Oral Susceptibility of Singapore *Aedes (Stegomyia)* aegypti (Linnaeus) to Zika Virus

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Abstract

**Background:** Zika virus (ZIKV) is a little known flavivirus that caused a major outbreak in 2007, in the South-western Pacific Island of Yap. It causes dengue-like syndromes but with milder symptoms. In Africa, where it was first isolated, ZIKV is mainly transmitted by sylvatic *Aedes* mosquitoes. The virus has also been isolated from *Ae. aegypti* and it is considered to be the vector involved in the urban transmission of the virus. Transmission of the virus by an African strain of *Ae. aegypti* has also been demonstrated under laboratory conditions. The aim of the present study is to describe the oral susceptibility of a Singapore strain of *Ae. aegypti* to ZIKV, under conditions that simulate local climate.

**Methodology/Principal Findings:** To assess the receptivity of Singapore’s *Ae. aegypti* to the virus, we orally exposed a local mosquito strain to a Ugandan strain of ZIKV. Upon exposure, fully engorged mosquitoes were maintained in an environmental chamber set at 29°C and 70–75% RH. Eight mosquitoes were then sampled daily from day 1 to day 7, and subsequently on days 10 and 14 post exposure (pe). The virus titer of the midgut and salivary glands of each mosquito were determined using a tissue culture infectious dose50 (TCID50) assay. High midgut infection and salivary gland dissemination rates were observed. By day 5 after the infectious blood meal, ZIKV was found in the salivary glands of more than half of the mosquitoes tested (62%); and by day 10, all mosquitoes were potentially infective.

**Conclusions/Significance:** This study showed that Singapore’s urban *Ae. aegypti* are susceptible and are potentially capable of transmitting ZIKV. The virus could be established in Singapore should it be introduced. Nevertheless, Singapore’s current dengue control strategy is applicable to control ZIKV.

Citation: Li Mi, Wong PSJ, Ng LC, Tan CH (2012) Oral Susceptibility of Singapore *Aedes (Stegomyia)* aegypti (Linnaeus) to Zika Virus. PLoS Negl Trop Dis 6(8): e1792. doi:10.1371/journal.pntd.0001792

Editor: Michael J. Turell, USAMRIID, United States of America

Received January 9, 2012; Accepted July 11, 2012; Published August 28, 2012

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Funding: The Ministry of Environment and Water Resources provided funding for the study. The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

Competing Interests: The authors have declared that no competing interests exist.

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Introduction

Zika virus (ZIKV) is an emerging mosquito-borne pathogen belonging to the genus *Flavivirus* of the Family *Flaviviridae* [1]. It is a positive single stranded RNA virus with a 10,794 nucleotide genome that is closely related to the Spondweni virus (*Flavivirus, Family Flaviviridae*) [2,3]. The virus was first isolated in 1947 from a febrile rhesus monkey in the Zika forest of Uganda [4]. Non-human primates were implicated as the reservoir host of ZIKV in Africa and Asia [5].

In humans, ZIKV causes a mild infection manifested by a rash, fever, joint and muscle pain, headache and peri-orbital pain, which are characteristic signs and symptoms of flavivirus infections [6,7]. The first human ZIKV infection was reported in Uganda in 1964 [6]. Although the isolation of ZIKV has so far been confined to the African continent [8,9], serological evidence has shown widespread distribution of the virus even in Asian countries such as Malaysia, India, Philippines, Thailand, Vietnam, Indonesia, and Pakistan [10,11,12,13,14,15]. The first major outbreak of human ZIKV infection was reported in the Pacific island of Yap and its adjoining islands in the Federated State of Micronesia in 2007 [3,7,16,17]. The outbreak lasted four months infecting approximately 73% of the islands’ population [7]. In 2011, ZIKV was first reported in the western hemisphere in travellers returning from Senegal [18]. Most recently, ZIKV was isolated from a 3-year old boy in Cambodia in 2010 [19].

ZIKV is transmitted to humans by *Aedes* spp. mosquitoes. The earliest evidence of ZIKV in a pool of *Ae. africus* from Uganda in 1948 coincides with its first isolation from a rhesus monkey in the same location [4]. Subsequent documents reported further isolation of the virus from *Ae. africanus* and *Ae. aegypti* caught in the Zika forest [20,21,22]; from *Ae. luteocephalus* in Nigeria in 1969 [23]; and from *Ae. albopictus, Ae. furcifer*, and *Ae. aegypti* in Ivory Coast in 1999 [24]. High prevalence of ZIKV antibodies in the urban population of Nigeria has led Fagbamii [23] to suspect that *Ae. aegypti* may play an important role in the urban transmission of ZIKV. Further evidence came from Asia, when ZIKV was isolated from a pool of *Ae. aegypti* caught in Bentong, Peninsular Malaysia [25]. This finding provided evidence of ZIKV transmission outside Africa. In Indonesia, the peak of human ZIKV infections coincides with peak *Ae. aegypti* population which is by the end of rainy season [14]. Apart from field surveillance data, early experimental studies conducted
Author Summary

Zika virus (ZIKV) is an emerging mosquito-borne zoonotic pathogen that causes dengue-like syndromes. Although the isolation of the virus was confined to the African continent, serological evidences have shown the widespread distribution of ZIKV, particularly in Asia. In 2007, it caused a major outbreak on the Pacific Island of Yap, infecting more than 70% of the island’s inhabitants. The propensity of the virus to spread outside its known geographical range was again demonstrated when it was detected in the US from travellers coming back from endemic countries. Several species of Aedes spp. mosquitoes have been incriminated as vectors of ZIKV, including Ae. aegypti. The current study showed that local Ae. aegypti are highly susceptible to ZIKV and by day 5 post-infectious blood meal, more than 50% of mosquitoes were potentially indeed. Singapore being a tourist and a business hub, coupled with the presence of susceptible vector and a population that is immunologically naïve and vulnerable, the local transmission of the ZIKV is plausible. Nevertheless, Singapore’s current dengue control strategy is applicable to control ZIKV.

by Boorman and Porterfield [26] and Cornet et al. [27] have also demonstrated the competency of Ae. aegypti to transmit ZIKV. Considering the geographic spread and the possible impact on susceptible human populations, mosquito-borne diseases are currently considered as a major threat to global health in both developing and developed world [28,29]. According to Gushulak et al. [30], the threat of emerging infectious diseases is mainly influenced by the migration and mobility of the human populations. The dengue, chikungunya and malaria situations in Singapore clearly demonstrate the role of importation in shaping the epidemiology of these diseases [31,32,33]. Introduction of ZIKV into Singapore, a travel and trading hub, coupled with the presence of susceptible vector and a population that is immunologically naïve and vulnerable, the local transmission of the virus is likely. Furthermore, as ZIKV has never been reported in Singapore, the local population is presumed to be immunologically naïve and vulnerable to the infection.

Although experimental studies conducted in the past have shown that Ae. aegypti is a competent vector for ZIKV, these studies used African strains of Ae. aegypti that were caught in Nigeria [26] and Senegal [27] and had been maintained in the laboratory for years. Furthermore, experimental methods used in these studies differed from those of the current study. Although Boorman and Porterfield [26] infected the mosquitoes using the oral route, the average incubation temperature was 24°C, which is low in the tropical context and resulted in an extrinsic incubation period that suggested low vectorial capacity. While Cornet et al. [27] incubated their infected mosquitoes between 27 to 28°C, the method of infection was by intrathoracic route which can artifically lead to shorter extrinsic incubation period and higher number of mosquitoes infected. In addition, the geographical variations in terms of oral susceptibility of mosquitoes to different strains are also well documented [34,35,36,37,38,39]. The present study describes the oral susceptibility of a Singapore field strain Ae. aegypti to ZIKV, under condition that simulate local climate.

Materials and Methods

Mosquitoes

Ae. aegypti, used for the experimental infection, were derived from eggs collected in the Western part of Singapore during a weekly ovitrap surveillance study to determine mosquito popula-

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Virus

Ugandan MR766 ZIKV strain obtained from the American Type Culture Collection (Manassas, VA, USA) was used to expose the mosquitoes to ZIKV. This virus was originally isolated from the blood of an experimental sentinel rhesus monkey in 1947 [4] and passaged in suckling mouse brains. The stock virus used in the current study has been passaged thrice in Vero cells prior to the infectious feed.

Oral infection of mosquitoes

Five- to 7-day-old female mosquitoes (n = 120) were transferred to 0.5 L containers and starved for 24 hours prior to the infectious blood meal. The blood meal consisted of 1:1 100% swine-packed RBC (Innovative Research, USA) and fresh virus suspension at a final concentration of 7.0 Log10 titer tissue culture infectious dose50 (TCID50)/mL. Adenosine Triphosphate (Fermentas, USA), at a final concentration of 3 mM, was added to the blood meal as a phagostimulant. Mosquitoes were fed with an infectious blood meal that was constantly warmed to 37°C using a Hemotek membrane feeding system (Discovery Workshops) housed in a feeding chamber. Thirty minutes after exposure to the infectious blood meal, mosquitoes were cold anesthetized at −20°C. Fully engorged females were transferred to 300 mL cartons and were maintained in an environmental chamber (Sanyo, Japan) at 29°C and 70–75% RH with a photoperiod of 12h:12h light:dark (L:D) cycles. They were allowed to mate randomly and fed with pathogen-free pig’s blood (A*Star Biomedical Resource Center, Singapore) using a Hemotek membrane feeding system (Discovery Workshops, Lancashire, United Kingdom). F1 eggs were collected using filter paper (Whatman, USA). Eggs were then allowed to hatch using de-chlorinated water and larvae were reared in 25 cm x 30 cm x 9 cm enamel pans containing 900 mL of water and fed with crushed dog food. Pupae were placed in 30 cm x 30 cm x 30 cm (HxWxL) cages before emergence. Prior to the infectious feed, adult mosquitoes were provided with 10% sugar/Vitamin B complex solution ad libitum.

Mosquito processing

To determine the ZIKV infection and dissemination rates in Ae. aegypti, eight mosquitoes were sampled daily from day 1 to day 7, and subsequently on days 10 and 14 post exposure (PE). To prevent cross-contamination of virus between midgut and salivary glands of each mosquito, these organs were carefully dissected using different dissecting needles and the organs were rinsed in Medium 199 (M199) (Gibco, USA) supplemented with amphotericin B (Sigma Aldrich, USA). The midguts and salivary glands from each mosquito were individually transferred to 2 mL microtubes containing 250 µL of M199. These organs were then homogenized using five mm stainless steel grinding balls (Retsch, Germany) in a MM301 mixer mill (Retsch, Germany) set at frequency of 12/sec for 1 min. The supernatant of the homogenate was applied in the viral titer assay. All dissecting needles were dipped in 80% ethanol and cleaned before being re-used. All experiments were conducted inside an ACL-2 facility.
Tissue Culture Infectious Dose50 Assay

Viral titers in this study were determined with a tissue culture infectious dose50 assay, an endpoint dilution technique, using Vero cells as described by Higgs et al. [40]. Briefly, 100 μL of 10-fold serial dilutions of each sample were titrated (in duplicate) in 96-well microtiter plates and incubated with Vero cells at 37°C and 5% CO2. At the end of day-7 incubation, the cells were examined microscopically for ZIKV-induced cytopathic effect (CPE). A well is scored positive if any CPE is observed compared to the uninfected control cells. All virus titers were expressed as Log10 TCID50/mL.

Statistical analysis

Proportion infected was calculated by dividing the number of infected midguts (or salivary glands) by the total number of midguts (or salivary glands) sampled. To compare viral titers at different time points, raw data was subjected to a normality test using SPSS Ver 18 (IBM, USA). Data that passed the normality test were analyzed by analysis of variance using the above mentioned software.

Results

Oral susceptibility of Ae. aegypti to ZIKV

Presence or absence of blood in the midgut was verified during dissection under a Stereoscope (Olympus, USA). By Day 3, when blood had been completely digested, seven (87.5%) of the analyzed mosquitoes were positive for ZIKV (Figure 1). From day 6 pe onwards, all midguts were positive for ZIKV except for one of the mosquitoes that was negative for the virus at day 7 pe. The presence of viable ZIKV in the salivary glands (n = 1) was first observed on day 4 pe (Figure 1) and 62% of mosquitoes sampled on day 5-pe showed detectable virus in the salivary glands. ZIKV was observed in salivary glands of all infected mosquitoes sampled at days 10 and 14 pe.

ZIKV midgut and salivary gland titers

Figure 2 presents ZIKV midgut titers at different days pe. Although remaining blood meal in midgut was not removed, an eclipse phase typically associated with low virus midgut titer can be seen on day 1 pe, with only one of the midgut showing detectable ZIKV. Virus titers in day 2 pe were higher than that observed for day 1 pe, mirroring the results obtained on percentage of midguts infected (Figure 1). These suggest that midgut ZIKV titer observed during day 2 pe was most probably due to virus replication in the midgut rather than to the remaining amount of blood observed in some of the mosquitoes. A significant increase (P<0.026) in mean viral titer was observed between days 3 pe (3.9 Log10 TCID50/mL) and day 5 pe (5.6 Log10 TCID50/mL). From day 6 pe onwards, mean viral titers showed a decreasing trend from fluctuated between 5.4 Log10 TCID50/mL and 5.9 Log10 TCID50/mL but the differences observed were not statistically significant (P>0.91).

ZIKV titers in the salivary glands increased from day 4 pe onwards (Figure 3). Although the difference in mean viral titers from day 5 pe (2.7 Log10 TCID50/mL) to day 7 pe (3.7 Log10 TCID50/mL) was not significant (P = 0.68), the mean viral load increased significantly (P<0.001) by day 10 pe (6.4 Log10 TCID50/mL), achieving the highest mean viral load of >8.0 Log10 TCID50/mL by day 14 pe.

Discussion

Recent unprecedented spread of chikungunya virus (CHIKV) in many parts of the world, with millions of people affected, exemplifies how arboviruses can adapt and affect human health on a global scale [31]. Singapore’s vulnerability to emerging and re-emerging arboviruses is accentuated by the country’s location as a popular tourist and business hub, high dependency on migrant workers, tropical climate, dense population, and the presence of potential mosquito vectors. An outbreak of chikungunya in Singapore during the 2008–09 period attests the country’s vulnerability to mosquito-borne diseases [31,41]. The outbreaks of ZIKV on Yap Island and the worldwide spread of CHIKV have shown the propensity of arboviruses to spread outside their known geographical range and their potential to cause large-scale epidemics.
Unlike CHIKV which has received much scientific attention, ZIKV is a little-known flavivirus despite its outbreak potential [42]. Most studies on ZIKV were conducted more than two decades ago and there is a dearth of information on mosquito-ZIKV interactions that are salient to a better understand virus transmission. In 1956, Boorman and Porterfield [26] successfully transmitted the virus to both mice and monkeys using ZIKV-infected laboratory strains of *Ae. aegypti*. Cornet et al. [27] further demonstrated that a high percentage (88%) of intrathoracically infected *Ae. aegypti* can transmit ZIKV to experimental mice within 7 days and transmission rates increased up to 95% on day 21 pe. The current study, using a field strain of mosquitoes, showed that Singapore’s *Ae. aegypti* are highly susceptible to ZIKV, with high midgut infection and salivary gland dissemination rates. By day 5 pe, 62% of the mosquitoes had detectable ZIKV in their salivary glands and by day 10 pe all mosquitoes were potentially infective. Based on the studies of Cornet et al. [27], nearly all mosquitoes with ZIKV in their salivary glands are assumed to be able to transmit the virus. This is supported by previous studies that have shown oral transmission of dengue (DENV) [43,44] and West Nile (WNV) [45] viruses were correlated with the proportion of mosquitoes with infected salivary glands.

The decrease in midgut viral titer at day 14 pe observed in our study was consistent with other published DENV and WNV studies [46,47,48] and were probably due to virus clearance by the mosquito immune system [47,49,50]. Despite a decrease in midgut
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viral titer, ZIKV infection in salivary glands was found to be higher than that observed in midgut. This suggests that the proliferation of ZIKV in Ae. aegypti salivary glands is not attributed to direct dissemination from the midgut, but rather a result of viral dissemination and amplification within the glands or other organs or tissues such as hemocytes, ganglion, fat bodies etc [19,51,52]. Salivary gland dissemination rates obtained from our current study is similar to that observed for a local highly epidemic DENV-2 in the same strain of Ae. aegypti (Tan et al., unpublished data).

A phylogenetic analysis, based on the NS5 region, of ZIKV revealed three branches: West African (Nigeria), East African (Uganda) and those from Yap island (ZIKV 2007 EC), with the latter virus being the most distally related [17]. The strain used in our current study, MR766, is the Ugandan prototype strain and the only strain available to our laboratory. It would be very interesting to study and compare the recent epidemic ZIKV 2007 EC strain in Ae. aegypti, especially in the light of a four amino acid motif found in the viral envelope genes of the ZIKV 2007 EC strain that are absent in the MR766 strain [17]. Unfortunately, no ZIKV 2007 EC was isolated during the outbreak in Yap Island. The four amino acid motif found in the ZIKV 2007 EC strain correspond to an envelope protein 154 glycosylation motif and the loss of this motif in the ZIKV prototype strain is thought to have been due to extensive passage in mice [17]. Studies have showed that loss of glycosylation motif due to mutation has been found to affect the replication rates of tick-borne encephalitis virus, DENV, and WNV in both vector hosts and insect cell lines and the dissemination rate of WNV in different Culex spp. mosquitoes [53,54,55,56]. Despite the absence of this aa 154 glycan, the latter virus being the most distally related [17]. The strain used in...
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