P &lt; 0.001</td>
<td>439 (35.2%)</td>
<td>809 (64.8%)</td>
<td>P &lt; 0.001</td>
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<tr>
<td>&gt; = 50</td>
<td>120 (40.5%)</td>
<td>176 (59.5%)</td>
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<td>198 (66.9%)</td>
<td>98 (33.1%)</td>
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</table>

*RT-PCR = reverse transcriptase-polymerase chain reaction; ELISA = enzyme-linked immunosorbent assay.
†P values calculated using Pearson χ² testing, with significance set at α = 0.05.

Table 3

| Clinical predictors of successful virus isolation and RT-PCR positivity, bivariate analysis* |
|----------------|---------|---------|----------|-----------|-----------|
| Virus isolation | Isol neg | Isol pos | P value* | Level of viremia by RT-PCR | Low viremia | High viremia | P value† |
| Illness-day of blood draw | | | | | | | |
| <= 4 days | 159 (14.7%) | 926 (85.3%) | P < 0.001 | 344 (31.7%) | 741 (68.3%) | P < 0.001 |
| > 4 days | 159 (34.6%) | 300 (65.4%) | | 293 (63.8%) | 166 (36.2%) | |
| DF or DHF‡ | DF | 70 (14.3%) | 418 (85.7%) | P < 0.001 | 168 (34.4%) | 320 (65.6%) | P < 0.001 |
| DHF | 228 (23.7%) | 731 (76.3%) | | 427 (44.5%) | 532 (55.5%) | |
| Temperature | < 38.0°C | 162 (26.4%) | 452 (73.6%) | P < 0.001 | 278 (45.3%) | 532 (54.7%) | P = 0.010 |
| >= 38.0°C | 156 (16.8%) | 774 (83.2%) | | 359 (38.6%) | 571 (61.4%) | |
| Primary or secondary dengue | Primary | 19 (9.0%) | 192 (91.0%) | P < 0.001 | 86 (40.8%) | 125 (59.2%) | P = 0.940 |
| Secondary | 299 (22.4%) | 1034 (77.6%) | | 551 (41.3%) | 782 (58.7%) | |

*RT-PCR = reverse transcriptase-polymerase chain reaction; DF = dengue fever; DHF = dengue hemorrhagic fever.
†P values calculated using Pearson χ² testing, with significance set at α = 0.05.

ability to isolate virus. The number of samples from patients experiencing DHF or DF was 959 and 488, respectively, and 107 samples did not receive a final diagnosis because of incomplete laboratory results or non-compliance with physician’s orders to return for evaluation during the recovery phase. Samples from patients experiencing DF were more likely to have DENV isolated from their blood then patients who experienced DHF (85.7% versus 76.3%, P = 0.03, Table 3). Among samples from secondary cases, 799 (60%) of 1,333 were recovered by direct C6/36 cell culture inoculation. It was found that 737 of 799 (92%) total samples recovered by C6/36 inoculation had anti-dengue IgM less than 40 U, and that 592 (80%) had anti-dengue IgG less than 50 U. Virus was recoverable after mosquito amplification in 235 (40%) of the 534 samples that failed C6/36 direct isolation. One hundred ninety-three (82%) of these had anti-dengue IgM less than 40 U and 103 (54%) had anti-dengue IgG less than 50 U (data not shown).

Multivariate analysis. All laboratory and clinical predictors had P values less than 0.10 in multivariate analysis and therefore all were included in the model. Controlling for the other variables in the model, a diagnosis of DF was borderline significantly associated with a lower rate of virus isolation as compared with DHF, (P = 0.053, Table 4). Blood specimens collected on or after the fourth day of illness were 68% less likely to be isolation positive than specimens collected on Days 0 to 3 (P < 0.001). DENV-1, DENV-2, and DENV-3 were all significantly less likely to be isolated than DENV-4, with DENV-3 being the least likely serotype to be successfully isolated (77% less than DENV-4) (P < 0.001). A higher body temperature was associated with a 66% increase in the likelihood of isolation (P = 0.001). A secondary DENV infection was 61% less likely to result in successful isolation than a primary infection (P = 0.002). Higher IgM and IgG titers were associated with decreases in the likelihood of virus isolation, with IgG titers exhibiting the most dramatic

level of 40 units) of anti-dengue IgM were much more likely to be isolated by either method (P < 0.001) (Table 3).
(87% decrease in isolation positivity with high IgG) \((P < 0.001\) for both).

### DISCUSSION

Clinical and epidemiological studies require identification of dengue serotypes. The PCR is the most sensitive and rapid method for the detection of dengue virus in the early stages of disease.\(^*\) However, viral culture has important benefits. Longitudinal collections of dengue viruses provide material for studies of pathogenesis, phenotypic characterization, and antigenic drift. Viral stocks are essential for studies that associate virulence with genotypes and clearly play an important role for the selection of parent strains for attenuated vaccines. Viral strains are also necessary for the development of diagnostic tests, including antigens for serological assays and reference stains for neutralization tests.

The samples in this study used a large randomized sample set collected in 2000–2002 from Queen Sirikit National Institute of Children’s Health in Bangkok, Thailand. The samples within this study were collected from both hospitalized and outpatient treated patients. We believe that the samples and results herein to be representative of dengue patients and viruses in Bangkok. The results show that clinical and routine laboratory data can be used to increase the likelihood of obtaining amplified viral stocks for further characterization of viruses. This is important when attempting to amplify viruses with low viral loads and may be particularly important to obtain viruses that do not cause serious infections and are potentially interesting candidates for studying factors that influence pathogenesis.

Our analysis used randomized 1,544 serological-confirmed positive PCR samples to allow the determination of sensitivity of direct C6/36 culture and mosquito amplification. These data confirmed the higher sensitivity of PCR over virus isolation previously reported by Deubel and others\(^{25}\) and Henchal and others,\(^{26}\) who also showed that greater than 75% of positive PCR samples were positive by isolation using the combination of C6/36 culture and mosquito amplification.

Viral load in clinical samples could play a critical role in amplifying virus in cell cultures or mosquitoes. To determine if conventional PCR could be used as a predictive factor for viral amplification in cell culture, we associated a positive band in the first round of a nested PCR reaction as a higher viral load than a positive band only in the second round. Analysis of these samples revealed that if the first round of PCR was positive, 80% of the samples could be isolated in C6/36 cells. However, for samples that could not be amplified in C6/36 cells the likelihood of amplifying first round PCR-positive samples in mosquitoes was modest with only 45% of these samples isolated in culture. These data are in agreement with the work of Oliveira De Paula and others\(^{27}\) who showed that 78% of clinical samples could be isolated by C6/36 cells with positive first round (35 cycle) RT-PCR.

In this study, the majority of samples selected were from hospitalized cases with secondary dengue infections.\(^{26,29}\) Fourteen percent of samples in this study are from primary infections and these were more likely to be isolated in C6/36 cells or mosquito inoculations, as shown in Table 2. Low levels of circulating antibodies appear to play a major factor in this as anti-dengue IgM units in 110 of 167 (66%) positive isolated samples were below the diagnostic level of 40 U of anti-dengue IgM in primary infection samples. This hypothesis was consistent in the analysis of secondary infections. Low levels of dengue IgM and IgG (below diagnostic levels) were significantly associated with higher isolation rates. High levels of anti-dengue IgM and/or IgG were associated with low rates of virus isolation. This might suggest that circulating antibodies bind to infectious virus particle preventing the attachment and thus infection of target cells in the cell cultures or within the mosquito.

This study also revealed that samples collected from DHF patients were less likely to have virus isolated. Despite DHF samples exhibiting increased viral load, the decreased isolation rates are likely caused by antibody complex development and circulating antibodies neutralizing free virus. A study by Vaughn and others\(^{29}\) in 2000 showed that viremia from DHF patients cleared more rapidly than viremia from patients experiencing DF. Viremia in this study was determined by the mosquito infectious dose. Studies that use PCR as an indicator for dengue virus detection show that viremia is higher in DHF versus DF patients.\(^{30,33}\) Although antibody-virus complexes and neutralized virus would impact viral biological assays such as viral isolation, they would not likely impact PCR readouts.

Clinical data can also be useful in evaluating viral isolation potential. Often the febrile status of patients is associated with severity of disease and increases in viremia. In this analysis we stratified all cases with fever as greater than 38°C or less than 38°C. Both direct C6/36 isolation and/or mosquito inoculations were significantly increased in cases who presented with fever at the time of blood collection. As shown by Vaughn and others\(^{34}\), fever is well correlated with viremia as assessed by mosquito inoculation and IFA staining. They showed that the percentage of positive-infected mosquitoes decreased as the day of defervescence approached. This suggests that fever is associated with viremia, which is likely the reason for the increase in viral isolation shown in this current study.
The final factor that was considered in this analysis was the day of disease that each patient presented to the hospital for blood collection. It is intuitive that after the peak of viremia the likelihood of isolating virus decreases as circulating anti-dengue antibodies increase. Our results show that during the first 4 days of infection greater than 85% of the samples can be isolated using the combination of the two methods and reduced to 65% for samples collect after Day 5. This is consistent with Yamada and others who reported that dengue viruses were isolated from 28 of 32 serum samples collected on disease Day 5 or earlier.

In this present analysis, we can conclude that PCR is the most sensitive and rapid method for virus detection, especially as a diagnostic assay. However, definitive biological assays for the detection of live virus must use virus isolation techniques. As virus isolation is necessary for further research studies, from our analysis, factors that predict the recovery of virus included viremia, level of anti-dengue IgM and IgG, and days after fever onset. In laboratories that process a large number of samples and must be selective in viruses that are amplified and stored, those clinical samples from patients presenting with fever with high viral load and low antibody levels collected within the first 4 days from the first symptoms would have a high probability of isolating virus by C6/36 cell culture. These rates could be further increased if this method was used in combination with mosquito inoculation. Successful isolation using these predictive indicators is consistent with dengue biology. Excluding DHF, dengue isolation is much more permissive when blood is collected in the early acute phase, during peak viral load, and often when the patient experiences the highest fever. As the patient defervesces, and the virus is cleared by the immune response, resulting in a significantly lower viral titer and a much greater potential to be complexed with neutralizing antibodies and thus lowering the isolation rates.

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