Infection and dissemination of two dengue type-2 viruses isolated from patients exhibiting different disease severity in orally infected *Aedes aegypti* from different geographic origin

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**Abstract:** The capacity for oral infection and dissemination of two dengue type-2 viruses, ThNH7/93 and ThNHpll/93, isolated from patients exhibiting different disease severity, dengue shock syndrome (DSS) and classical dengue fever (DF), respectively, was compared in two strains of *Aedes aegypti* from different geographic origin. The capacity for oral infection and dissemination to the head tissues of the isolate ThNH7/93 (DSS) was significantly higher in the *Ae. aegypti* strain from Nakhon Phanom, Thailand, than that in the strain from Escuintla, Guatemala, \(P < 0.05, \chi^2 = 6.833, df = 1\), whereas for the isolate ThNHpll/93 (DF) the capacity was not significantly different between the *Ae. aegypti* strains \(P > 0.05, \chi^2 = 0.557, df = 1\). The *Ae. aegypti* strain from the location where the viruses were isolated (Nakhon Phanom, Thailand) showed significantly higher susceptibility to the isolate ThNH7/93 strain than to the isolate ThNHpll/93 \(P < 0.05, \chi^2 = 13.283, df = 1\), while the susceptibility to both isolate virus strains was not significantly different in the *Ae. aegypti* strain from Guatemala \(P > 0.05, \chi^2 = 0.049, df = 1\). These results suggested that the efficacy of dengue virus circulation was likely to vary according to combination of the virus strains and origin of the vector mosquitoes.

**INTRODUCTION**

Dengue (DEN) viruses with 4 different serotypes, causing dengue fever (DF), dengue haemorrhagic fever (DHF), and dengue shock syndrome (DSS) are a medically important arthropod-borne virus affecting humans in terms of morbidity (Monath and Heinz, 1996; WHO, 2000). DF/DHF and DSS have re-emerged mainly in tropical and subtropical regions, and DHF has become the leading cause of death and hospitalization among children in some Southeast Asian countries during the last two decades (Gubler and Clark, 1995; Monath, 1994).

Inter-population variation in vector competence of mosquito species for arbovirus has been well documented: *Ae. albopictus* and chikungunya virus (Tesh et al., 1976), *Ae. aegypti* and yellow fever (Aitken et al., 1977), and *Ae. triseriatus* and La Crosse virus (Grimstand et al., 1977). As for flavivirus, significant variation in oral susceptibility among geographic strains of *Ae. albopictus* and *Ae. aegypti* for the 4 dengue serotypes has been reported
(Gubler and Rosen, 1976; Gubler et al., 1979; Rosen et al., 1985; Tardieux et al., 1990). Differences have also been reported among *Ae. albopictus* strains in the rates of dengue virus infection, dissemination, and transmission (Boromisa et al., 1987). However, the mechanisms responsible for intra and interspecific variation in the ability of *Aedes* subgenus *Stegomyia* mosquitoes for infection and transmission of dengue (DEN) viruses are not well understood. Some previous works proposed that it was genetically controlled (Gubler and Rosen, 1976; Gubler et al., 1979), while others considered that the variability of the oral infection rate was attributed to the mesenteronal barrier, since no significant differences were noted when several strains of *Ae. aegypti* and *Ae. albopictus* were infected by parenteral inoculation (Rosen et al., 1985).

Pandy and Igarashi (2000) in a molecular study on dengue type-2 viruses has classified the virus of the Southeast Asian genotype isolated from patients exhibiting different disease severity into 3 subtypes according to non-synonymous amino acid replacements. In parallel, *in vitro* experiments on growth rate of dengue type-2 viruses have shown that representative strains from each of the 3 different amino acid sequence subtypes of the virus showed different infection rates to a primary culture of human peripheral blood leukocytes; hence, it was concluded that the infection rate correlated with the disease severity (Mangada and Igarashi, 1998). In addition, Morens et al. (1991) has reported that the observed cytopathic effects in C6/36 cells correlated with disease severity.

Despite all these observations, there was no document on the rates of susceptibility of *Ae. aegypti* to oral infection with virus isolated from patients exhibiting different disease severity. Thus, in this study the infection and dissemination rates of two strains (ThNH7/93 and ThNHpl/93) of dengue type-2 virus isolated from patients exhibiting different disease severity during the same epidemic season in the same epidemic area were evaluated in orally infected *Ae. aegypti* from different geographic origin.

The aim of this study was to evaluate the oral infection and dissemination of two dengue type-2 virus isolated from patients exhibiting different disease severity in *Ae. aegypti* of different geographic origin, one of which was originated from Guatemala where the dengue type-2 virus of Southeast Asian genotype had not been reported to be prevalent.

### MATERIALS AND METHODS

**Mosquito strains**

Two geographically different *Ae. aegypti* strains were used in this study. The dengue epidemiological background of the site where the mosquitoes were collected is presented in Table 1. The strain from Thailand was derived from eggs oviaposited by field-collected adults, while the strain from Guatemala was derived from field-collected eggs. The mosquitoes were reared in plastic pans (33 cm × 25 cm × 11 cm high approximately) containing 3 l of

<table>
<thead>
<tr>
<th>Geographic origin</th>
<th>Year of colonization</th>
<th>Dengue epidemiology background</th>
</tr>
</thead>
<tbody>
<tr>
<td>Nakong Phanon Thailand</td>
<td>January 1997</td>
<td>Dengue endemic area, with previous report of DSS, DHF cases.</td>
</tr>
<tr>
<td>Escuintla* Guatemala</td>
<td>December 1991</td>
<td>Dengue endemic area with a recent report of DHF cases (Sept. 2000)</td>
</tr>
</tbody>
</table>

* Supplied by Dr. I. Tanaka from Department of Environmental Biology, Japan Environmental Sanitation Center.
aged tap water with a density of 180–210 larvae per container. The larvae were fed on a diet of yeast extract with mouse food powder and maintained at 25°C. Adults were provided with 10% sucrose solution.

Virus strains

Virus isolates were originally obtained from the sera of patients in Nakhon Phanom Provincial hospital in Northeastern Thailand, during the dengue outbreak in 1993. The serotypes were determined as dengue 2 by reverse transcription-polymerase chain reaction (RT-PCR). Clinical information, serological response of the patients, in vitro infectivity and amino acid (AA) subtyping of coded strains used are shown in Table 2. Each of the virus isolates was obtained as a seed virus from the Department of Virology, Institute of Tropical Medicine, Nagasaki University.

Virus assay

A high titer of each isolate was prepared from the seed virus by inoculation into a monolayer culture of Ae. albopictus clone C6/36 cell line and incubated at 28°C for 8 days in Eagle's medium supplement with 2% heat-inactivated fetal calf serum (FCS) and 0.2 mM each nonessential amino acids. The infected culture fluid was harvested 8 days after inoculation, aliquoted, and stored at −80°C until used. Thus, the virus titer of the isolates was measured by the focus formation test by using BHK-21 cells on 96-well plates. Then three different titers were prepared by dilution with Eagle's medium supplement with 2% heat-inactivated fetal calf serum (FCS) and 0.2 mM each nonessential amino acids, and used for preparation of the infectious meal, blood virus sucrose solution (BVS).

**Oral infection**

To minimize age factors, only 4–5 day-old female mosquitoes were used. About 60 females were placed in cylindrical pint cardboard cages covered at one end with fine non-wettable nylon mesh. These females were deprived of food for 24–36 hrs. prior to the infectious meal. The females were allowed to feed with the infectious meal (BVS) consisting of equal volumes of isolate virus suspension, washed rabbit erythrocytes, and 10% sucrose solution. Drops of the infectious meal were placed on the mesh covering the cardboard cage containing mosquitoes as previously described (Gubler et al., 1979). Feeding time was limited to 1–2 hrs, engorged mosquitoes were collected with an aspirator at 1 hr. intervals and transferred into clean cartons and maintained for up to 14 days at 30 ± 1°C.

**Detection of virus infection and dissemination**

Mosquito head and abdomen were severed and homogenized with a pellet mixer in a microtube containing Eagle's medium supplement with 2% heat-inactivated fetal calf serum (FCS) and 0.2 mM each nonessential amino acid. An aliquot of 150 µl of homogenized mosquito was clarified by centrifugation. The supernatant was filtrated through a 0.22 µm filter and then inoculated into a monolayer culture of Ae. albopictus clone C6/36 cell line and incubated at 28°C for 8 days in Eagle's medium supplement with 2% heat-inactivated fetal calf serum (FCS) and 0.2 mM each nonessential amino acids. After the culture fluid was harvested on the 8th day, the presence of the viral antigen was assayed by antigen detection-ELISA.
as described previously (Bundo-Morita, 1989). The detection of viral antigen in the homogenized abdomen was interpreted to indicate the mosquito midgut had become infected. Detection of viral antigen in the homogenized head indicated that the midgut had become infected and that the virus had subsequently disseminated to secondary target organs.

**Determination of Oral ID50**

The amount of virus to infect 50% of *Ae. aegypti* (oral ID50) was estimated as previously described (Gubler et al., 1979). Briefly, *Ae. aegypti* strains were fed on a suspension of virus strains of which titers were $4 \times 10^4$, $4 \times 10^3$, and $2 \times 10^2$ FFU/ml.

Then the percentage of mosquitoes infected was plotted against the virus titers of blood virus sucrose solution (BVS) used as the infectious meal.

**Statistical analysis**

The statistical analysis was done using Systat 7.0 software for Windows. A multiple comparison Chi-square analysis (with Yate's correction) was used to determine the significant difference among the infection and dissemination rates of the different isolate virus in each mosquito strain.

**RESULTS**

**Screening for susceptibility**

Significant variation in susceptibility

<table>
<thead>
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<th>Titer of feeding suspension$^a$</th>
<th>Isolate virus strain</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>ThNH7/93</td>
</tr>
<tr>
<td></td>
<td>Infection$^b$</td>
</tr>
<tr>
<td>$4 \times 10^4$ FFU/ml</td>
<td>16/28(57)</td>
</tr>
<tr>
<td>$4 \times 10^3$ FFU/ml</td>
<td>13/33(39)</td>
</tr>
<tr>
<td>$2 \times 10^2$ FFU/ml</td>
<td>3/33(9)</td>
</tr>
<tr>
<td>Total</td>
<td>32/94(34)</td>
</tr>
<tr>
<td>Oral ID50$^d$</td>
<td>3.51$\times 10^4$</td>
</tr>
</tbody>
</table>

$^a$ Determined by infectivity titration by focus formation in BHK-21 cells. $^b$ Number of mosquitoes positive for dengue type-2 viral antigen in abdomen/number tested. $^c$ Number of mosquitoes positive for dengue type-2 viral antigen in head tissues/number tested. $^d$ Titer of isolate suspension required to infect 50% of mosquitoes (FFU/ml). The numbers in parentheses show percentage.

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</tr>
<tr>
<td></td>
<td>Infection$^b$</td>
</tr>
<tr>
<td>$4 \times 10^4$ FFU/ml</td>
<td>5/44(11)</td>
</tr>
<tr>
<td>$4 \times 10^3$ FFU/ml</td>
<td>2/21(9)</td>
</tr>
<tr>
<td>$2 \times 10^2$ FFU/ml</td>
<td>2/35 (6)</td>
</tr>
<tr>
<td>Total</td>
<td>9/100(9)</td>
</tr>
<tr>
<td>Oral ID50$^d$</td>
<td>1.82$\times 10^5$</td>
</tr>
</tbody>
</table>

$^a$ Determined by infectivity titration by focus formation in BHK-21 cells. $^b$ Number of mosquitoes positive for dengue type-2 viral antigen in abdomen/number tested. $^c$ Number of mosquitoes positive for dengue type-2 viral antigen in head tissues/number tested. $^d$ Titer of isolate suspension required to infect 50% of mosquitoes (FFU/ml). The numbers in parentheses show percentage.
for the oral infection with the two isolates was observed. The overall infection rate for the two isolate virus in mosquitoes from Thailand ranged from 12.9–34.0% (Table 3), and 9.0–11.6% in mosquitoes from Guatemala (Table 4). The Ae. aegypti strain from Thailand (Nakhon Phanom) showed the highest susceptibility, and was more susceptible to the isolate ThNH7/93 (DSS) than to the isolate ThNHpll/93 (DF), $P<0.05$, $\chi^2=13.283$, df = 1; whereas the Ae. aegypti strain from Guatemala (Escuintla) had a slightly lower infection rate, and a significant variation in susceptibility for the oral infection was not observed with either of the two isolate viruses used, $P>0.05$, $\chi^2=0.049$, df = 1. The results demonstrate that the mosquito strain from Thailand is more susceptible to the oral infection with either isolate virus strain than the mosquito strain from Guatemala.

**Ascending the infectivity**

Variation in the infectivity rate between Ae. aegypti strains was observed for the virus strains. For the isolate ThNH7/93 (DSS), infection and dissemination rates ranged from 9–34% and 8–32%, respectively; and for the isolate ThNHpll/93 (DF) ranged from 11–13 and 10–11%, respectively (Tables 3 and 4). Infection and dissemination rates of the isolate ThNH7/93 (DSS) were significantly higher in the Ae. aegypti strain from Thailand (Nakhon Phanom) than those in the strain from Guatemala (Escuintla), $P<0.05$, $\chi^2=6.833$, df = 1; whereas for the isolate ThNHpll/93 (DF), even though slightly higher in the Ae. aegypti strain from Thailand than those in the strain from Guatemala, the observed rates were not significantly different, $P>0.05$, $\chi^2=0.557$, df = 1. Furthermore, in a parallel experiment at 5th day of post-infection the ThNH7/93 (DSS) showed an infection and dissemination rate significantly higher than that of ThNHpll/93 (DF) in either Ae. aegypti strain, $P<0.05$, $\chi^2=9.167$, df = 1 (data not shown). On the other hand, when the amount of each isolate virus to infect orally 50% of mosquitoes (oral ID50) was determined for each mosquito strain as described in Materials and Methods, the results showed that oral ID50 (FFU/ml) of ThNH7/93 and ThNHpll/93 for Ae. aegypti from Thailand were $3.51 \times 10^4$ and $1.0 \times 10^5$ respectively (Table 3); whereas oral ID50 of ThNH7/93 and ThNHpll/93 for Ae. aegypti from Guatemala were $1.82 \times 10^5$ and $1.18 \times 10^5$ respectively (Tables 3 and 4).

**Discussion**

Variability in susceptibility to experimental oral infection with all 4 serotypes of dengue virus is well known for *Aedes* sp. and strains of a given species (Tesh et al., 1976; Aitken et al., 1977; Grimstand et al., 1977; Tardieux et al., 1990). Despite all these observations, the susceptibility, infection and dissemination rate in orally infected Ae. aegypti originating from different geographic localities with dengue virus isolated from patients exhibiting different disease severity has not been described yet. Therefore, in the present study the capacity of two different isolates of dengue type-2 virus to infect and disseminate in orally infected Ae. aegypti strains was ascertained.

The present study on the isolate viruses ThNH7/93 and ThNHpll/93 which were isolated from patients with clinical manifestation of dengue shock syndrome (DSS) and classical dengue fever (DF), respectively, has shown that significant variation in susceptibility to oral infection with virus isolates occurred between strains of Ae. aegypti originating from different geographic localities. Even though the infection rate for the two isolate virus strains in the Ae. aegypti strain from Guatemala was lower than that in the strain from Thailand (Tables 3 and 4), the observed infection rates are consistent with previous works that have reported infection rates for dengue type-2 virus of 3–57% (Tesh et al., 1976; Grimstand et al., 1977;
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ThNH7/93.
The

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ThNHpl1/93,

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(DSS).

Therefore,
even

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Ae.

egypti

strain

from

Guatemala

did

not

show

a

significant

difference

in

susceptibility

to

oral

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two

isolate

viruses,
similar

susceptibility

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the

Ae.

egypti

strain

from

Thailand

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Ae.

egypti

in

Guatemala

if

the

dengue

type-2

virus

of

Southeast

Asian

genotype

happens

to

invade

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the

country.

Mangada

and

Igarashi

(1998)
in

their

study

on

biological

in

vivo

properties

reported

that

the

ThNH7/93

isolate

produced

significantly

larger

plaques

than

the

ThNHpl1/93.

It

was

speculated

that

the

difference

might

be

due

to

certain

genetic

elements

of

the

isolate

virus.

Furthermore,
Pandy

and

Igarashi

(2000)
classified

the

isolates

ThNH7/93

and

ThNHpl1/93

into

amino

codon

(AA)

subtypes

I

and

III,

respectively,

and

reported

a

possible

correlation

between

the

amino

codon

sequence

subtypes

of

the

virus

strain

and

clinical

severity

of

the

patient

from

whom

each

virus

strain

was

isolated.

In

the

present

study,
infection

and

dissemination

rate

for

the

isolates

ThNH7/93

and

ThNHpl1/93

were

not

significantly

different

in

Ae.

egypti

from

Guatemala.

However,
in

Ae.

egypti

from

Thailand

it

was

observed

that

the

isolate

ThNH7/93

seemed

to

be

more

infective

than

the

isolate

ThNHpl1/93,

which

correlated

with

severity

of

disease

exhibited

by

the

patients

from

whom

each

isolate

virus

strain

was

isolated.

As

in

the

studies

previously

mentioned

(Mangada

and

Igarashi,

1998;
Pandy

and

Igarashi,

2000),

the

differences

in

susceptibility,

infection

and

dissemination

rates

of

both

isolate

viruses

between

the

Ae.

egypti

strains

observed

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the

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be

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elements

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the

isolate

virus.

In

Guatemala,
even

though

DSS

cases

have

not

been

reported

up

to

the

present

time,

apparently

are

being

experienced

similar

continued

cycles

of

dengue

infections,

as

previously

observed

in

countries

in

Southeast

Asia

(PAHO,

2000).

Our

findings

showed

that

the

Ae.

egypti

strain

originated

from

Guatemala

(Escuintla)

was

susceptible

to

oral

infection

with

the

virus

isolated

from

patients

originated

from

a

Southeast

Asian

country

exhibiting

either

DSS

or

DF

symptoms.

Thus,

eventual

introduction

of

the

dengue

type-2

virus

Southeast

Asian

genotype,

which

is

associated

with

severe

clinical

manifestations,

and

subsequently

possible

DSS

manifestations,

should

be

taken

into

consideration

as

a

potential

risk

factor

in

Guatemala.

Acknowledgments

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authors

acknowledge

the

technical

assistance

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also

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for

statistical

support

and

N.

Phumala

and

M.

Pascucci

for

critically

reading

the

manuscript.

The

first

author

is

a

recipient

of

a

Mombusho

scholarship

from

the

Ministry

of

Education,

Science,

Sports

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Culture

of

Japan

for

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Tropical

Medicine,

Nagasaki

University.

References

Aitken,

T.

H.

G.,

Downs,

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Shope,

R.

E.

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Aedes

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J.

Trop.

Med.

Hyg.,

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Boromisa,

R.

D.,

Rai,

K.

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Grimstad,

P.

R.

1987.

Variation

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Aedes

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for

dengue

1

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J.