<table>
<thead>
<tr>
<th><strong>Title</strong></th>
<th>Aedes (Stegomyia) albopictus (Skuse) : a potential vector of Zika virus in Singapore.</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Author(s)</strong></td>
<td>Wong, Pei-Sze Jeslyn.; Li, Mei-zhi Irene.; Chong, Chee-Seng.; Ng, Lee-Ching.; Tan, Cheong-Huat.</td>
</tr>
<tr>
<td><strong>Citation</strong></td>
<td>Wong, P.-S. J., Li, M.-z. I., Chong, C.-S., Ng, L.-C., &amp; Tan, C.-H. (2013). Aedes (Stegomyia) albopictus (Skuse) : a potential vector of Zika virus in Singapore. PLoS neglected tropical diseases, 7(8), e2348-.</td>
</tr>
<tr>
<td><strong>Date</strong></td>
<td>2013</td>
</tr>
<tr>
<td><strong>URL</strong></td>
<td><a href="http://hdl.handle.net/10220/18420">http://hdl.handle.net/10220/18420</a></td>
</tr>
<tr>
<td><strong>Rights</strong></td>
<td>© 2013 The Authors. This paper was published in PLoS Neglected Tropical Diseases and is made available as an electronic reprint (preprint) with permission of the authors. The paper can be found at the following official DOI: [<a href="http://dx.doi.org/10.1371/journal.pntd.0002348">http://dx.doi.org/10.1371/journal.pntd.0002348</a>]. One print or electronic copy may be made for personal use only. Systematic or multiple reproduction, distribution to multiple locations via electronic or other means, duplication of any material in this paper for a fee or for commercial purposes, or modification of the content of the paper is prohibited and is subject to penalties under law.</td>
</tr>
</tbody>
</table>
Aedes (Stegomyia) albopictus (Skuse): A Potential Vector of Zika Virus in Singapore

Pei-Sze Jesly Wong¹, Mei-zhi Irene Li¹, Chee-Seng Chong¹, Lee-Ching Ng¹,², Cheong-Huat Tan¹*

¹Environmental Health Institute, National Environment Agency, Singapore, Singapore, ²School of Biological Sciences, Nanyang Technological University, Singapore, Singapore

Abstract

Background: Zika virus (ZIKV) is a little known arbovirus until it caused a major outbreak in the Pacific Island of Yap in 2007. Although the virus has a wide geographic distribution, most of the known vectors are sylvatic Aedes mosquitoes from Africa where the virus was first isolated. Presently, Ae. aegypti is the only known vector to transmit the virus outside the African continent, though Ae. albopictus has long been a suspected vector. Currently, Ae. albopictus has been shown capable of transmitting more than 20 arboviruses and its notoriety as an important vector came to light during the recent chikungunya pandemic. The vulnerability of Singapore to emerging infectious arboviruses has stimulated our interest to determine the competence of local Ae. albopictus to transmit ZIKV.

Methodology/Principal Findings: To determine the competence of Ae. albopictus to ZIKV, we orally infected local mosquito strains to a Ugandan strain virus. Fully engorged mosquitoes were maintained in an environmental chamber set at 29°C and 80–85%RH. Twelve mosquitoes were then sampled daily from day one to seven and on day 10 and 14 post infection (pi). Zika virus titre in the midgut and salivary glands of each mosquito were determined using tissue culture infectious dose_{50} assay, while transmissibility of the virus was determined by detecting viral antigen in the mosquito saliva by qRT-PCR. High dissemination and transmission rate of ZIKV were observed. By day 7-pi, all mosquitoes have disseminated infection and 73% of these mosquitoes have ZIKV in their saliva. By day 10-pi, all mosquitoes were potentially infectious.

Conclusions/Significance: The study highlighted the potential of Ae. albopictus to transmit ZIKV and the possibility that the virus could be established locally. Nonetheless, the threat of ZIKV can be mitigated by existing dengue and chikungunya control program being implemented in Singapore.

Introduction

The Asian tiger mosquito, Aedes (Stegomyia) albopictus (Skuse), is considered a vector or potential vector of several pathogens of human and veterinary importance. Viral isolation and vector competence studies have shown that this mosquito is an efficient vector of more than 20 arboviruses [1]. Due to its biological and ecological plasticity, this notoriously invasive species has a wide geographic distribution. At present, aside from its tropical Asian home, Ae. albopictus can be found in temperate Asian countries, in tropical and temperate Americas, Europe, Middle East, the Pacific islands, Australia and Africa [2,3,4,5]. The global spread of Ae. albopictus is mainly caused by human activities, such as increase in intercontinental trade, especially in the last three decades [1].

In places where Ae. aegypti and Ae. albopictus co-exist, Ae. albopictus was considered second only to Ae. aegypti in terms of importance as vector of dengue and chikungunya [6,7]. However, its notoriety as an important vector came to light during the recent unprecedented global outbreak of chikungunya. According to Tsatsarkin et al. [8], a mutation in the envelope gene of chikungunya virus (CHIKV) enhances the virus infectivity and transmissibility in Ae. albopictus. The continual global expansion of Ae. albopictus is a growing concern as this mosquito may alter the transmission dynamics of arboviral diseases and increase the risks of humans to mosquito-borne viral infections [3,9]. This has stimulated increased interest to determine the extent of pathogens this mosquito can transmit.

Zika virus (ZIKV), a little known arbovirus, has gained prominence when it caused a large scale epidemic in the Pacific Island in 2007 [10,11]. It is a member of the genus Flavivirus of the Family Flaviviridae [12]. The virus is a positive single stranded RNA virus with a 10,794 nucleotide genome that is closely related to the Spondweni virus [13,14,15]. It was first isolated from a febrile sentinel monkey in Uganda in 1947 [15], but human ZIKV infection was first reported in 1964 [16]. The virus causes dengue-like syndromes such as rash, fever, arthralgia, headache and peri-orbital pain [11,16].

To date, only Aedes mosquitoes have been known to transmit ZIKV. In Africa, the virus was isolated from both sylvatic and peri-domestic mosquitoes: Ae. africanus, Ae. apicocongensis, Ae. luteocephalus, Ae. furcifer, Ae. vitattus and Ae. aegypti [17,18,19,20,21,22]. In Asia, ZIKV was only isolated from a pool...
Author Summary

The prominence of *Ae. albopictus* as a major vector came to light during the global pandemic of chikungunya. The continual global expansion of *Ae. albopictus* coupled with data from competence studies that show its ability to transmit more than 20 arboviruses has fuelled growing concerns that this mosquito may alter the epidemiology of arbovirus worldwide and increase the risks of humans to mosquito-borne diseases. This has stimulated our interest to determine the extent of pathogens *Ae. albopictus* can transmit, especially arboviruses with high epidemic potential. The current study showed that local *Ae. albopictus* are susceptible to ZIKV. By day 7 pi, all mosquitoes that were orally infected were found to have disseminated infections and 73% have ZIKV in their saliva. This study highlighted the potential of *Ae. albopictus* to transmit ZIKV. The local transmission of ZIKV in Singapore is plausible due to the presence of susceptible vectors, an immunologically naive and vulnerable human population, and the travel hub status of Singapore. Nonetheless, the threat of ZIKV can be mitigated by existing dengue and chikungunya control program being implemented in Singapore.

of *Ae. aegypti* caught from shop houses in the State of Pahang in Peninsular Malaysia [22]. During the ZIKV outbreak in Yap Island in 2007, no virus was isolated from any of the mosquitoes caught. However, based on epidemiological evidence, *Ae. hensili* was suspected to be the vector responsible for the outbreak [11]. In 2010, a case of ZIKV involving a three year old child was reported in Kampong Speu Province in Cambodia, however, the vector responsible was not known [23].

Identification of vectors and potential vectors of ZIKV or any other mosquito-borne diseases in a given geographical area has important implication when it comes to disease outbreak control. It is imperative that vectors are identified, so that a holistic and sound vector control program can be formulated. To date, *Ae. aegypti* is the only vector of ZIKV identified in Southeast Asia [22] and data on mosquito-ZIKV interactions have also been confined to this mosquito [24,25]. Our recent study has also showed that local *Ae. aegypti* strains are highly susceptible to ZIKV and viral dissemination rates reflect that observed for a local, highly epidemic DENV-2 strain [20].

In the light of continuous global niche expansion of *Ae. albopictus*, coupled with its catholic feeding behaviour, ecological adaptability and propensity to support a wide range of arboviruses, it is important to determine its competence to transmit pathogens with high epidemic potential such as ZIKV. The current study describes ZIKV infection in our local *Ae. albopictus*.

Materials and Methods

Mosquitoes

*Aedes albopictus*, used for the experimental infection, was derived from eggs collected during weekly ovitrap surveillance study as previously described [26]. Mosquitoes were colonized under standard insectary conditions as described by Li and colleagues [26]. In order to obtain enough number of mosquitoes for the study, F3 generations were used.

Virus

Ugandan MR766 ZIKV strain obtained from the American Type Culture Collection (Manassas, VA, USA) was used to infect the mosquitoes. The stock virus used in the current study was passaged thrice in Vero cell line prior to the infectious feed [26].

Oral infection of mosquitoes

Five to seven-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 a fresh ZIKV suspension, at a final concentration of 7.5 Log10 tissue culture infectious dose50/mL ((Log10TCID50/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 warmed to 37°C using a Hemotec membrane feeding system (Discovery Workshops, Lancashire, United Kingdom) housed. After thirty minutes, mosquitoes were cold anesthetized and fully engorged females were transferred to 300 ml ca. paper cups and were maintained in an environmental chamber (Sanyo, Japan) at 29°C and 70–75% RH with a 12 h/12 h L:D cycle and provided with 10% sugar/vitamin B complex ad libitum. All experiments were carried out in an arthropod containment level 2 (ACL-2) facility.

Mosquito processing

To determine the ZIKV infection and dissemination rates in *Ae. albopictus*, 12 mosquitoes were sampled daily from day one to seven, and subsequently on day 10 and 14 post infection (pi). Saliva was collected using the forced salivation technique as previously described [27] with modification. The proboscis of each mosquito, with its legs and wings, was inserted into a micropipette tip containing 10 μl of M199 and allowed to salivate. After 45 minutes, the Medium 199 containing the saliva from each mosquito was transferred into microcentrifuge tubes containing 100 μl of M199. The midgut and salivary glands of each mosquito were processed as described by Li and colleagues [26]. Briefly, the midgut and the salivary glands were homogenized using 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 titre assay. All disecting needles were dipped in 80% ethanol and cleaned before being re-used. All experiments were conducted inside an ACL-2 facility.

Quantitative reverse transcriptase-polymerase chain reaction (qRT-PCR) assay

Total RNA was isolated from saliva using the QIAamp Viral Mini Kit (Qiagen, Germany) following manufacturer’s recommendations. ZIKV in saliva was detected using a one-step real-time reverse transcriptase-polymerase chain reaction (qRT-PCR) as previously described [10].

Tissue Culture Infectious Dose50 (TCID50) assay

Viral titres from the midgut and salivary glands were determined with tissue culture infectious dose50 assay, an endpoint dilution technique, using Vero cells as described by Higgs et al. [28].

Data analysis

The infection rate at each sampling day was determined by the percentage of infected midguts, while dissemination rate was calculated by dividing the number of infected salivary glands by the total number of mosquitoes with infected midguts. On the other hand, transmission rate was calculated by dividing the number of positive saliva by the number of infected salivary
glands. Kolmogorov-Smirnov tests indicated that the data did not conform to conditions of normality, hence non-parametric analyses were performed. Kruskal-Wallis tests were used to determine differences in viral titres in midguts and salivary glands between days post-infection. When a significant difference was detected, Mann-Whitney U-tests were performed to determine which day differed. All analyses were performed in Minitab.

Results

Oral susceptibility of Ae. albopictus to ZIKV

From day 3-pi onwards, when blood had been completely digested, all midguts were positive for ZIKV, except for day 7- and 10-pi (Table 1). The presence of viable ZIKV in the salivary glands was first observed on day 3-pi in three mosquitoes. By day 5-pi, half of the mosquitoes sampled showed disseminated infection. From day 7-pi onwards, all mosquitoes were found to have ZIKV in their salivary glands (Table 1).

Transmission was first observed on day 4 after the infectious blood meal and transmission rates were observed to increase at each sampling days. By day 10-pi onwards, ZIKV RNA was found in all saliva tested (Table 1).

ZIKV midgut and salivary gland titres

Figure 1 presents ZIKV midgut and salivary gland titres at different days pi. A significant difference in midgut ZIKV titres was observed between different days pi (Kruskal-Wallis test, $P = 0.017$). Midgut viral titres at days 3-, 4-, and 5-pi ($>5.15 \log_{10} \text{TCID}_{50}/\text{ml}$) were found to be significantly higher when compared to viral titre at day 14-pi (Mann-Whitney test, $P < 0.05$) (Figure 1a). From days 6-pi onwards, viral titres fluctuated between $4.94 \log_{10} \text{TCID}_{50}/\text{ml}$ to $4.21 \log_{10} \text{TCID}_{50}/\text{ml}$ with no significant difference observed (Kruskal-Wallis test: $P = 0.81$).

In contrast, increasing ZIKV titre was observed in the salivary glands of Ae. albopictus (Figure 1b) (Kruskal-Wallis test, $P < 0.0001$). Although the differences in ZIKV salivary gland titres from day 3-pi ($2.54 \log_{10} \text{TCID}_{50}/\text{ml}$) to day 6-pi ($3.26 \log_{10} \text{TCID}_{50}/\text{ml}$) were comparable (Mann-Whitney test, $P = 0.09$), the viral load increased significantly (Mann-Whitney test, $P < 0.05$) by day 10-pi onwards ($>5.96 \log_{10} \text{TCID}_{50}/\text{ml}$).

Discussion

Emerging and re-emerging mosquito-borne diseases are considered to be major threats to global health in both developing and developed countries. Their tendency of spreading outside their known geographic range and causing large scale epidemics has been clearly demonstrated during the recent global epidemic of CHIKV [29].

Zika is a neglected tropical disease, and like CHIKV, interest in ZIKV epidemiology was limited until recently, when its high epidemic potential was demonstrated during a large-scale outbreak in the Pacific Island of Yap in 2007 [10,11,13,30].

Table 1. Infection, dissemination and transmission rates for Ae. albopictus orally fed with ZIKV and held at 29°C at various days post-infection.

<table>
<thead>
<tr>
<th>Days post-infection</th>
<th>Infection rate</th>
<th>Dissemination rate</th>
<th>Transmission rate</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>No. positive MG (number sampled)</td>
<td>Percent</td>
<td>No. positive SG (number sampled)</td>
</tr>
<tr>
<td>3</td>
<td>12 (12)</td>
<td>100</td>
<td>3 (12)</td>
</tr>
<tr>
<td>4</td>
<td>12 (12)</td>
<td>100</td>
<td>7 (12)</td>
</tr>
<tr>
<td>5</td>
<td>12 (12)</td>
<td>100</td>
<td>6 (12)</td>
</tr>
<tr>
<td>6</td>
<td>12 (12)</td>
<td>100</td>
<td>9 (12)</td>
</tr>
<tr>
<td>7</td>
<td>11 (12)</td>
<td>91.7</td>
<td>11 (11)</td>
</tr>
<tr>
<td>10</td>
<td>10 (12)</td>
<td>83.3</td>
<td>10 (10)</td>
</tr>
<tr>
<td>14</td>
<td>12 (12)</td>
<td>100</td>
<td>12 (12)</td>
</tr>
</tbody>
</table>

MG = midgut; SG = salivary gland.
doi:10.1371/journal.pntd.0002348.t001
During the outbreak that lasted four months, more than 70% of the island’s population was affected [11]. Like many vector-borne diseases, the absence of vaccines and specific treatment against ZIKV means prevention and control relies heavily on vector control. Therefore, key information such as the identity of the vector, its bionomics, distribution, and density are needed in order to develop and implement sound mosquito control programs. To date, little is known about the vectors of ZIKV outside Africa, except for Ae. aegypti.

The overlapping geographic distribution of Ae. albopictus with that of ZIKV has stimulated our interest to determine the potential of this mosquito to transmit ZIKV. Furthermore, the widespread distribution of Ae. albopictus in Singapore and large-scale local outbreaks of chikungunya in 2008–09 attest to the country’s vulnerability to emerging mosquito-borne diseases [31]. The potential of these diseases to be established locally is accentuated by the country’s reputation as a popular commercial and tourist hub, high dependency on migrant workers, tropical climate, dense human population, and the presence of potential mosquito vectors. Recently, we have shown the potential for ZIKV outbreak in Singapore by Ae. aegypti [26]. The study showed local Ae. aegypti are highly susceptible to ZIKV, with a short extrinsic incubation period (EIP) of five days.

Our current study showed that Singapore’s Ae. albopictus mosquitoes are susceptible to ZIKV, with high dissemination and transmission rates observed. By day 4-pi, 58% (n = 7/12) of the infected mosquitoes have disseminated infection and of these, three (43%) had ZIKV in their saliva. By day 7-pi, all infected mosquitoes are capable of transmitting the virus. A short EIP and high ZIKV salivary gland titres were also observed when we infect our local Ae. aegypti with ZIKV [26]. In that study, it took five and ten days post infectious blood meal to achieve a 62% and 100% dissemination rate, respectively. However, it does not mean that Ae. albopictus is more susceptible to ZIKV than Ae. aegypti, rather it could be due the amount of virus used to infect Ae. aegypti was lower (6.95 Log10 TCID50/mL) compared to the current study (7.52 Log10 TCID50/mL).

References

Acknowledgments
We thank Dr. Elizabeth McGraw (Monash University) and our colleague Dr. Chanditha Hapuarachchi for their critical reading of this article.

Author Contributions
Conceived and designed the experiments: CHT LCN. Performed the experiments: CHT PSJW MZIL CSC. Analyzed the data: CHT PSJW MZIL CSC. Contributed reagents/materials/analysis tools: CHT PSJW MZIL CSC. Wrote the paper: CHT PSJW.