# Effects of Temperature, Relative Humidity, and DEN-2 Virus Transovarial Infection on Viability of *Aedes aegypti*

## Pengaruh Temperatur, Kelembaban Relatif, dan Infeksi Transovarial Virus DEN-2 terhadap Viabilitas *Aedes aegypti*

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**Abstract**

Environmental changes influenced survival life and virus transmission of dengue virus (DEN) in a mosquito. The purpose of the present study was to define DEN-2 virus transmission dynamic and effect of temperature, relative humidity (RH), and DEN-2 virus infection on viability of *Aedes aegypti (Ae. aegypti)*. This experimental study with pretest-posttest control group design was conducted at the Laboratory of Center for Tropical Medicine, Faculty of Medicine, Gadjah Mada University (UGM), Yogyakarta. Seventh-days old female *Ae. aegypti* (F0) were infected DEN-2 via oral membrane and kept until F2 generation by transovarial transmission, number of eggs produced and hatched was recorded. After 14-day incubation was found that transovarial transmission rate of DEN-2 virus infection in F0 and F1 were 93.3% and 82.2%, respectively. Egg production, hatching rates from infected and uninfected mosquitoes F0 were 68% and 85%; and F1 were 72.6% and 76%, respectively. At defined room condition tests, 7 day adult mosquitoes in dark and humid environment produced highest number of eggs, compared normal environment and in incubated without $CO_2$. In fourteenth day old mosquitoes at dark and humid produced highest number of eggs, compare normal environment condition, and in incubated without $CO_2$. DEN-2 virus was able to infect *Ae. aegypti* by transovarial transmission where the infection rate in F0 was higher than F1 generation. Temperature and humidity affected the ability of *Ae. aegypti* eggs to live and grow to adulthood.

**Keywords**: *Aedes aegypti*, DEN-2 virus, humidity, temperature, transovarial

**Abstrak**

Perubahan lingkungan memengaruhi hidup dan transmisi virus dengue dalam tubuh nyamuk. Tujuan penelitian ini adalah mengetahui pengaruh suhu, kelembaban udara (RH), terhadap transmisi virus DEN-2 pada nyamuk *Aedes aegypti*. Studi eksperimental dengan desain *pre* dan *post tes control* group dilakukan di laboratorium pusat kedokteran tropis, Fakultas Kedokteran, Universitas Gadjah Mada, Yogyakarta. Penelitian ini dilakukan pada kelompok *Ae. aegypti* betina umur 7 hari (F0). Virus DEN-2 diinfeksikan secara transovarial cara membran oral sampai generasi F2. Kelompok lain sebagai kontrol di inkubator temperatur dan suhu tertentu, waktu tertentu, jumlah telur yang dihasilkan, yang menetas dan mengandung virus dicatat. Hasil penelitian menemukan indeks transmisi transovarial generasi F0 dan F1 selama 14 hari masa inkubasi adalah 93,3% dan 82,2%, laju tetas telur dari nyamuk F0 yang terinfeksi dan tidak terinfeksi masing-masing 68% dan 85%, sedangkan laju tetas telur dari nyamuk F1 yang terinfeksi dan tidak terinfeksi masing-masing 72,6% dan 76%. Pada tiga kondisi ruang uji, nyamuk berumur 7 hari dalam ruang gelap dan lembab menghasilkan telur paling banyak dibandingkan pada kondisi normal dan pada inkubasi tanpa $CO_2$. Nyamuk umur 14 hari menghasilkan telur tertinggi dalam ruang gelap dan lembab, dibandingkan pada kondisi ruang normal dan dalam inkubasi tanpa $CO_2$. Virus DEN-2 dapat menginfeksi *Ae. aegypti* secara transovarial dengan laju infeksi lebih tinggi pada F0 daripada F1. Suhu dan kelembaban mempengaruhi kemampuan produksi telur *Ae. aegypti* untuk hidup dan tumbuh.

**Kata kunci**: *Aedes aegypti,* virus DEN-2, kelembaban, suhu, transovarial

## Introduction

Dengue has become a global health problem, particularly in tropical and subtropical countries. As such, global warming along with climate changes significantly affects the mechanism of dengue transmission. Changes in air temperature and relative humidity may reduce the viability of Dengue virus (DEN) in the mosquito's body.[1] To maintain its existence in nature, DEN virus has two mechanisms i.e. by horizontal transmission between ver-

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tebrate viremia which is transmitted by *Aedes* mosquitoes and by vertical transmission (transovarial) from the next generation of infective female.[2] Of these transmissions, transovarial mechanism is an important aspect in maintaining the viability of DEN virus during the inter-epidemic period in nature.

There are four types dengue virus namely DEN-1, DEN-2, DEN-3, and DEN-4, one of most potent is DEN-2 virus has been isolated from larvae of *Ae. aegypti* in Yangon, Myanmar, and was expected that transmission occurs by vertical mechanism.[3] Transovarial transmission can take place until the 7th generation of mosquitoes that have been infected by DEN virus parentally.[4] Eggs from infective female mosquitoes, which hatched after an incubation period of several weeks at room temperatures, increased the percentage of transovarial transmission.[5] At temperature ranges from 12 to 36ºC, with maximum and minimum limits of 35ºC and < 12ºC, respectively, egg viability was greater than 80%.[6]

The existence of this nature phenomenon is important for *Aedes* mosquitoes in supporting and maintaining the vector endemic capacity of a region. This indirectly affects the incidence of dengue in the community which shows a complete Dengue transmission mechanism. So, for better understanding of the transmission mechanism, it is necessary to know the contributing components of the dynamic transmission. The purposes of this study was to prove the existence of transovarial transmission of DEN-2 virus in *Ae. aegypti*, to know the number of eggs produced by mosquitoes *Ae. aegypti* infected with DEN-2 virus, and to investigate the effect of temperature and humidity on the hatchability of eggs of transovarial DEN-2 virus-infected *Ae. aegypti* after the eggs are stored for 7 days and 14 days.

## Methods

This experimental study with pre-posttest control group design was conducted at the laboratory of Center for Tropical Medicine, Faculty of Medicine, Gadjah Mada University (UGM), Yogyakarta. Adult female *Ae. aegypti* mosquitoes aged 7 days were used as subject. The mosquitoes were infected by DEN-2 virus through oral membrane feeding (fresh rat skin that had been sheared fur).[7] The DEN-2 viruses used in this experiment have been colonized in *Ae. aegypti* since 2004 at the Insectariums of the Center for Tropical Medicine, UGM. DEN virus suspension consisting of C6/36 cell culture supernatant of infected mosquitoes, which was previously infected by DEN-2 virus (Hawaiian isolate) for 6 days incubation, was mixed with erythrocytes of healthy persons containing anti-coagulant heparin and sucrose 10% with a ratio of 1 : 1 : 1.12. The mixture was placed in the room at 25ºC ± 3ºC and relative humidity (RH) 70% ± 5%. On the third day, the mosquitoes were blood-fed on healthy rats. Further, mosquito eggs were collected from the parent (F0) of DEN-2 infected group and control group mosquitoes after 10 days incubation period (*Gonotrophic* II). Meanwhile, mosquito eggs from the progeny (F1) from the infected and control parent (F0) mosquitoes were collected after 10-day-old mosquitoes (*Gonotrophic* II). The eggs were collected by confining each individual female mosquito in paper cups covered by nylon netting on the top and bottom parts. Cotton pads, covered by wet filter paper (ovistrip) on its upper, were placed in the paper cups for egg laying. After the egg is released, the female parent was tested for the presence of DEN virus using head squash Immunocytochemical SBPC method with monoclonal DSSC7 as primary antibodies.[8,9]

Eggs from F1 *Ae. aegypti* with positive DEN-2 virus transmitted transovarially after 3-day embryonization were referred as group 1 (K1) and group 2 (K2) for 7-day and 14-day incubations, respectively. The K1 and K2 were then categorized into three groups of room temperature and RH: ambient condition at 25ºC ± 5ºC and RH 80% ± 5% ($T_1RH_1$), humid dark room at 20ºC ± 5ºC and RH 85% ± 5% ($T_2RH_2$), and incubated without $CO_2$ at 30ºC ± 5ºC and RH 70% ± 5% ($T_3RH_3$). Sample of 100 mosquitoes eggs in each group and attached to the *ovistrip*. The temperature and humidity were measured using thermohygrometer and were observed twice a day in the morning (07:00 to 08:00 pm), hereinafter referred to as P1, and in the afternoon (12:00 am to 1.00 pm), hereinafter referred as P2. F1 mosquito eggs, after being kept into adult mosquitoes in 3 different treatments, were colonized until F2 generation. The results were analyzed statistically with the Mann-Whitney test and ANOVA. Mean differences between treatments revealed a significant effect if the critical value F0 ≥ critical value F table at $\alpha = 0.05$.[10]

## Results

The results of DEN-2 virus antigen test of females (F0) *Ae. aegypti*, which were infected orally by DEN-2 virus in 14 day incubation period and their progeny (F1) adult of 14 days of age are presented in Table

**Table 1. Antigen Test of DEN-2 Virus in Preparations Mosquito Head Squash Parent (F0) and the Progeny (F1) Adult Stage**

| Mosquitoes Generation | Groups of Mosquitoes Tested | Antigen Virus DEN-2 | | | Infection Rate (%) |
|---|---|---|---|---|---|
| | | Positive | Negative | Total | |
| F0 | Infected by DEN-2 virus | 56 | 4 | 60 | 93.3 |
| | Not infected | 0 | 30 | 30 | 0 |
| F1 | Infected by DEN-2 virus | 74 | 16 | 90 | 82.2 |
| | Not infected | 0 | 30 | 30 | 0 |

1. It reveals the DEN-2 virus infection rate in F0 and F1 generation mosquitoes. Number of eggs produced from F0 and F1 *Ae. aegypti* mosquitoes are presented in Table 2. It describes the number of hatching and not hatching eggs from infected and non infected mosquitoes.

Results of colonization of eggs from F1 mosquitoes, which were positively infected by DEN-2 virus transovarially, are presented in Table 3, whereas colonization results of uninfected F1 mosquitoes are presented in Table 4. Both tables cover 7-day and 14-day test periods for 3 different temperature and RH experimental conditions.

During laboratory colonization, the average air temperature and RH were 24.5ºC and 65.5%, respectively. At this condition, eggs hatched into larvae within 1 – 3 days, larva stage lasted 8 – 12 days to become pupae, and pupal stage lasted 2 – 3 days to become adult mosquito.

## Discussion

Table 1 indicates that local strains of *Ae. aegypti*, which has been colonized since 2004 at the Center for Tropical Medicine UGM, is susceptible to DEN-2 virus infection. It was found 75% infection rates in first colony by immunocytochemistry SBPC head squash methods.[9] These results have good agreement with previous research that the mosquitoes were categorized as vulnerable if after infected orally the DEN virus, found positive results on brain tissue, based on head squash preparations immunofluorescence method in 2-week incubation.[11] Vulnerability of *Ae. aegypti* against DEN virus was associated with vertical transmission rate because higher transovarial transmission in the offspring may be caused by the high number of infected females and vulnerability.[4]

**Table 2. Number of Hatching and Not Hatching Eggs from DEN-2 Virus-Infected and Uninfected (Control) F0 and Progeny (F1) of *Ae. aegypti* Mosquitoes**

| Mosquito Generation | Group of Test | Hatching and Not Hatching Eggs | | | | Total | Total Eggs Produced | Average Eggs* |
|---|---|---|---|---|---|---|---|---|
| | | Hutched (+) | | Hutched (-) | | | | |
| | | n | (%) | n | (%) | | | |
| F0 | Infected by DEN-2 virus | 68 | 68 | 32 | 32 | 100 | 4,235 | 62 |
| | Not infected | 85 | 85 | 15 | 15 | 100 | 6,688 | 79 |
| F1 | Infected by DEN-2 virus | 109 | 72.6 | 41 | 27.4 | 150 | 7,990 | 73 |
| | Not infected | 114 | 76 | 36 | 24 | 150 | 9,459 | 83 |

Notes:
*Per each mosquitos

**Table 3. Colonization Results of Eggs of Transovarially DEN-2 virus-infected F1 *Ae. aegypti* After 7-day and 14-day Treatment at 3 Different Room Temperature and Humidity Conditions.**

| Test Period | Test Group | Repetition | Eggs Tested | Adult Mosquito Live | | Total | Average |
|---|---|---|---|---|---|---|---|
| | | | | Male | Female | | |
| 7-day | $T_1RH_1$ | 1 | 100 | 33 | 22 | 55 | 52 |
| | | 2 | 100 | 25 | 28 | 53 | |
| | | 3 | 100 | 25 | 23 | 48 | |
| | $T_2RH_2$ | 1 | 100 | 40 | 34 | 74 | 62 |
| | | 2 | 100 | 23 | 31 | 54 | |
| | | 3 | 100 | 17 | 42 | 59 | |
| | $T_3RH_3$ | 1 | 100 | 28 | 17 | 45 | 42 |
| | | 2 | 100 | 19 | 22 | 41 | |
| | | 3 | 100 | 18 | 21 | 39 | |
| 14-day | $T_1$ $RH_1$ | 1 | 100 | 14 | 20 | 34 | 36 |
| | | 2 | 100 | 12 | 27 | 39 | |
| | | 3 | 100 | 10 | 25 | 35 | |
| | $T_2RH_2$ | 1 | 100 | 23 | 21 | 44 | 46 |
| | | 2 | 100 | 15 | 32 | 47 | |
| | | 3 | 100 | 22 | 26 | 48 | |
| | $T_3RH_3$ | 1 | 100 | 22 | 18 | 40 | 40 |
| | | 2 | 100 | 23 | 14 | 37 | |
| | | 3 | 100 | 19 | 23 | 42 | |

Notes:

$T_1RH_1$ = Normal room conditions (temperature 25 ± 5ºC, relative humidity (RH) 80 ± 5%); $T_2RH_2$ = Dark and humid room (temperature 20 ± 5 ºC, RH 85 ± 5%); $T_3RH_3$ = incubated without $CO_2$ (temperature 30 ºC ± 5ºC, RH 70% ± 5%)

**Table 4. Colonization Results of Eggs of Uninfected F1 *Ae. aegypti* After 7-day and 14-day Treatment at 3 Different Room Temperature and Humidity Conditions**

| Test Period | Test Group | Repetition | Eggs Tested | Adult Mosquito Live | | Total | Average |
|---|---|---|---|---|---|---|---|
| | | | | Male | Female | | |
| 7-day | $T_1RH_1$ | 1 | 100 | 51 | 39 | 90 | 79 |
| | | 2 | 100 | 45 | 33 | 78 | |
| | | 3 | 100 | 21 | 48 | 69 | |
| | $T_2RH_2$ | 1 | 100 | 46 | 42 | 88 | 86 |
| | | 2 | 100 | 47 | 36 | 83 | |
| | | 3 | 100 | 52 | 34 | 86 | |
| | $T_3RH_3$ | 1 | 100 | 31 | 37 | 68 | 72 |
| | | 2 | 100 | 41 | 31 | 72 | |
| | | 3 | 100 | 3 | 36 | 75 | |
| 14-day | $T_1RH_1$ | 1 | 100 | 32 | 36 | 68 | 58 |
| | | 2 | 100 | 34 | 25 | 59 | |
| | | 3 | 100 | 28 | 19 | 47 | |
| | $T_2RH_2$ | 1 | 100 | 43 | 29 | 72 | 66 |
| | | 2 | 100 | 41 | 21 | 62 | |
| | | 3 | 100 | 27 | 37 | 64 | |
| | $T_3RH_3$ | 1 | 100 | 23 | 34 | 57 | 59 |
| | | 2 | 100 | 19 | 35 | 54 | |
| | | 3 | 100 | 36 | 29 | 65 | |

Notes:
$T_1RH_1$ = normal room conditions (temperature 25 ºC ± 5ºC and relative humidity (RH) 80% ± 5%); $T_2RH_2$ = dark and humid room at temperature 20 ºC ± 5 ºC and RH 85% ± 5%; $T_3RH_3$ = incubator without $CO_2$ at temperature 30 ºC ± 5ºC and RH 70% ± 5%).

Mosquitoes are more vulnerable to viruses due to variation in inter (genetic) and between species in inhibiting the infection and spreading out of the virus leading to differences in the infection rate and transmission. In susceptible mosquitoes, the titer will increase, possibly due to replication in secondary target organs, while the mechanism of vertical transmission of arbovirus in the mosquito's body will produce progeny with infection rates exceeding 80%.[11]

Susceptibility of *Ae. aegypti* against DEN-2 virus infection provides good mechanism for the survival of the virus and mosquitoes in nature. The present study shows that the control group mosquitoes produced more eggs than the infected ones. As shown in Table 2, the uninfected parent (F0) *Ae. aegypti* produced 6,688 eggs from 85 mosquitoes (79 eggs per mosquito in average). This figure was significantly different (p = 0.001) with infected F0 which produced only 4,235 eggs from 68 mosquitoes (62 eggs per mosquito in average). The same figure was found in the progeny (F1), that the uninfected mosquitoes significantly produced higher eggs (9,459 eggs) than the infected ones (7,990 eggs) (p = 0.009).

In one gonotrophic cycle, a female *Ae. aegypti* is able to produce as many as 100 to 400 eggs.[12] Based on this figure, the number of eggs and mean number of eggs produced by F0 and F1 mosquitoes of both infected and uninfected were lower (Table 2). These possibly due to room temperature and humidity and lack of food intake (blood) which is a source of protein for growth and development of eggs. In the life of mosquito after feed the blood to be continued produce eggs, thus the number of eggs were deposited. The difference between the control (uninfected) and infected mosquitoes probably due to the effects of DEN-2 virus infection during embryogenesis. Virus multiplication in different organs during embryogenesis or in the final stages of mosquito may vary due to the tissue tropism, descendant of the virus, and host genetic creation. These factors, either alone or together, can contribute to the duration of larvae, productivity, fertility, and mortality is different in the infected mosquito transovarium.[4]

Temperature and humidity have significant effect on *Ae. aegypti* in producing eggs. As shown in Table 3 and Table 4, infected and uninfected F1 mosquitoes kept 7 days in dark and humid room ($T_2RH_2$: 22 – 26ºC, RH 87 – 92%) produced in average 62 and 86 eggs, respectively, contrast to 52 and 79 eggs at ambient condition ($T_1RH_1$: 25.5 – 28ºC, RH 79 – 87%) and 42 and 72 eggs in incubator without $CO_2$ ($T_3RH_3$: 33 – 35ºC, RH 68 – 72%). The same pattern was observed in 14-day period. These results show a decline number of eggs hatching and growing into adult mosquitoes live where 14-day treatment was worse than 7-day treatment.

The effect of temperature, humidity, and DEN-2 virus infection was observed in the offspring of *Ae. aegypti*. As shown in Table 3 and Table 4, the number of survival eggs hatching into adult mosquitoes was different among temperature and humidity conditions.

The low hatchability of eggs incubated without $CO_2$ ($T_3RH_3$) was likely influenced by temperature and humidity of the incubator. During the treatment, the average incubator temperature (34.5°C) was above the optimum temperature (20 – 30°C) at which generally mosquitoes lay their eggs. The optimum temperature for growth and developments of mosquitoes is 25 – 27°C, and the mosquito growth stops when temperature is lower than 10°C or higher than 40°C.[11]

Mosquito eggs are easily broken and some embryonic processes are crashed in condition not optimum or placed in a moistened environment. Those reared in a high moisture environment hatched at a high rate compared with their to a drier environment.[13] In the present study, the average air RH in all three treatment groups ranged 69.8 – 90.3%. This was within the average range of optimum humidity for mosquito development of 70 – 90% and, therefore, air humidity during treatment did not affect much on the growth and development of mosquito eggs. The experimental condition of room humidity was optimum for the embryonic processes and embryo survival resulting in longer mosquito lifespan.[11]

DEN virus affected to the ability of life and growth and development of progeny. It was indicated by the difference of the number of hatching eggs into adult mosquitoes. As shown in Table 2, hatching eggs of infected mosquitoes (68% of F0 and 72.6% of F1) were smaller than the uninfected ones (85% of F0 and 76% of F1). Previous research showed that hatching rate of eggs from DEN-3 virus mosquitoes was only 30 – 68.1%.[14]

In the present study, factors associated with pathogenesis of DEN-2 virus in mosquito system or other factors cannot be explained because the presence of DEN antigen and viral titer of egg, larva, and pupa were not checked. However, it is known that the pathogenesis of the virus also relies on the offspring, where less adaptable offspring causes higher pathogenesis. Meanwhile, mosquitoes infected with DEN-3 virus showed higher mortality compared with control mosquitoes. This differs from other finding that classically arbovirus has no disturbing effect on the vector.[11] As a result, DEN virus breeding in cells of *Ae. albopictus* does not always cause CPE (cytopathogenic effect) so that the infection is persistent. This may be analogous to the presence of natural DEN-2 virus in *Ae. aegypti* where the virus replicates did not cause the mosquito dead due to the absence of CPE.

Effect of DEN-2 virus transmitted transovarially can be regarded as one of biological controlling factors over the reproductive potency of *Ae. Aegypti*. *Ae. aegypti* eggs, which still survive and hatch up to become adult mosquitoes, is probably the best mosquito progeny of genetic superiority and important transovarial dynamic transmission of dengue for the next generation.

## Conclusions

The DEN-2 virus was able to infect *Ae. aegypti* by transovarial transmission, where infection rate in F0 was higher than that in F1 generation. In both generations, DEN-2 virus-infected *Ae. aegypti* mosquitoes produced smaller number of eggs than those of uninfected mosquitoes. Temperature and humidity affected the ability of *Ae. aegypti* eggs to live and grow to adulthood, when longer storage resulted in smaller survival rates.

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