SUSCEPTIBILITY AND TRANSOVARIAL TRANSMISSION OF DENGUE VIRUS IN *AEDES AEGYPTI*: A PRELIMINARY STUDY OF MORPHOLOGICAL VARIATIONS

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Abstract. Two types of morphological variants, the dark form and the pale form of *Aedes aegypti* were selected from wild-caught mosquitos. Ascertaining any differences between the two forms for susceptibility to dengue type 2 virus was performed by oral feeding. Transovarial transmission was further determined from the progenies of infected mosquitos by tracing them to the third generation. Significant differences in oral infection were not observed between these two forms of mosquitos. Transovarial transmission was found in the progenies of infected females of both forms, and the filial infection rates (FIRs) were also similar. However, there was a trend of declining FIR in the later generation. In order to achieve an accurate result, more tests are currently underway to obtain a larger number of progeny. Although the FIR was low in the present study under laboratory conditions, higher rates might occur under field conditions, which could have a significant impact on the maintenance of dengue viruses in nature.

INTRODUCTION

Dengue is the most important arthropod-borne viral disease in Southeast Asia, where it occurs in both endemic and epidemic forms (WHO, 2000). Dengue fever (DF) and dengue hemorrhagic fever (DHF) are caused by one of the four serotypes (DEN-1, DEN-2, DEN-3, and DEN-4), which are members of the genus *Flavivirus*. Their natural history suggests that the biology of these viruses is highly adapted to their mosquito hosts and they were most likely mosquito viruses prior to becoming adapted to lower primates and humans (Gubler *et al*, 1979). These viruses are maintained in a "human-mosquito-human" cycle. *Aedes* (*Stegomyia*) mosquitos are the primary mosquito vectors. The cyclic nature of dengue epidemics, and how the virus is maintained during interepidemic periods in areas where epidemics have occurred previously, pose questions which have led to studies to evaluate the importance of transovarial transmission in dengue virus maintenance.

The principal vector of dengue virus is *Ae. aegypti.* Variable susceptibility to dengue virus has been documented among populations and strains of this species (Gubler *et al*, 1979). Differences in vector potential within a mosquito species have traditionally been associated with the presence of subspecies and significant geographic or ecological separation. Mattingly (1958) recognized 3 subspecies of *Ae. aegypti* (Linnaeus). These were differentiated by the color of the scale on the abdomen; ssp *formosus* (Walker), a black form; the type form, an intermediate form; and var *queenslandensis* (Theobald), a pale form.

In each of the above situations, a single species of mosquito has been described from a broad geographical range and variety of ecologies. Investigation of susceptibility to DEN-2, between the type form and ssp *formosus*, found that the type form was more susceptible to DEN-2 than ssp *formosus* (Bosio *et al*, 1998). However, the variations in vector efficiency of this species may not only be associated with a broad geographic or ecological separation, but may exist within localized populations as well (Gubler *et al*, 1979).

In Thailand, morphological variations of *Ae. aegypti* have also been observed. The populations consisted of the dark form and the pale form (Mogi *et al*, 1989; Sucharit and Surathin, 1994). In addition, laboratory infection with DEN-2 of the dark form and the pale form from different areas (Phrae and Chanthaburi provinces), showed that the susceptibility to DEN-2 virus of the two forms was similar (Sucharit *et al*, 1997).

Nevertheless, the possible association of morphological variations with dengue susceptibility has not yet been resolved in *Ae. aegypti* populations from the same, or other, geographical areas. Thus, this

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was studied to confirm the susceptibility to DEN-2 virus of the dark and pale forms which were selected from *Ae. aegypti* originating from Chiang Rai and Satun provinces, respectively. It is also interesting for further investigation of the possibility of the transovarial transmission of both forms.

The purpose of this study was twofold. The first was to compare the susceptibility of two forms of *Ae. aegypti* to infection with DEN-2 virus and the second was to determine the possibility of transovarial transmission of DEN-2 virus in these *Ae. aegypti*.

MATERIALS AND METHODS

Field observation of morphological variations of *Ae. aegypti*

To determine the presence of the dark form and the pale form in nature, the collection of *Ae. aegypti* larvae from breeding sites was carried out in various places. The immature stages of living samples were brought to the laboratory and raised until adult. The dark form and the pale form were classified and the percentages were recorded.

Mosquitos

The dark and pale forms were obtained by the laboratory selection of *Ae. aegypti* originating from Chiang Rai and Satun provinces, respectively. Mosquitos from the ninth laboratory selection were used to determine susceptibility and transovarial transmission. In addition, *Toxorhynchites splendens* were used for viral assay. The dark form and pale form were examined and selected following Mattingly *et al* (1957) and McClelland (1960). Standard rearing conditions were 28°C and a 12L: 12D photoperiod. Larvae were reared at no more than 300 per liter of tap water, and were fed on guinea-pig pellets. Adults were provided with 10% sugar solution and blood meals from uninfected hamsters (Limsuwan *et al*, 1987).

Viruses

DEN-2 16681 viruses were obtained from the Center for Vaccine Research and Development, Mahidol University at Salaya, Nakhon Pathom, Thailand. The viruses had been passed and propagated in *Toxorhynchites* mosquitos. Dengue virus-infected *Toxorhynchites* mosquitos were triturated and a virus suspension was made in PBS, pH 7.5, with heat-inactivated fetal calf serum. This was employed as the source of virus for oral infection experiments.

Preparation of infectious blood meal and oral infection of mosquitos

Infectious blood meals were prepared by mixing 2 parts virus suspension, 2 parts 10% sugar solution and 1 part washed human erythrocytes. Approximately 5-7 days' old mosquitos were deprived of food for 36-48 hours and then were orally infected with DEN-2 by artificial membrane feeding via parafilm membrane, as described by Harada *et al* (1996). The fully engorged female mosquitos were selected for experimental trials.

Virus assay

Virus assay for titration of infectious blood meal was done by intrathoracic inoculation of *Tx. splendens* mosquitos, as described by Rosen and Gubler (1974). In brief, five *Tx. splendens* examined from each sample received an inoculum of 10-fold serial dilutions of an infectious blood meal, after incubation at 32 °C for 14 days. The presence of viral antigen was determined using the indirect fluorescence antibody test (IFAT) on the head squash. Viral titer was calculated using the method of Reed and Meunch (1938), and expressed as 50% mosquito infectious dose (MID50) log 10/ml.

Raising of progeny from transovarially-infected mosquitos

The egg batch from each infected female was allowed to hatch. The F1 progenies were reared to adult and retained for 5-7 days after emergence to ensure mating. Female mosquitos were allowed to feed on fresh uninfected human blood by artificial membrane feeding via parafilm membrane, and were confined individually for egg-laying. Head squashes of the surviving mosquitos were tested for the presence of dengue antigen. Eggs of the females positive with virus antigen were selected and kept for 1 month. The F2 progenies were reared similarly. The mosquitos were raised to the third generation.

RESULTS

Field observations

Morphological variations used in the present study were observed in the field. The collection of *Ae. aegypti* in the field was carried out from 176 breeding containers in 13 different localities. More than 2,487 living offspring of *Ae. aegypti* were examined and it was found that the dark form (70.88%) had wider distribution than the pale form (29.11%).

Selection in the laboratory

The developmental rates of the dark form and pale form appeared to be similar, and up to the ninth laboratory selection, the pure dark form and pale form appeared in each colony. Therefore, mosquitos from the ninth laboratory selection were used to determine susceptibility and transovarial transmission of the vector mosquitos.

Susceptibility to DEN-2 virus

In a preliminary oral infection trial with DEN-2 virus, with the two forms of *Ae. aegypti* mosquitos, the infection rates among individuals of both forms were observed by an indirect fluorescence antibody technique (IFAT).

The comparative susceptibility to DEN-2 of both forms is summarized in Table 1. The infection rates of the dark form and the pale form were 20.63% and 26.82%, respectively. There were no significant differences in the percentage infections of these forms ($p \geq 0.05$).

The filial infection rate (FIR) of both forms of *Ae. aegypti* showed that transovarial transmission (TOT) was present in the progenies of infected parental females. For the dark form, the FIRs in F1 to F3 progeny were 3.7%, 3.3%, and 1.4%, respectively (Fig 1). For the pale form, the progenies were able to be maintained to the F1 generation only, of which its FIR (2.7%) was slightly lower than that of the dark form of the same generation (3.7%) (Fig 1).

Table 1
Infection rates of *Aedes aegypti* (dark form and pale form) after oral infection with DEN-2 16681 virus.

Aedes aegypti	Titer of blood meal log 10 MID50/ml	No. of infected / no. tested (%)
Dark form	7.8	13 / 63 (20.63)
Pale form	7.8	11 / 41 (26.82)

The results of the present study indicated that dengue virus could persist in mosquito generations through transovarial passage.

DISCUSSION

The availability of morphological markers was associated with virus transmission (Mattingly, 1958). There are numerous reports of the morphological variations of sibling species complex and subspecies among populations of *Aedes* (*Stegomyia*); for instance, the variation in tarsal banding patterns on the midtarsomeres of the *Ae. simpsoni* complex (Lutwama and Mukwaya, 1994), the variation in scale-color pattern of palps, halters and metatarsi (Vande Hey *et al*, 1978), and the variation in scale-color pattern of the abdominal tergite (Mattingly, 1957; McClelland, 1960). In addition, relationships between morphological variations and vector competence have also been reported by various authors (Warren *et al*, 1977; Chan *et al*, 1994; Sucharit *et al*, 1997; Adak *et al*, 1999).

The present study indicated that morphological variants of *Ae. aegypti*, the dark form and the pale form from Chiang Rai and Satun provinces, were not associated with oral susceptibility to DEN-2 virus. These findings are similar to those of Sucharit *et al* (1997), who used two phenotypes of *Ae. aegypti* from Phrae and Chanthaburi provinces. These investigations strongly support no relationship between the susceptibility to DEN-2 virus and the dark form and the pale form.

The amount of virus required to infect a significant

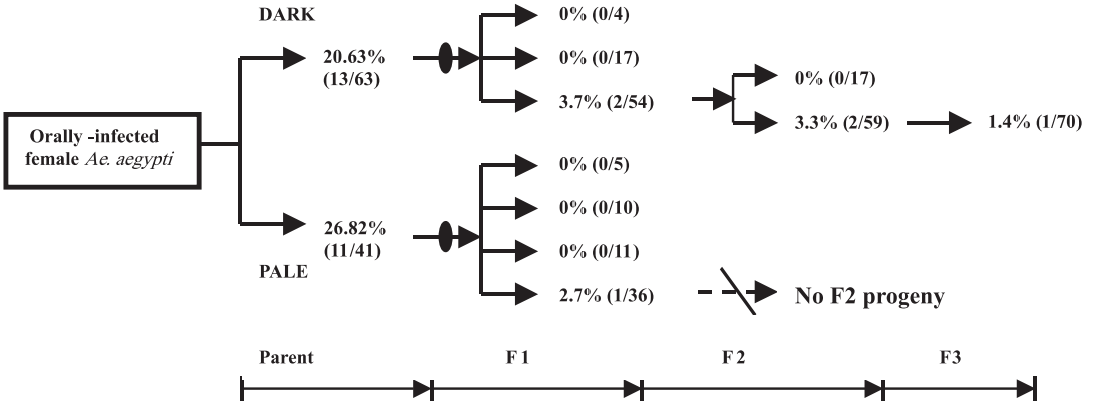

Fig 1- Transovarial transmission of dengue-2 virus tracing 3 generations of *Ae. aegypti*: dark form and pale form. Infection rates of their offspring given as percentage infection (sample size in parentheses).

proportion of the population is an important quantitative factor that affects vector competence. Gubler *et al* (1979) reported that *Ae. aegypti* could be infected with dengue virus when feeding on virus suspension ranging in titer from 7.3 to 9.0 log10 MID50/ml. From our results, the infection rates for DEN-2 of both forms of *Ae. aegypti* were slightly low with a feeding suspension of 7.8 log 10 MID50/ml. However, other investigators found no significant differences in infection rates using a virus titer of 8.0 log 10MID/ml (Gubler *et al*, 1979; Sucharit *et al*, 1997).

Transovarial transmission of dengue viruses in *Ae. aegypti* and *Ae. albopictus* from field and laboratory experiments have been reported (Khin and Than, 1983; Rosen *et al*, 1983; Shroyer, 1990; Ahmad *et al*, 1997). The isolation of dengue viruses from field collections has suggested that transovarial transmission of dengue virus occurs in nature (Khin and Than, 1983; Hull *et al*, 1984; Joshi *et al*, 1996).

The pedigree presented here showed that the dark form and the pale form possess efficient transovarial transmission if only a selection of the transovarially infected females was maintained.

Regarding differences in filial infection rates in the dark form, the FIR gradually declined with each generation and the virus could be detected up to the third generation. In the pale form, only transovarially infected F1 progeny could be obtained which showed that where a FIR was slightly lower, dengue virus could also be transmitted to F1 progeny.

Rosen *et al* (1983) showed that the transovarial transmission of dengue viruses by *Ae. aegypti* varies extensively, depending on dengue serotype, strain of virus, and strain of mosquito. Ahmad *et al* (1997) demonstrated transovarial transmission of DEN-1 virus in *Ae. aegypti* with low FIR. In addition, in the orally-infected mosquitos with DEN-1 virus, it was observed that the filial infection rate decreased with successive generations (Rosen *et al*, 1983). However, the study of Joshi *et al* (2002) demonstrated the efficient transovarial transmission of DEN-3 by *Ae. aegypti* females, and suggested that the virus could be detected up to the seventh generation. An increase in the number of infected mosquitos in the initial generation up to F2, which then stabilized in subsequent generations, was observed.

The preliminary study result showed that some of the infected female mosquitos did not suck blood, infertility among the mosquitos, and the number of eggs were reduced, with longer periods of storage (1 month), and that the decrease in the larval hatching rate was due to aging. The failure to detect transovarial transmission in subsequent (F2-F3) generations of the pale form was due to its being weaker than the dark form, and reproductive disadvantage may occur during the process of selection.

Furthermore, under laboratory conditions, individuals possessing the required extreme genotypes for laboratory selection may be at a reproductive disadvantage. Therefore, further investigation is required into hatching, emergence and mortality rates.

The data presented here suggest that the persistence of transovarial transmission in successive generations of mosquitos is an important mechanism in the inter-epidemic maintenance of dengue virus. However, in the present preliminary study, the failure to detect transovarial transmission in some of the tests may have been due to insufficient sample size, especially when the rates of transovarial transmission and filial infection were low. Therefore, morphological variations of *Ae. aegypti* should be tested further by examination of a larger sample size to obtain a more precise comparison between the dark and pale forms. The present data suggest that these vectors could serve as reservoirs of dengue viruses in nature. This may be one factor supporting the prediction of dengue outbreaks in specific areas.

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