

Epidemiological Factors Relating to the Successful Isolation of Dengue Viruses

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OBJECTIVES : To determine the serotypes of dengue viruses associated with human infections and to determine the relation of certain aspects of infection to the isolation of dengue viruses.

BACKGROUND : Epidemics of dengue hemorrhagic fever (DHF) have been documented in Bangkok, Thailand, since the early 1960's. Clinical and virological studies have shown considerable fluctuation in the types of infection and in the types of dengue viruses associated with human infections. According to data presented elsewhere in this Annual Progress Report (2), dengue virus type-2 has been isolated from DHF patients during every epidemic that was studied. The other serotypes have exhibited a sporadic pattern of association with human disease and the frequency of isolation has been lower than that observed for dengue virus type-2. This report describes results of virological and serological studies conducted on patients of the Children's Hospital during the 1978 dengue epidemic.

METHODS : The subjects of this study were patients treated at the out-patient clinic and/or admitted to the Children's Hospital, Bangkok, during 1978. Out-patients had febrile illnesses and admitted patients had signs and symptoms consistent with those of dengue and/or dengue hemorrhagic fever (DHF). The day of onset of fever was recorded as the first day of disease. Heparinized blood (7 ml) was obtained from patients during acute phase of illness and a second blood sample (3 ml) was obtained, when possible, 2 to 3 weeks later. Clinical histories of patients were recorded on standardized forms.

One ml of blood per patient was submitted for hemogram analysis and the remainder was centrifuged at 2000 rpm for 15 minutes. Each plasma fraction

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was transferred to each of 2 vials, and then stored at -20°C and -70°C for serological and for virological testing, respectively. The cellular fraction was separated into platelet and leukocyte subpopulations for virus assay as described elsewhere (3).

Plasma and cellular fractions were assayed for virus by the mosquito inoculation technique employing *Toxorhynchites splendens*, the direct and delayed plaque assay and an infectious center assay using LLC-Mk2 cells (3,4). Viruses were identified by plaque reduction neutralization tests employing dengue virus types 1, 2, 3, and 4 monospecific antisera prepared in rhesus monkeys.

Acute and convalescent plasma of patients was acetone extracted and tested for (HI) antibody by the micro-hemagglutination inhibition test. Acetone extracted mouse brain antigens of dengue virus types 1, 2, 3 and 4 were used in the test, 8 hemagglutinating units per antigen. Confirmation of dengue virus infection of patients was based on a clinical diagnosis of dengue and/or DHF accompanied by a virus specific immune response and/or isolation of virus as described in results section (Table 1).

Except for modifications presented in results section, the diagnostic criteria employed for classification of the type of infection were those of the World Health Organization (WHO) (5). Illnesses with an antibody titer of < 20 before the 4th day of disease and a four-fold or greater increase in titer, but not to greater than 1:1280 one to 4 weeks later, were classified as primary infections. A secondary infection was recorded for patients with antibody titers < 20 in plasma obtained before the 5th day of disease with a titer ≥ 2560 in convalescent plasma, or titers ≥ 20 in plasma obtained before the 5th day a after onset of disease, with a four fold or greater increase in titer for convalescent plasma. An HI antibody titer of ≥ 1280 in acute and convalescent plasma without a four-fold difference was considered to be a presumptive secondary infection.

RESULTS : A total of 178 patients who were examined at the hospital and/or the out-patient department during 1978 were included in virus isolation studies. Of the 178 patients, 138 were clinically diagnosed dengue fever and/or dengue hemorrhagic fever cases. The remaining 40 were patients with febrile illness, the majority of whom were selected from the out-patient department to obtain preliminary data and/or virus isolations for other investigations. Of the 138 patients with a clinical diagnosis of DHF, presumptive or definite evidence of dengue virus infection was found by virus isolation and/or by serology in 118 cases. Of the 20 cases with unproven dengue etiology, paired plasma for diagnostic tests were available for 11. A correct diagnosis was thus made for 91.5% (118/129) of the patients. Dengue virus infections was diagnosed in 38% (10/32) of the patients with undifferentiated pyrexia with paired plasma specimens. A summary of the distribution of the 178 patients by etiology and clinical presentation of illness is presented in Table 1.

One hundred and twenty four of the 128 confirmed or presumptive dengue virus infected patients were classified as primary or secondary infections. Table 2 presents the distribution of the types of infections by sex and age. On the basis of the criteria employed, only two patients (1.6%) had primary infections. An additional 5 patients (2 males and 3 females) had possible

primary infections. Studies are in progress to confirm the type of infection. One hundred and twenty-two patients (95.3%) had secondary infections and 4 (3.1%) infections could not be classified. The unclassifiable infections were based on the isolation of dengue viruses from unpaired plasma specimens with antibody titer for 3 of 4 equal to or less than 1:20.

No apparent difference was noted between male and female for the different types of infections. The age of dengue virus infected patients ranged from less than one year to 16 year of age, with infections being most common in the 4 to 7 year age group (Table 3).

Table 4 summarizes the types of dengue viruses isolated from plasma and cellular components of the blood of dengue patients by type of infection. Seven additional isolates were detected, but were lost while attempting to increase infectivity titers for identification tests. Dengue virus types 2, 3 and 4 were isolated with 86.5% of the isolates being dengue virus type 2.

The distribution of dengue virus isolations according to age and sex of patients is presented in Table 5. Overall, the rate was slightly higher for males (31%) than for females (27%). The rate of virus isolation varied for different age groups and tended to be higher for patients 7 years of age and older. The apparent increase in virus isolation rates for older children mentioned above suggested that they reported to the hospital earlier than younger children infected with dengue viruses. An analysis of the data showed the mean reporting day to be approximately the same in each age group, but the range in the reporting period indicated that the older children did (7-8 and 11-12 age groups) tend to seek medical attention sooner than younger children.

The rate of isolation of dengue viruses in relation to the day of disease and the antibody titer of dengue patients is shown in Figure 1. Virus was isolated from 100% of the patients during days 2 and 3 of illness. Subsequently rate declined significantly ($r = -0.958$, $p = 0.001$) until day 8 when virus could no longer be isolated from patients (Figure 2). The rate of virus isolation for patient with antibody titers that ranged from less than 10 to 160 was 74% (20/27) as compared to a 17% (17/99) rate for patients with antibody titers of 320 or greater. The association of a decrease in virus isolation with an increase in antibody titers of patients was significant ($r = 0.843$, $p = 0.001$) Figure 3. Multiple linear regression analysis of the data showing the relation of virus isolation to both the day of illness and the HI antibody titer of dengue patients is presented in Figure 4. On the basis of these results, the probability of obtaining dengue virus isolations from patients can be predicted with a high degree of certainty. Employment of these findings in future studies will save both technical input and time, as well as overall cost required to perform the assays.

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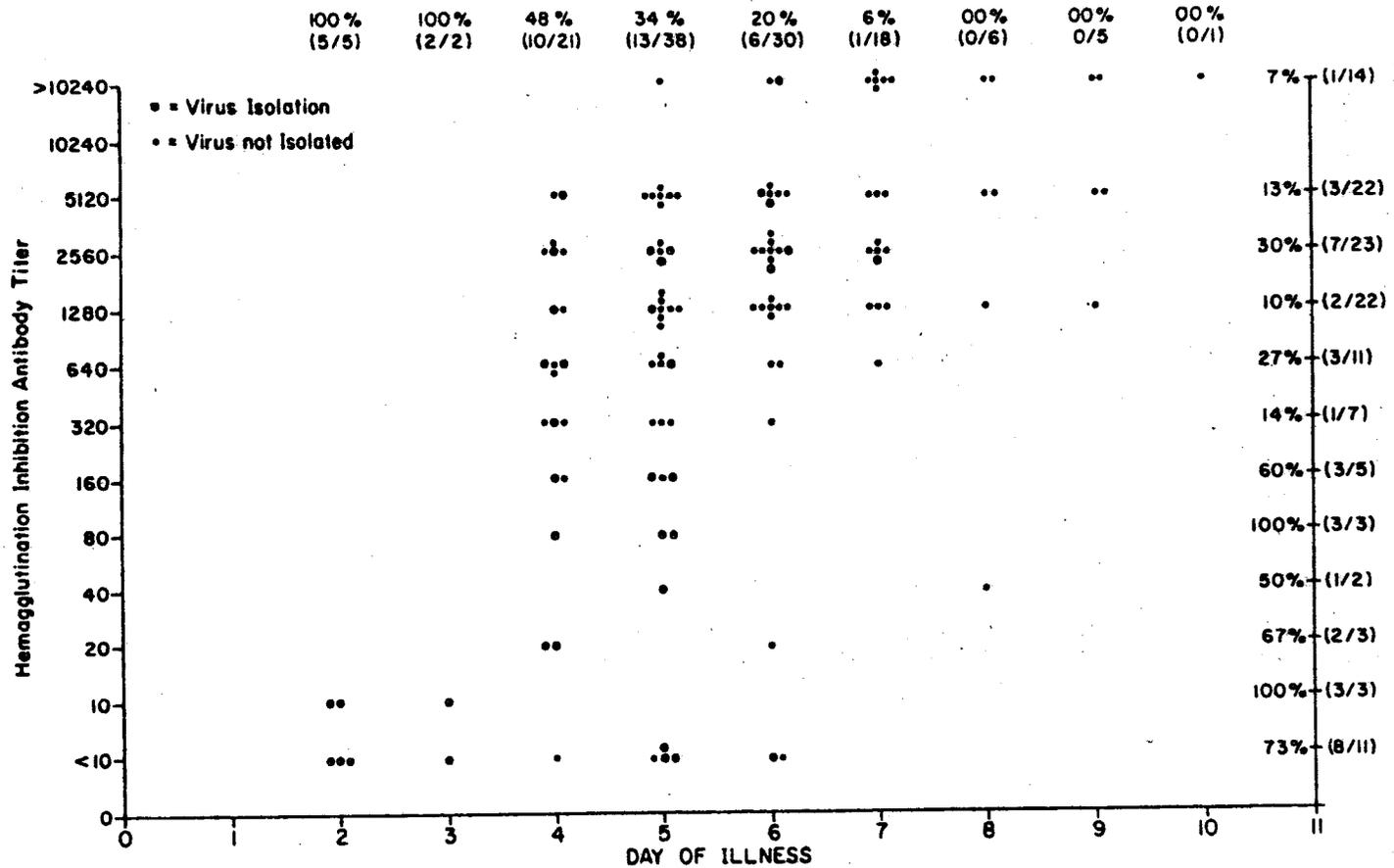


Figure 1. Distribution of dengue virus isolation in relation to hemagglutination inhibition antibody titer and of the day of illness of dengue patients.

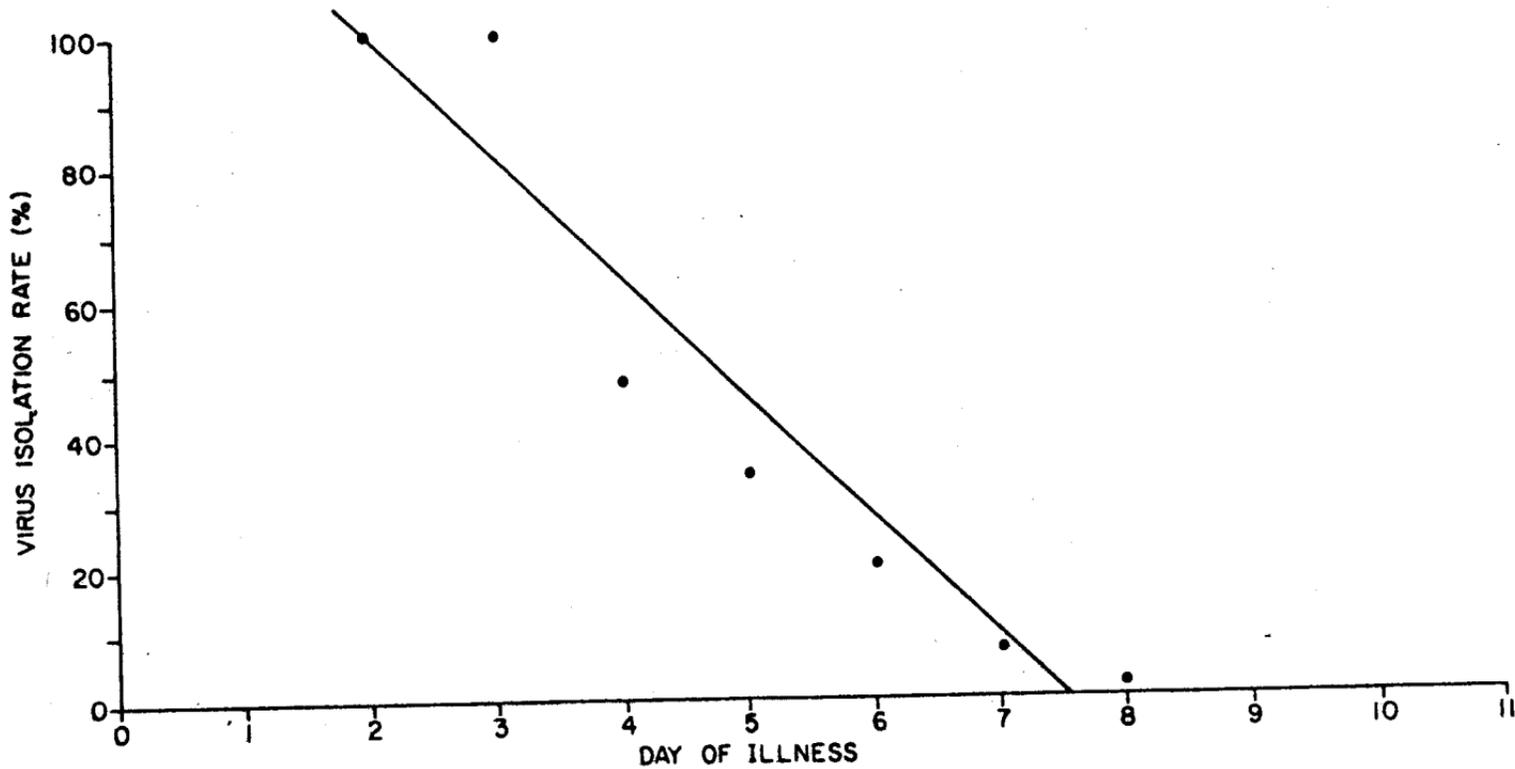


Figure 2. Correlation of the isolation rate of dengue viruses with the day of illness of dengue patients ($r = 0.958$, $p = 0.001$)

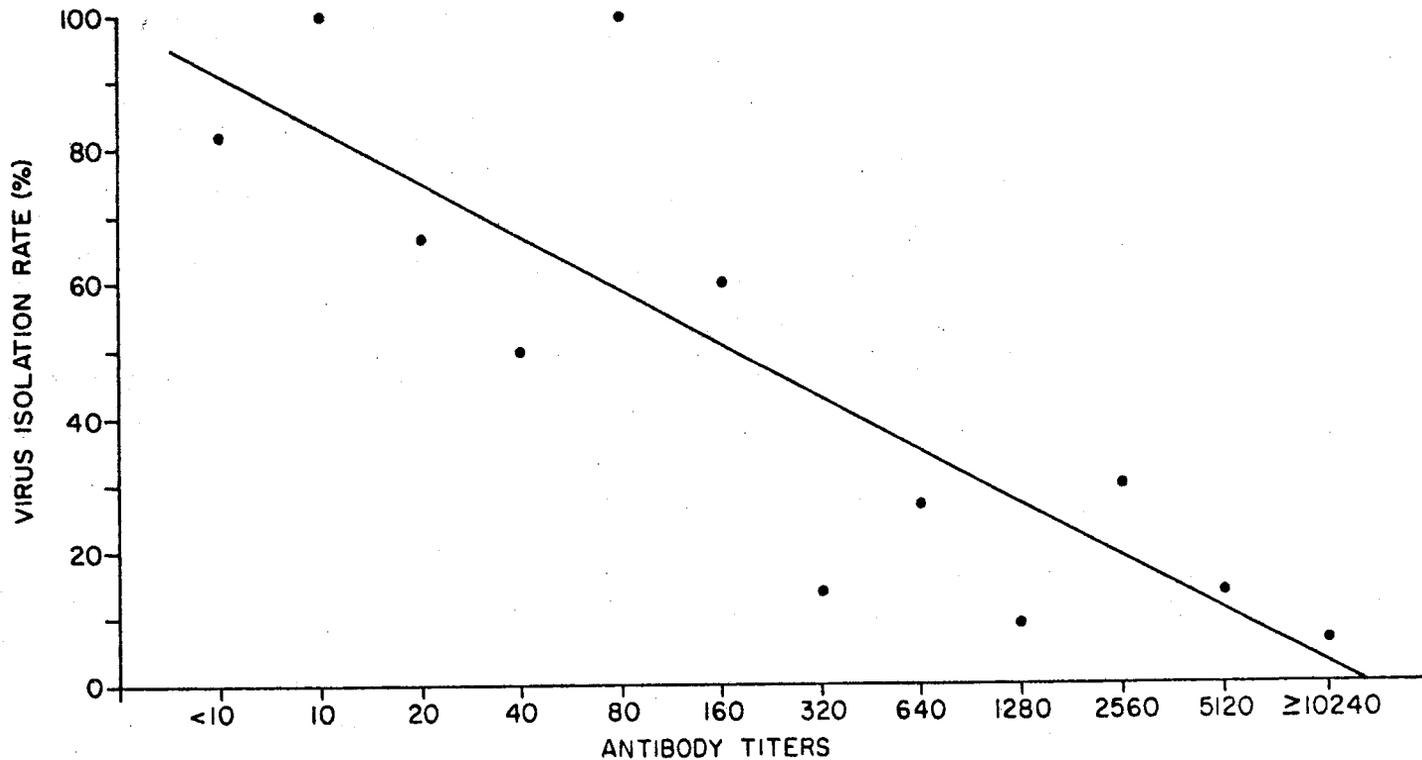


Figure 3. Correlation of the isolation rate of dengue virus with hemagglutination antibody titer of dengue patients ($r=0.843$, $p=0.001$)

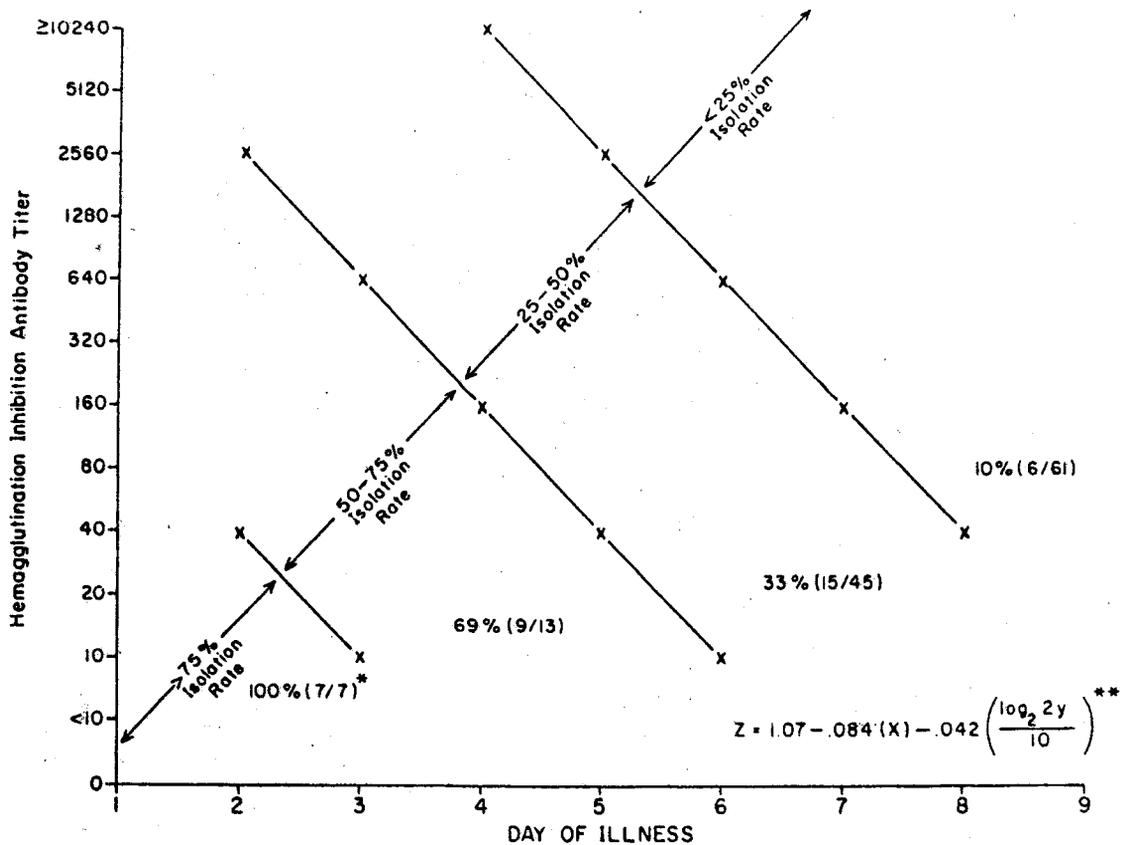


Figure 4. Dengue virus isolation rates in comparison to predicted rates determined by multiple linear regression analysis of actual virus isolation data in relation to the day of disease and the hemagglutination inhibition antibody titers of dengue patients

* actual virus isolation rate

** z = probability of isolating virus

x = day of illness

y = hemagglutination inhibition antibody titer