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Similar vertical transmission rates of dengue and chikungunya viruses in a transgenic and a non-transformed *Aedes aegypti* (L.) laboratory strain

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Abstract. The increase of the burden of dengue and chikungunya and the relative failure of traditional vector control strategies have highlighted the need to develop new control methods. RIDL-SIT, a vector control method based on the release of engineered male mosquitoes, has shown promising results from field trials conducted in the Cayman Islands and Brazil. In large scale use, a small proportion of females might be released along with the males. Such females are potential virus vectors; here we investigate the vertical transmission of dengue and chikungunya of homozygous OX513A females.We provided females of OX513A-My1 and a wild type comparator strain with blood meals artificially infected with dengue serotype 1, 2, 3, 4 or chikungunya viruses. For 14 days post-feeding, eggs laid by females were collected. Larvae and their mothers were first tested by qRT-PCR, then by inoculation on cell cultures to search for infectious viral particles. We found no significant difference between the minimum infection rate of OX513A-My1 and wild type females. We also discussed the potential number of females being released, a fraction of the female wild population. Consequently, we conclude that there are no evidence that OX513A-My females, if released into the environment, would cause more harm than their wild counterparts.

INTRODUCTION

Dengue and, to a lesser extent chikungunya, are major public health threats throughout the tropics with an estimated 50 to 400 million new dengue cases yearly (WHO, 2009, Bhatt *et al.*, 2013). The recent outbreak of chikungunya in Italy (Bonilauri *et al.*, 2008) and local foci of transmission of chikungunya in France (Gould *et al.*, 2010), and of dengue in France (Gould *et al.*, 2010, La Ruche *et al.*, 2010) and Croatia (Schmidt-Chanasit *et al.*, 2010, Gjenero-Margan *et al.*, 2011) have demonstrated that temperate European countries are now also at risk due to the presence and spread of *Aedes albopictus* (Scholte & Schaffner, 2007). However, the dominant primary vector remains *Aedes aegypti* (Lambrechts *et al.*, 2010), at least for dengue the most widespread of this two arboviral diseases. In the absence of effective

vaccines or specific therapeutic or prophylactic drugs against these viruses, control relies entirely on mosquito control. Unfortunately, the rise in dengue in the past 50 years (WHO-TDR, 2006) and the recent epidemics of chikungunya (Lahariya & Pradhan, 2006, Flahault, 2007, Pulmanausahakul *et al.*, 2011, Singh *et al.*, 2012) have highlighted the limitations of the traditional control methods and the need for innovative methods for mosquito control to be tested and integrated into vector control programs.

New control methods based on genetic engineering either by population suppression or population replacement are under development (Thomas *et al.*, 2000, Dobson *et al.*, 2002, Burt, 2003, Sinkins & Gould, 2006, James, 2007, Alphey *et al.*, 2008, Walker *et al.*, 2011, Wilke & Marrelli, 2012). Here, we focus on an improvement of the classical Sterile Insect Technique (SIT) known as RIDL® (Release of Insects carrying a Dominant Lethal gene (Thomas *et al.*, 2000) which has now entered field trials. Engineered pink bollworm, *Pectinophoragossypiella* (Saunders), within a radiation-based SIT programme and 'genetically sterile' RIDL *Ae. aegypti* mosquitoes have been released and have proven the ability of engineered sterile males to mate with wild females (Harris *et al.*, 2011, Simmons *et al.*, 2011); such mating between modified males and wild females is critical to most genetic strategies. Furthermore, sustained releases of RIDL males strongly suppressed wild *Ae. aegypti* populations in the Cayman Islands (Harris *et al.*, 2012) and Brazil (McKemey, personal communication).

A RIDL programme only requires the release of male mosquitoes; since male mosquitoes do not bite they would have no possibility of transmitting arboviruses. However, it is possible that some females might inadvertently be released; indeed since no sex separation is perfect, the presence of a small proportion of females in the release populations should be expected and anticipated. Data from actual field trials has quantified this: 0.066% (95% CI: 0.050% – 0.081%) females in Cayman Islands, (Harris *et al.*, 2012) and 0.02% (95% CI: 0.014% – 0.026%) females in Brazil (Carvalho *et al.*, 2014). This raises the question of whether such transgenic females pose any increased risk to human health compared to wild females, which are presumably already present at the site in much larger numbers. Particularly, vertical transmission is a mechanism that may be important for the maintenance of viruses between epidemics and during dry seasons (Shroyer, 1990, Joshi *et al.*, 2002). We therefore investigated the vertical transmission of dengue and chikungunya of OX513A female mosquitoes, to address two risk hypotheses: (i) are OX513A-My1 females no more competent for vertical transmission of dengue and chikungunya viruses than females from a wild type strain (My1) from which they were derived and (ii)will release of OX513A-My1 females enhance the potential rate of vertical transmission.

In the present study, to determine the impact of the RIDL transgene on vertical transmission for the four dengue serotypes and chikungunya, we compared the vertical transmission of orally infected females of a transgenic strain (OX513A-My1) to its Malaysian parental laboratory strain (My1).

MATERIALS AND METHODS

Facilities

The experiments were conducted in the Institute for Medical Research (IMR), Kuala Lumpur, Malaysia. Import permit for the OX513A strain was obtained from the Malaysian Ministry of Natural Resources and Environment for contained studies of transgenic mosquitoes in Arthropod Containment Level-2 facilities (Permit reference: Ref NRE.62. 140.020.001/004 Jld. 18). Furthermore, facilities were also approved for infected mosquitoes studies in Biosafety Containment Level-2 laboratories (Arthropod Containment Level 2 Permit: IMR/P/15/1502/0005). The research protocol of this study was reviewed and approved by the Research Review Committee of the IMR (Project number: JPP-IMR-06-053).

Mosquitoes

A laboratory strain originating from Jinjang, Kuala Lumpur, Malaysia, that has been reared in the IMR since the 1960s (referred as My1 strain), and the OX513A-My1 strain derived from it as previously described (Lacroix *et al.*, 2012) were used for this study. The OX513A-My1 strain was constructed by making a line homozygous for the OX513A insertion after introgressing the insertion from its original Rockefeller strain background (Phuc *et al.*, 2007) into the My1 strain by backcrossing for 5 generations such that ~ 97% of the genome of the resulting strain, termed OX513A-My1, is expected to derive from the Jinjang strain. Consequently, the two strains are closely related and differences in vector competence, if any, are likely to be due to the transgenic insertion.

Mosquito rearing

Mosquitoes were reared at 26°C (±1°C), 70% (±10%) humidity and 12:12 photoperiod. Eggs were hatched under vacuum. Larvae were reared in trays (23 x 30.5 x 8 cm) containing 1L of water and fed daily with Vipan fish food (Sera®, Heinsberg, Germany). Females and males were separated and allowed to mature in separated cages (20 x 20 x 20 cm) for 3–4 days before 100 males and 100 females were put together in a cage for mating purposes, six such cages were set for each strain of mosquito. After 24h, all males were removed from the cages.

Viruses

DENV-1, 3 and 4 strains were prototype strains obtained from the Virology Unit, IMR while DENV-2 strain was obtained from the Department of Medical Microbiology, University of Malaya. DENV-1 strain originated from Hawaii (Hawaii strain, isolated in 1944), DENV-2 from New Guinea (New Guinea C strain, isolated in 1944), DENV-3 from the Philippines (H-87, isolated in 1956) and DENV-4 from the Philippines (H-241, isolated in 1956)and CHIKV from Malaysia (Bagan Panchor strain, isolated in 2009). All viruses had been passaged at 25°C on *Ae. albopictus* C6/36 cell line prior to the study; four times for DENV-1, 3 and 4, two times for DENV-2 and CHIKV. All the strains were maintained in the Unit of Medical Entomology, IMR, for the duration of the study.

Oral infection

The following procedures were repeated for the 5 viruses. Fresh blood was obtained from a single healthy male human volunteer after he signed an informed consentform (WHO/CDS/NTD/WHOPES/GCDPP/2006.3). The volunteer was tested by RT-PCR for dengue and chikungunya prior to collecting the blood to ensure he was dengue and chikungunya free. For each mosquito strain, three cages of females were fed with infected blood (titer 10^7pfu/µl, determined by plaque assay before the blood meal) while the three remaining cages were offered uninfected blood. Females were blood fed using the Hemotek™ system (Hemotek, Accrington, United Kingdom) at 38°C (±1°C)(Hagen & Grunewald, 1990, Cosgrove *et al.*, 1994) after being starved overnight. Fully engorged females from each cage were individually isolated in tubes where a wet cotton wool had been set at the bottom to provide oviposition surface (over 200 females per treatment for each mosquito strain and virus). The females were provided with 10% sucrose solution *ad libitum*.

From day 0 (blood feeding day) today 14 post infection, 10 females were sacrificed daily and tested for virus infection by qRT-PCR: (i) five for whole body and (ii) five for their dissected salivary glands.

Detection of virus in larvae

Eggs were counted and collected after sacrifice of the females or at the end of the study, dried for at least 3 days, hatched within a week and reared following standard protocol. Eggs were stored in individual covered containers at insectarium conditions (Temperature 26°C (±1°C), Humidity 70% (±10%)). Eggs for DENV-1 were kept 4 weeks before hatching due to logistics. Eggs from each female were reared separately. Pools of 20 third and fourth instar larvae from each female were tested for virus presence by RT-PCR. For positive specimens, further analysis by C6/C36 cell culture was performed to confirm that the virus was infectious and thus able to multiply and make the adult females

infective to human hosts. Samples positive for virus by cell culture assay were considered to indicate a positive vertical virus transmission. Not all blood-fed females could be classified as positive or negative for vertical transmission as not all females provided larvae (these females either laid no eggs or the eggs failed to hatch).

Wing measurement

For each selected specimen, one wing was carefully removed and mounted on a microscope slide. Digital micrographs of mounted wings were obtained using a Nikon DSFi1™ camera (Nikon, Tokyo, Japan). Wing length was measured using the ImageJ® software package (Schneider *et al.*, 2012). Wing length was defined as the linear distance from the axillary incision to the apical margin, excluding the fringe (Harbach & Knight, 1980, Maciel-De-Freitas *et al.*, 2007).

Quantitative RT-PCR

RNA extraction

For CHIKV, larvae samples were homogenized in chilled micro-centrifuge tubes with 1.0 ml of the maintenance medium (Hank's MEM medium) supplemented with 2% fetal bovine serum (FBS), 1M HEPES buffer (pH = 7-7.4), 7.5% (v/v) sodium bicarbonate and antibiotics (Lee *et al.*, 1997). For dengue, samples were homogenised in a sterile homogeniser. For all viruses, samples were centrifuged for 15 minutes at 835 *g* at 10ºC (Lee *et al.*, 1997) (Eppendorf Centrifuge 5415R, Hamburg, Germany) and RNA was extracted using the AccuPrep® Viral RNA Extraction Kit (Cat. No. K-3033), according to the manufacturer's protocol. A sample of the supernatant was reserved for virus isolation. The remainder was filtered through a 0.22 µm filter unit, centrifuged at 371 *g* for 5 minutes and then used for RNA extraction. The eluted RNA was stored at -70°C.

RT-PCR

For dengue viruses, a one step RT-PCR was carried out with the Access Quick RT-PCR kit (Promega, USA) with a forward primer (Dcon 5'AGT TGT TAG TCT ACG TAC GTG GAC CGA CA') which anneals to a conserved sequence in all four dengue serotypes, with a reverse primer specific to each serotype, as follows: Den1 reverse primer, 5'CCC CGT AAC ACT TTG ATC GCT CCA TT' giving a 342bp product; Den2 Reverse primer, 5'CGC CAC AAG GGC CAT GAA CAG' giving a 251bp product; Den3 Reverse primer, 5'GCA CAT GT TGA TTC CAG AGG GTG TC' giving a 538bp product; Den 4 Reverse primer, 5'GTT TCC AAT CCC ATT CCT GAA TGT GG TGT' giving a 754bp product; CHIKV sense CHIK/E1/10367/+ (CTC ATA CCG CAT CCG CAT CAG) and anti-sense Chik/E1/10495/+ (ACA TTG GCC CCA CAA TGA ATT TG) giving a 129bp product. The PCR was carried out on a thermocycler (Mastercycler gradient machine, Eppendorf, Germany), the programme consisted of a 30 minutes reverse transcription step at 50°C, 15 minutes of denaturation at 95°C, followed by 40 cycles of denaturation at 95°C for 30 seconds, annealing at 55.8°C for 45 seconds, extension at 72°C for 60 seconds and then a final extension at 72°C for 10 minutes. The reaction products were analysed by gel electrophoresis to score positive and negative samples.

TaqMan real time RT-PCR

The One-step TaqMan Probe real-time RT-PCR was carried out using an iCycler Real-time PCR machine (BioRad, Hercules, California, USA) using QuantiTect Probe RT-PCR Kit (Qiagen) following procedure of Kong *et al.* (Kong *et al.*, 2006). Samples were assayed in a 25 µl reaction containing 5 µl of extracted RNA, 0.25 µl of RNA transcriptase, 12.5 µl of RT-PCR master mix, 0.5 µl TaqMan probe and 0.5 µl of each primer according to the recommendations of the kit's manufacturer. The primer sequences used were similar to the ones used for RT-PCR. After analysis of the reaction products by gel electrophoresis, the fluorogenicTaq-man probes used were the following: DENV-1, 5' (FAM) CTC AGA GAC ATA TCA AAG ATT CCC GGG (BHQ1); DENV-2, 5' [Texas Red] TAA GAG ACG TGA GCA AGA AAG AGG GAG GAG [BHQ2]; DENV-3, 5' (Cy5) ACA TTT CCA AGA TAC CCG GAG GAG (BHQ3); DENV-4, 5' (HEX) CCT AGA GGA CAT AGA CAA AAA GGA AGG AGA CC (BHQ1);

CHIKVChik/E1/10469/+ [Hex]TCC TTA ACT GTG ACG GCA TGG TCG CC [BHQ]. The thermal cycling profile for this assay comprised 30 minutes reverse transcriptase step at 50°C, 15 minutes of Taq polymerase activation at 95°C, followed by 40 cycles of amplification steps of denaturation at 95°C for 30 seconds, then annealing and extension at 60°C (58.7°C for CHIKV) for 60 seconds, followed by a final extension at 72°C for 10 minutes. Virus RNA solutions ranging from 10^{10} to 10^{0} used to construct the standard curve for the qRT-PCR, were prepared from the initial solution whose concentration was determined by spectrophotometry (Biophotometer, Eppendorf AG, Hamburg, Germany). Infection rates were determined by analysis of the obtained results.

Virus isolation

The RT-PCR assay detects the presence of nucleic acid, e.g. RNA, with sequence related to that of dengue or chikungunya. However, it does not indicate that the source material is intact, viable virus. In order to further assess the status of PCR-positive samples, a cell culture-based assay was used, based on the protocol of Chakravarti *et al.* (Chakravarti *et al.*, 2006), which uses the *Ae. albopictus* C6/36 cell line. After virus inoculation, cells were incubated at room temperature (range 25°C–30°C) in L-15 medium supplemented with 2% heat-activated fetal calf serum and 10% (v/v) of tryptose phosphate broth (TPB) (Ummul Haninah *et al.*, 2010). Once the prominent cytopathic effect (CPE) was observed by cell structure and size changes under microscope (Eclipse TS100-F, Nikon, Tokyo, Japan), the infected culture fluids were harvested by the freeze-thaw method, centrifuged and supernatants were passed through 0.22 µm filter units. The cells were checked daily and considered negative after 14 days without positive observation. In the final results only the pools of larvae that showed positive virus isolation by this cell culture assay were considered as a demonstration of vertical transmission.

Data analysis

Data was analyzed using the R software (R Core Team, Vienna, Austria). The proportion of females for which vertical transmission was detected – at least one offspring larvae positive for the virus during the cell culture assay – was tested with the Fisher's Exact test using the Bonferroni multiple testing correction:since five viruses were tested the error of the first kind α was chosen as 0.01 (0.05/5). The 95% confidence intervals for these rates were calculated by the binomial exact test. The Minimum Infection Rate (MIR) and their 95% confidence interval were calculated using PoledInfRateaddon in Excel™ (Microsoft, Redmond, USA) (Biggerstaff, 2006) and the cell culture assay. The number of eggs laid per female were tested using Mann-Whitney-Wilcoxon test as the data did not follow a normal distribution (Shapiro-Wilk: $p < 0.05$). As previously, the Bonferroni multiple testing correction was used to take into account the test repetition: the test between strains involved five repetitions so the error of the first kind α was chosen as 0.01 (0.05/5) and for the tests between infected and non-infected blood meals since 2 strains were tested for 5 viruses the error of the first kind α was chosen as 0.005 (0.05/10).

RESULTS

Infection rate

Females infection rates were as follow for OX513A-My1 and My1 strains respectively: DENV-1 (65% vs. 60%), DENV-2 (77% vs. 81%), DENV-3 (40% vs. 44%), DENV-4 (62% vs. 41%) and CHIKV (68% vs. 61%). Details for infection rate results analysis are described in Chandru *et al.* (Submitted to Transgenic Research). All the females that had virus positive progeny had been found positive for the same virus strain during horizontal transmission studies. Since the infection rate of blood fed females was relatively high we were able to run the vertical transmission study on a reasonable number of samples for all viruses.

Vertical transmission

Larvae were tested by batches of 20 (or less when not enough larvae hatched or remained) by qRT-PCR and, when positive,

by cell culture. Progeny from 29 to 130 females fed with infected blood were tested for each virus. The proportion of females for which vertical transmission was detected by cell culture is presented in figure 1. None of the samples of DENV-2 and CHIKV were positive for either mosquito strains. The highest vertical transmission rate was observed for DENV-1 (> 10 %) while DENV-3 and 4 were both below 5% (Figure 1). Whether from the total number of females that were offered an infected meal or from the total number of adult females positive for the virus for either whole body or salivary glands, vertical transmission rate was not significantly different between the OX513A-My1 and My1 strains for any of the viruses tested in the study (Fisher's exact test: $p > 0.2$ for all viruses). The Minimum Infection Rates (MIR) ranged between 0.45 to 10.55 per 1,000 larvae (Figure 2). DENV-1 had the highest MIR (> 5) while DENV-3 and 4 were below 5, this difference was significant for My1 females only (PooledInf-Rate 95% CI did not include 0). The MIR considering females which received an infected meal or only virus positive females were not significantly different from each other. OX513A-My1 offspring MIR did not differ significantly from My1 offspring for all viruses (PooledInf-Rate 95% CI included 0 for all pairwise tests).

The RT-PCR detected false positive that did not develop during the cell culture procedure. For DENV-1, only 36% (OX513A-My1) and 38% (My1) of the samples positive for RT-PCR developed during the cell culture. This rate was higher for DENV-3 (OX513A-My1: 60%; My1: 67%) and DENV-4 (OX513A-My1: 100%; My1: 50%) but stayed relatively consistent between mosquito strains for each virus.

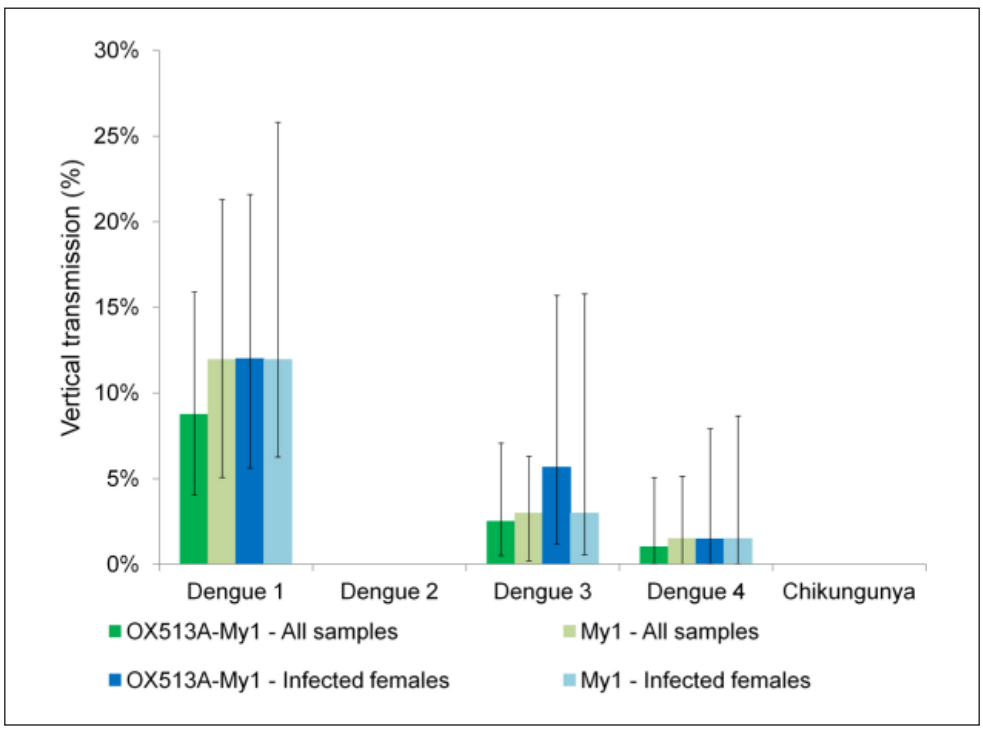

Figure 1. Vertical transmission. Using cell culture assays, the proportion of females for which vertical transmission to at least one of their progeny was detected either from the total number of females that were offered an infected meal (All samples) or from the total number of adult females positive for the virus (Infected females) for OX513A-My1 and My1 strains. Error bars show the 95% confidence interval.

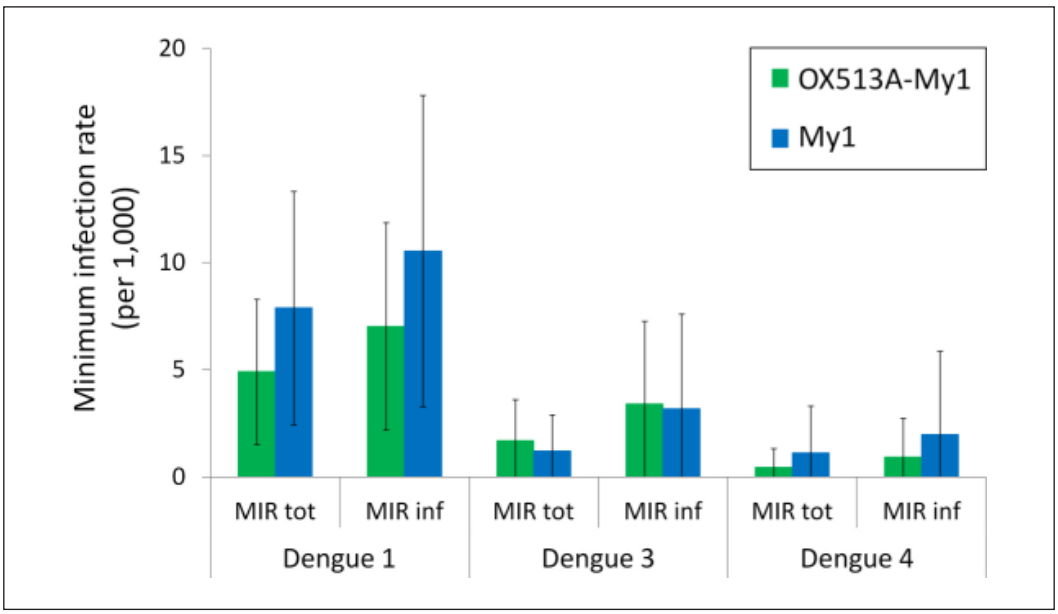

Figure 2. Minimum infection rate. Using the cell culture assays, the minimum infection rate per thousand larvae (MIR) is presented for females fed with infected blood (MIR tot) and females positive for virus by PCR (MIR inf); error bars are the 95% confidence interval.

Fecundity

The number of eggs laid was recorded for each individual female; the average number of eggs laid per female is presented in Table 1. There were no significant differences in the number of eggs laid by OX513A-My1 and My1 females fed on infectious blood (Mann-Whitney-Wilcoxon with the Bonferroni multiple testing correction: $p > 0.05$) except for DENV-4 for which OX513A-My1 females laid significantly more eggs (Mann-Whitney-Wilcoxon with the Bonferroni multiple testing correction: $p < 0.001$ – but also more eggs in the non-infected control). Females fed with non-infected blood produced significantly more eggs than those fed on infected meals in five tests while the latter produced significantly more eggs in three tests; the two remaining tests showed no significant difference.

DISCUSSION

There were no observed differences in vertical transmission rates between OX513A-My1 and My1 females for DENV-1, 2, 3, 4, and CHIKV. Presence of the transgene had no effect on the infection of progeny of females infected with the viruses. The genes introduced in OX513A-My1 encode tTAV and DsRed2 (Phuc *et al.*, 2007) which are not related to the mosquito immune system. It would therefore be reasonable to expect that the OX513A insertion would have little effect on vector competence; this study provides direct evidence to support that expectation for transovarial transmission. This study supports the risk hypothesis that OX513A-My1 mosquitoes are no more able to transmit dengue and chikungunya to their progeny than the My1 strain from which they are derived.

DENV-1 presented a higher vertical transmission rate than DENV-3 and 4. This may be due to strain rather than serotype variation; several strains of each serotype would need to be tested to distinguish between these possibilities. The time between egg laying and hatching has been reported to increase the observed infection rate of the eggs; progeny from 30 day old eggs had a 50% increase in infection rate compared to progeny from 1 week old eggs (Mourya *et al.*, 2001). In the present study, the eggs from DENV-1 infected females were kept one month before hatching due to logistic

Table 1. Number of eggs laid per female. The average number of eggs laid per female is presented below for each mosquito strain either fed on infected or non-infected blood. In brackets is the standard error of the means. A Bonferroni multiple testing correction was applied to the error of the first kind α so an asterisk (*) indicates a significant difference at 99.5% level (p-value < 0.005) or 99% level (p-value < 0.01)

Virus	Strain	Eggs/female Infected blood	Non-infected blood	p-value Blood meal effect (α=0.005)	Strain effect (α=0.01)
CHIKV	OX513A-My1	24 (± 2.7)	33 (± 2.7)	< 0.001*	0.342
	My1	25 (± 2.4)	33 (± 2.6)	0.03	
DENV-1	OX513A-My1	48 (± 3.6)	24 (± 3.6)	< 0.001*	0.218
	My1	42 (± 2.6)	46 (± 2.0)	0.1	
DENV-2	OX513A-My1	23 (± 2.0)	34 (± 2.8)	< 0.001*	0.890
	My1	21 (± 2.0)	30 (± 2.5)	0.002*	
DENV-3	OX513A-My1	39 (± 2.4)	50 (± 2.2)	< 0.001*	0.480
	My1	38 (± 1.7)	46 (± 1.7)	< 0.001*	
DENV-4	OX513A-My1	44 (± 2.2)	26 (± 2.5)	0.0048*	< 0.001*
	My1	29 (± 2.1)	12 (± 1.1)	< 0.001*	

reasons compared to 1 to 2 weeks for the other viruses; the higher observed vertical transmission rate for DENV-1 might relate to this difference. Vertical transmission of DENV-1, 2, 3 and 4 has been reported in the field and in the laboratory for DENV-1 (Rosen *et al.*, 1983) and DENV-3 (Joshi *et al.*, 2002) for *Ae. aegypti* and for all serotypes for *Ae. albopictus* (Rosen *et al.*, 1983, Shroyer, 1990). The MIR varies considerably from a study to another in the field (0.05% to 40%) and only a few studies managed to detect vertical transmission (1% to 20%). The MIR estimates in the present study were all in the lower range of the literature, being around or below 1%.

Vertical transmission of chikungunya was reported to be very low (Delatte *et al.*, 2008) or absent (Bellini *et al.*, 2012) in the field and low (Bellini *et al.*, 2012) or absent (Mourya, 1987, Vazeille *et al.*, 2009) in the laboratory. In the present study, no vertical transmission was detected for either of these viruses. Consequently, the OX513A insertion not only does not affect the vertical transmission when it occurs but was also not found to induce vertical transmission when it would otherwise not occur.

The level of vertical transmission has been reported to be subject to seasonal variation (Thongrungkiat *et al.*, 2003, Arunachalam *et al.*, 2008, Bina *et al.*, 2008, Mulyatno *et al.*, 2012) and might also be sex dependent (Mulyatno *et al.*, 2012). Vertical transmission may provide a mechanism for the virus to maintain itself in an area through the low mosquito season; this is further suggested by studies demonstrating the possibility of maintenance of the virus through several generations of *Ae. aegypti* (Joshi *et al.*, 2002) and *Ae. albopictus* (Shroyer, 1990) without further exposure to the virus. It is also interesting in this context to consider the reported increase in vertical transmission rates detected in older eggs, discussed above. Regarding OX513A-My1 females, as the majority of their progeny will die before emerging as adults and being able to reproduce or bite, it is very unlikely that the strain would play any role in the maintenance of a virus in the field.

OX513A-My1 and My1 females laid similar number of eggs whether fed on infected or non-infected blood with the exception of DENV-4 as previously reported for non-infected females (Lee *et al.*, 2009).

As the number of eggs laid per female *Ae. aegypti* usually shows high variability, at least 72 females laying eggs were used for each mosquito strain and virus strain to get consistent results. Nevertheless, the DENV-4 My1 females laid fewer eggs than their OX513A-My1 counterparts whether fed with infected blood or not; this could be due to rearing adaptation differences and thus difference in size as small females lay less eggs than large ones (Briegel, 1990, Farjana & Tuno, 2013). Indeed, for that virus, the wing length of My1 females was 33% lower than the OX513A-My1 females (2.05mm vs. 3.09mm) while the difference was less than 20% for other treatments. Previous studies have found that females fed on infected blood had a lower fertility for *Ae. aegypti* (Maciel-de-Freitas *et al.*, 2011) and on other parasite/host systems (Hurd, 2001). Our study does not provide strong support for such an effect, though more treatments resulted in lower egg numbers for females fed on infected blood than the contrary (6 treatments vs. 3 treatements; Table 1).

The immune response of *Ae. aegypti* to dengue infections is based on genotype by genotype interactions, meaning that the outcome of the infection will depend on the specific interaction between the two genotypes (Armstrong & Rico-Hesse, 2001, Lambrechts *et al.*, 2009, Fansiri *et al.*, 2013). Such specific mechanisms are common in host-pathogen interactions (Carius *et al.*, 2001, Schulenburg & Ewbank, 2004, Lambrechts *et al.*, 2005, Salvaudon *et al.*, 2007, de Roode & Altizer, 2010) and have been reported for chikungunya virus (Martin *et al.*, 2010, Bellini *et al.*, 2012) in which a single mutation in the virus enabled an increase in the vector competence of some strains of *Ae. albopictus* (Tsetsarkin *et al.*, 2007, Vazeille *et al.*, 2009). Presumably as a result, large variations in vertical transmission of dengue and chikungunya have been reported in Aedine species depending on the mosquito strain and virus strain (Mourya, 1987, Fouque & Carinci, 1996, Joshi *et al.*, 1996, Mourya *et al.*, 2001, Joshi *et al.*, 2002, Thongrungkiat *et al.*, 2003, Arunachalam *et al.*, 2008, Delatte *et al.*, 2008, Vazeille *et al.*, 2009, Bellini *et al.*, 2012, Martins *et al.*, 2012, Mulyatno *et al.*, 2012). Vertical transmission is also dependent on the susceptibility of the females which is highly variable as well (Nalim *et al.*, 1978, Gubler *et al.*, 1979, Tardieux *et al.*, 1990, 1991, Sumanochitrapon *et al.*, 1998, Tran *et al.*, 1999, Vazeille *et al.*, 1999, Armstrong & Rico-Hesse, 2001, Bennett *et al.*, 2002, Knox *et al.*, 2003, Lourenco-de-Oliveira *et al.*, 2004, Diallo *et al.*, 2008, Lambrechts *et al.*, 2009). While the results presented here obviously do not describe all the possible interactions between virus strains and the OX513A-My1 females, the lack of substantial differences relative to their background non-transformed counterpart indicates that the presence of transgene does not interact with major traits determining vector competence.

We investigated vertical transmission as far as larvae. The measured rate may be an overestimate of the frequency of production of infectious female offspring as the virus may fail to persist to later developmental stages, or to reach the salivary glands, for example. While it is possible that the frequency of loss at these later stages may be influenced by the genotype of the mosquito, infection rates following oral challenge did not differ significantly between OX513A-My1 and My1 females, indicating similar susceptibility, at least in adults. Moreover, in the environment, in the absence of tetracycline, the large majority of OX513A-My1 larvae will die before developing into functional adults (Phuc *et al.*, 2007).

Ratios as high as 50 to 100 OX513A males to 1 wild male are foreseen to be necessary to suppress wild populations of *Ae. aegypti*. Assuming broadly similar populations for both sexes, the few females released along the males (0.07% of the males (Harris *et al.*, 2012)) would represent a ratio of 0.03 to 0.07 to 1 wild female, hardly a substantial contribution to the wild population. Once the wild population is suppressed, ongoing releases of males to maintain the low population level or re-infestation would be at lower levels, consequently, the number of females will be correspondingly lower relative to the initial population. Furthermore, the reduced adult lifespan of OX513A adults compared to their wild type

counterpart in the laboratory (Massonnet-Bruneel *et al.*, 2013) is very likely to be translated to the field which would limit the vectorial capacity of released OX513A females in the field. In conclusion, due to the absence of effect of the gene on the vertical transmission and the relatively low number of released females, if any, both risk hypotheses concerning vertical transmission have been validated; RIDL-SIT programmes will not expose the human population to an increased risk of dengue or chikungunya maintenance due to the release of OX513A-My1 *Ae. aegypti*.

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