Australian Aedes aegypti mosquitoes are susceptible to infection with a highly divergent and sylvatic strain of dengue virus type 2 but are unlikely to transmit it

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Short report

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Abstract

Background: Humans are the primary hosts of dengue viruses (DENV). However, sylvatic cycles of transmission can occur among non-human primates and human encroachment into forested regions can be a source of emergence of new strains such as the highly divergent and sylvatic strain of DENV2, QML22, recovered from a dengue fever patient returning to Australia from Borneo. The objective of the present study was to evaluate the vector competence of Australian Aedes aegypti (A. aegypti) mosquitoes for this virus. Methods: Four day old mosquitoes from two strains of A. aegypti from Queensland, Australia, were feed a meal of sheep blood containing 10 8 50% cell culture infectious dose per ml (CCID 50 /ml) of either QML22 or an epidemic strain of DENV serotype 2 (QML16) isolated from a dengue fever patient in Australia in 2015. Mosquitoes were maintained at 28°C, 75% relative humidity and sampled at 7, 10 and 14 days post-infection (DPI). Live virions in mosquito bodies (abdomen/thorax), legs and wings and saliva expectorates from individual mosquitoes were quantified using a Cell Culture Enzyme-linked Immunosorbant Assay (CCELISA) to determine infection, dissemination and transmission rates. Findings: The infection and dissemination rates of the sylvatic DENV2 strain, QML22, were significantly lower than that for QML16. While the titres of virus in the bodies of mosquitoes infected with either of these viruses were similar, titres in legs and wings were significantly lower in mosquitoes infected with QML22 at most time points although they reached similar levels by 14 DPI. QML16 was detected in 16% (n = 25) and 28% (n = 25) of saliva expectorates at 10 and 14 DPI, respectively. In contrast, no virus was detected in the saliva expectorates of QML22 infected mosquitoes. Conclusions: Australia urban/peri-urban A. aegypti species are susceptible to infection by the sylvatic and highly divergent DENV2 virus QML22. Our results indicate that replication of QML22 is attenuated relative to the contemporary strain QML16. Alternatively a salivary gland infection or escape barrier acts to prevent infection of saliva, potentially preventing onward transmission of this highly divergent virus in Australia.

Background

Dengue viruses (DENV) have two ecologically and evolutionarily discrete transmission cycles, sylvatic and urban endemic/epidemic [1]. The sylvatic cycle employs non-human primates as hosts and several arboreal *Aedes* mosquito species as transmission vectors [2, 3]. In contrast, the urban endemic/epidemic cycle has humans as the host and the peri-domestic *Aedes aegypti (A. aegypti)* mosquito as the principal vector. These two cycles of DENV transmission are evolutionarily distinct and all four serotypes of endemic/epidemic DENV are considered to have evolved independently from the sylvatic DENV progenitors over the past 1,000 years. Whether sylvatic DENV strains can overcome adaptive barriers to infect peri-domestic *A. aegypti* mosquitoes, then enter the urban human-mosquito-human transmission cycle to cause secondary human infection (spillover epidemics), has been a source of debate for more than a decade [1, 9-11]. Sylvatic DENV1-4 strains from Malaysia and DENV2 from West Africa have been reported to be able to spill over to infect humans causing similar or relatively milder dengue symptoms compared with the classic endemic/epidemic DENV infections [4-8]. Previous assessments of the ability of sylvatic DENV strains to infect *A. aegypti* have produced a confusing picture in which the susceptibility

of *A. aegypti* to infection with sylvatic DENV2 has ranged from refractory to almost 100% [12-15]. Significantly, none of the viruses studied were recovered from patients, instead, sylvatic viruses had been isolated from non-human primates and/or mosquitoes. In addition, these studies used virus dissemination to mosquito legs, wings and heads as a proxy for virus transmission capability, based on the assumption that if the virus were able to disseminate from midgut to other tissues, that the virus would have infected the salivary glands and transmission could occur [15, 16]. The detection of infectious virus from mosquito saliva provides a more accurate proxy for transmission [17].

In 2016, we recovered a sylvatic strain of DENV2, QML22, from a patient returning to Australia from Borneo which was the most basal of all strains of DENV in phylogenetic trees and was divergent from Asian and West African lineages of sylvatic DENV2 [5]. It has been reported that susceptibility of Australian strains of *A. aegypti* to infection with DENV varied with the geographic locations from which the mosquitoes were obtained [18-20]. This study determined whether colonies derived from two different locations in northern Australia, where dengue outbreaks have occurred were likely to be able to transmit this sylvatic strain of DENV if it were to be introduced.

Methods

Cells, viruses and mosquitoes

C6-36 (*Ae. albopictus* mosquito) cells were purchased from the American Type Culture Collection (ATCC) and cultured in 10% v/v heat inactivated foetal calf serum (FCS, Life Technology, USA)/RPMI 1640 medium (Sigma-Aldrich, St. Louis, MO, USA). The QML16 strain of DENV2 was isolated from a dengue fever patient in Australia and strain QML22 was isolated from a dengue fever patient returning to Australia from Borneo [5]. The virus strains were passaged three times in C6-36 cells and the cell culture supernatant was harvested, aliquoted and stored at -80°C for further use. One vial of the viral stocks was thawed to determine virus titre ($CCID_{50}$/ml) using a Cell Culture Enzyme-linked Immunosorbant Assay (CCELISA) method. As required, remaining vials were removed from the -80°C freezer immediately thawed, diluted and mixed with blood to prepare artificial viremic blood meals as described previously [12].

Colonies of *A. aegypti* were established from collections in Townsville and Innisfail, north QLD, Australia, and maintained within the Australian Defence Force Malaria and Infectious Disease Institute and QIMR Berghofer Medical Research Institute insectaries, respectively. Both mosquito colonies were established before the release of *Wolbachia* in northern Australia. Larvae were reared at a density of 200 larvae in 3 L of water, prepared by reverse osmosis, in plastic trays (48x40x7 cm) and fed ground TetraMin tropical fish food flakes (Tetra, Melle, Germany) at a rate of 0.25 – 1.0 mg/larva/day as development progressed. Pupae were transferred to cages (30 × 30 × 30 cm) for adult emergence. Adults were provided with 10% w/v sucrose solution on cotton wool pledgets which were removed 24 hr prior to feeding.

Membrane feeding

Approximately one hundred 5 day-old mosquitoes were placed into 750 ml containers with gauze covering the opening. Stocks of DENV2 QML16 and QML22 strain viral stocks were thawed and immediately mixed with defibrinated sheep blood directly at a titre of 10^8 $CCID_{50}$/ml. The mosquitoes, in containers, were allowed 1 hr to feed on the blood/virus mixtures through bovine ceacum membrane using an artificial feeding apparatus maintained at 37 °C, as previously described [21]. After feeding, mosquitoes were anaesthetized using CO_2, placed on a petri dish on ice and fully engorged females were separated from unfed or partially fed mosquitoes. The engorged mosquitoes were placed into the gauze covered containers, provided with cotton balls soaked with 10% sugar solution, and maintained within an environmental chamber (Panasonic, Australia) set at 28 °C, 75% relative humidity and 12:12 h day : night light schedule with 30 min dawn : dusk periods.

In vitro transmission assays

At 7, 10 and 14 days post infection (DPI), female mosquitoes were anesthetized using CO_2 and placed in petri dishes on ice. Legs and wings were removed and their virus content used to determine the dissemination rate as described previously [22]. *In vitro* transmission assays were performed as previously described [23, 24]. For each mosquito, the proboscis was placed in a capillary tube containing 20µl of a 1:1 solution of 50% sucrose and FCS. After 30 min, the contents were expelled into 0.25ml MD (MD, 2% v/v FCS in RPMI 1640, 50µg/ml penicillin/streptomycin, 50µg/ml gentamycin, 2.5µg/ml Amphotericin B, 10mM HEPES). Mosquitoes were observed for abdominal contractions during the 30-min salivation period to confirm they had salivated. Those that did not appear to have salivated were discarded.

Determination virus titre

Legs, wings and bodies from individual mosquitoes were placed into separate 2 ml screw cap vials with 1ml MD with 4-5 zirconium silica beads. The samples were homogenized by shaking the tubes for 90 seconds in a chilled block using a MiniBeadbeater-96 sample homogenizer (Biospec Products, Bartlesville, OK, USA) followed by centrifugation at 17,000× g, 10 min, 4 °C. Supernatants were transferred to sterile tubes.

Virus stocks and virus in mosquito samples were titrated using a modification of the Cell Culture Enzyme Linked Immunosorbant Assay (CCELISA) procedure of Broom et al [25]. Briefly, virus stocks and samples were ten-fold serially diluted and inoculated onto monolayers of C6/36 cells grown in RPMI 1640 supplemented with L-glutamine, 5% heat inactivated FCS, 1% penicillin/streptomycin (Gibco Life Technologies, USA) and maintained at 30˚C, 5% CO_2. After 7 d incubation, cells were fixed in acetone: methanol (1:1) for 1 hr at 4˚C. Plates were air-dried and antigen was detected using a cocktail of anti-flavivirus monoclonal antibody hybridoma supernatants (4G2[26]:6B-6C1:3H5[27], at a ratio of 1:1:1), followed by horseradish peroxidase (HRP-) conjugated goat anti-mouse polyclonal antibody (DAKO, Carpinteria, CA, USA) (1:2000 in PBS-Tween). Antibodies bound to the cell monolayers were detected by the addition of 3,3′,5,5′-Tetramethylbenzidine (TMB) Liquid Substrate System for Membranes (Sigma-

Aldrich, St. Louis, MO, USA). The $CCID_{50}$ was determined from titration endpoints as described everywhere [28] and expressed as the C6/36 $CCID_{50}$/ml.

The infection rate was defined as the proportion of mosquitoes with bodies containing DENV divided by the total number of engorged mosquitoes. Dissemination and transmission rates were defined as the proportions of infected mosquitoes with legs/wings containing DENV and salivary secretions containing DENV divided by the total number of engorged mosquitoes. The Mann-Whitney *U*-test and Chi square tests were employed to compare virus titres in tissues and proportions of infected tissues.

Mosquito immunohistochemistry

Histological analysis of DENV infection within mosquitoes employed indirect immunofluorescence assays (IFA) as described previously [23]. Briefly, mosquitoes with legs and wings removed were fixed in 4% v/v paraformaldehyde/0.5% v/v Triton X-100 for 12 hr, dehydrated in xylol followed by a graded ethanol series, embedded in paraffin and 3-4 μM sections fixed to slides. Sections were incubated in Diva antibody retrieval solution (Biocare Medical, Concord, CA, USA) at 125°C for 5 min in a Biocare Medical Decloaking Chamber. Sections were cooled for 20 min and washed twice in 0.025% v/v Tween 20/PBS pH 7.2 for one minute each wash. Non-specific antibody binding was inhibited by incubating the sections in 2% w/v Bovine Serum Albumin (Sigma-Aldrich, St. Louis, MO, USA)/ Biocare Medical Background Sniper for 30 min. Excess Sniper/BSA was removed from the sections before they were incubated with anti-flavivirus monoclonal antibody, 4G2, for 2 hours at room temperature. Sections were washed three times with PBST and Alexa Fluor 488 donkey anti-mouse antibody diluted 1:300 in PBST applied for 30 minutes. Sections were washed three times with PBST before being counterstained with DAPI for 10 minutes,, washed several 4 times with PBST before being mounted.

Results

A smaller proportion of mosquitoes from the *A. aegypti* colony from Townsville became infected when fed DENV2 QML22 (38.7%, n = 75) than QML16 (75%, n = 75) (Figure 1a and b. Chi square test, $p < 0.0001$) and the proportion of bodies infected with each strain remained stable between 7 and 14 DPI. Although the proportion of mosquitoes infected with QML22 was lower than that for QML16, the titres of each virus in infected mosquitoes were not significantly different (Figure 1b) (Mann Whitney, $p > 0.05$).

DENV2 QML16 was detected in legs and wings as early as 7 DPI while QML22 was not detected in these tissues until 10 DPI. Furthermore, QML22 disseminated to legs and wings in fewer mosquitoes that QML16 and virus grew to lower titres than QML16 at 7 and 10 DPI (Figure 1a and b, $p<0.01$, chi squared test,). However, QML22 reached similar titers to QML16 in legs and wings by 14 DPI ($p>0.05$, chi square test, Figure 1b).

No live virus was detected in saliva expectorates of mosquitoes fed on blood meals containing QML22 strain at 7, 10 or 14 days post feeding. This was in contrast to mosquitoes fed on blood meals containing QML16, which resulted 16% (4/25) and 28% (7/25) of positive saliva samples containing virus at day 10

and 14 DPI, respectively. The titres of DENV in these samples reached a maximum of 1250 $CCID_{50}$/mosquito.

These investigations then were repeated using a colony of *A. aegypti* established from mosquitoes collected at Innisfail, 250 km from Townsville several years after the Townsville colony was established. The infection rate of QML22 in *A. aegypti* from Innisfail fed on the same concentrations as above was lower (16%, n=25 at 14 DPI) than that observed with the Townsville colony (above), while the rates of infection of mosquitoes from both *A. aegypti* colonies with QML16 were similar (72%, n=25 for Innisfail and 75%, n=75 for Townsville, $p < 0.001$, Figure 1a and b). In keeping with the first experiment, the titres of virus in the infected bodies was similar between mosquitoes fed QML16 and QML22 strains (~ 10^7 $CCID_{50}$/mosquito, Figure 1b). This suggested that the *A. aegypti* mosquitoes from Innisfail were less susceptible to infection with QML22 than those from Townsville. Low numbers of infected mosquitoes from Innisfail prevented statistical analyses.

Histological examination of limited numbers of mosquitoes infected with QML16 and QML22 supported the above results. (Figure. 2). Disseminated virus infection was observed in 88% (n = 25) mosquitoes ≥ 10 d after feeding on QML16 and infection was observed in 50% (n = 6) of the salivary glands of these insects. In contrast, no dissemination of virus could be detected beyond the midgut in any mosquitoes ≥ 10 d after feeding on QML22 (n = 13).

Discussion

The introduction of a pathogenic, transmissible and highly divergent DENV2 genotype into areas of Australia with a human population largely susceptible to DENV infection as well as the existence of the principal urban vector of DENV, *Ae. aegypti* [29], could have significant public health implications. However, while colonies of *A. aegypti* established from two population centres in northern Australia were found to be susceptible to infection with and able to transmit a contemporary epidemic strain of DENV2, they were much less susceptible to infection with the highly divergent and sylvatic strain, DENV2 QML22, and thus appear unlikely to be able to transmit it.

Variable competence of *A. aegypti* populations from around the world to act as vectors for sylvatic DENV have been reported. A sylvatic strain of DENV2 isolated from a mosquito in Senegal in 1965 was found to infect 50-91% of *A. aegypti* among eight different Senegalese *A. aegypti* populations. Moreover, these results were achieved from blood meals containing substantially less virus than used in this study (approximately 10^{6-7} PFU/ml virus)[14]. In contrast, infection rates of only 0-27% were observed in another study in which six Senegalese *A. aegypti* populations were fed 10^{6-7} PFU/ml of a sylvatic strain DENV2 isolated from Senegal in 1999 [12]. Our infection rates more closely resemble those achieved when *A. aegypti* collected from Galveston, United States and from Bolivia were fed on blood meals containing 10^8 to $10^{9.5}$ $TCID_{50}$/ml of strains of sylvatic DENV2 isolated from a mosquito in Burkina Faso, West Africa, and from a sentinel monkey in Malaysia [30]. All these studies used dissemination to distal body tissues as a measure of the potential to transmit these viruses based on an assumption that

mosquitos were capable of transmitting DENV if the virus had disseminated from the midgut into the hemocoel [15, 16]. In our experiments with QML22, the virus disseminated into legs and wings but could not be detected in saliva at any time point.

Our data reinforces the extensive DENV vector competency literature that demonstrates that no two strains of DENV can be assumed to behave in exactly the same way in *A. aegypti* from different localities. Investigations to determine the mechanisms underpinning the resistance of *A. aegypti* to infection with this sylvatic strain of DENV2 are likely to be complex given the enormous differences between the nucleotide and amino acid sequences of it and other strains of DENV2 for which *A. aegypti* is known to be able to be a competent vector [5]. Added to this is the additional complexity of host factors that this study observed in two colonies of mosquitoes derived from areas only 250 km apart.

In order to transmit the virus to an uninfected human, DENV must escape the mosquito innate immune system to replicate and disseminate through the mosquito before infecting saliva [31]. Several physiological 'barriers' to this dissemination have been hypothesised, including midgut infection and escape barriers (MIB and MEB) and salivary gland infection and escape barriers (SGIB and SGEB). Earlier studies have indicated that the MIB is a major determinant of vector competence for DENV [32, 33]. The lower body infection rate of QML22 (Figure 1a) suggested a MIB might be the first obstacle for the highly divergent QML22 where the virus/cell-receptor interaction and internalization into the midgut epithelial cells is occurring. When the MIB was overcome, QML22 replicated to titres similar to those for QML16 in body tissues (Figure 1b). Lower dissemination rates and slower replication rates for QML22 than QML16 in legs and wings would have a significant effect on transmission given the relatively short half-life of *A. aegypti* in nature. Failure to detect QML22 in mosquito saliva by CCELISA was not surprising given the inability to detect DENV in salivary glands from a small number of mosquitoes infected with QML22 (Figure 2). However, the difference between the proportion of QML16 infected mosquitoes with infected salivary glands and the proportion with virus in saliva (Figure 1 and 2), suggested SGEB plays a role in the VC on Australian *A. aegypti* mosquitoes. The relative importance of physiological infection barriers remains to be further determined.

The marked differences between the ability of colonies of *A. aegypti* to become infected with and to transmit this highly divergent/primitive strain of DENV 2 (QML22) and a conventional strain (QML16) suggest further studies with *A. albopictus* and arboreal strains of *Aedes* are warranted, if such colonies can be established, to determine whether other strains of *A. aegypti* also are poor vectors of QML22 or whether there is a gradient of competencies from arboreal to urban mosquitoes.

Conclusions

We demonstrated that *A. aegypti* mosquitoes from Townsville and Innisfail in northern Australia are highly susceptible to infection with and able to transmit a contemporary epidemic strain of DENV2 but are much less susceptible to infection with a highly divergent and sylvatic DENV2 QML22 and potentially

unable to transmit it. Our findings support a conclusion that sylvatic DENV is unlikely to enter urban human –mosquito-human transmission cycles in Australia [34].

Abbreviations

A. aegypti: *Aedes aegypti*; ATCC: American Type Culture Collection; CCELISA: Cell Culture Enzyme-linked Immunosorbant Assay; CCID50: 50% cell culture infectious dose; DENV: Dengue virus; DPI: Days post infection; FCS: Foetal calf serum; HRP: Horseradish peroxidase; IFA: indirect immunofluorescence assay; PBST: PBS+0.025% Tween 20; QLD: Queensland; VC: vector competence (VC); TMB: Tetramethylbenzidine.

Declarations

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Availability of data and material

The data supporting the conclusions of this article are included within the

article. Raw data and materials are available from the corresponding author

upon request.

Authors' contributions

Pickering and Hugo undertook the experiments and contributed equally to this study. GJD, JGA and WJL conceived and designed the study. All authors read, reviewed and approved the final manuscript.

Authors' information

PP and LH should be considered joint first authors.

Competing interests

The authors declare that they have no competing interests.

Consent for publication

Not applicable.

Ethics approval

Not applicable.

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Figures

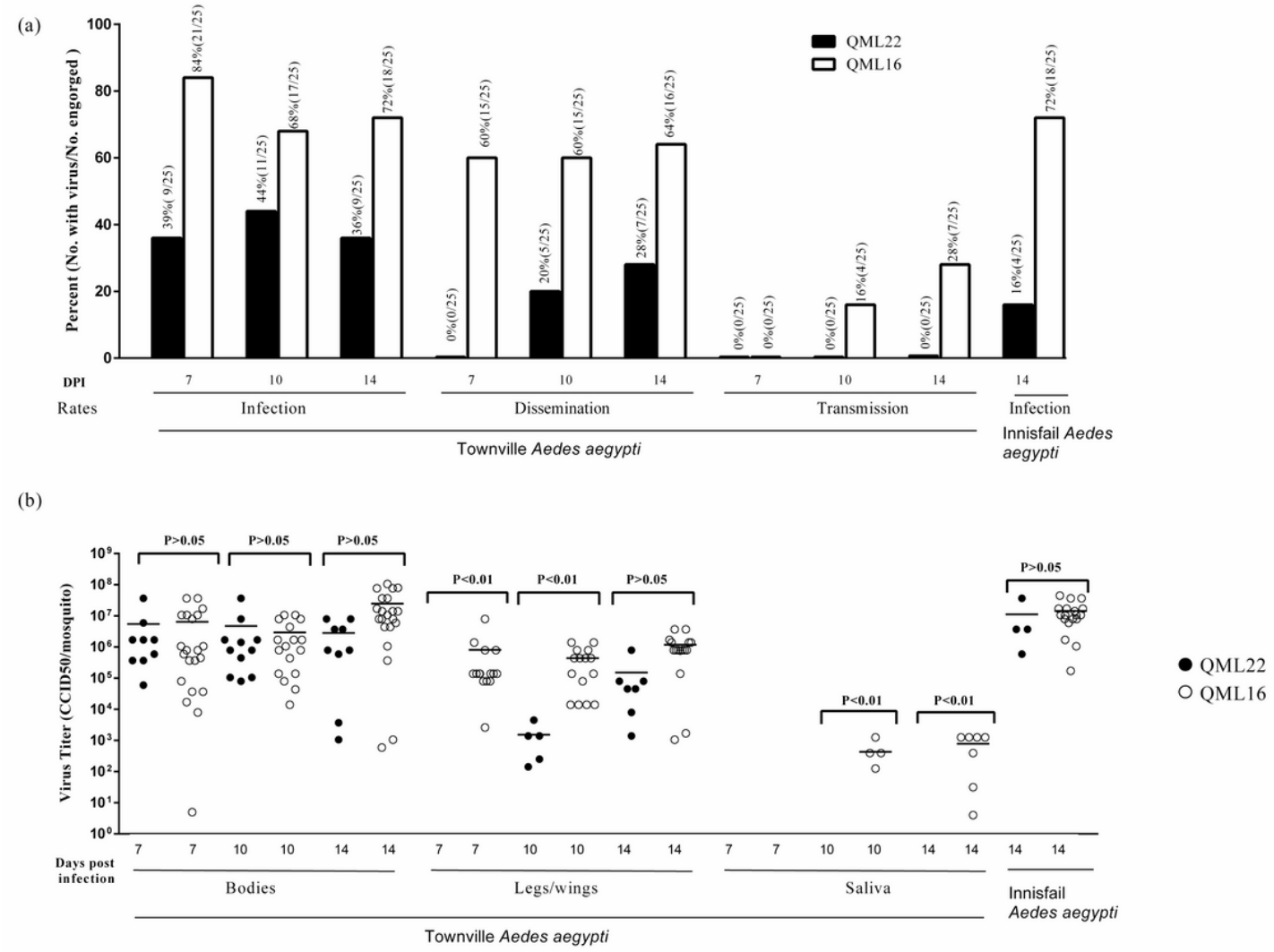

Figure 1

Infection rate, dissemination and transmission potential of DENV2 QML16 (open symbols) and DENV2 QML22 (closed symbols) in A. aegypti mosquitoes. (a) Infection, dissemination and transmission rates were calculated from the proportion of blood fed mosquitoes that developed a detectable DENV infection in the bodies, legs/wings and saliva, respectively. (b) Titres of virus in tissues from infected insects quantified by CCELISA in C6/36 mosquito cells.

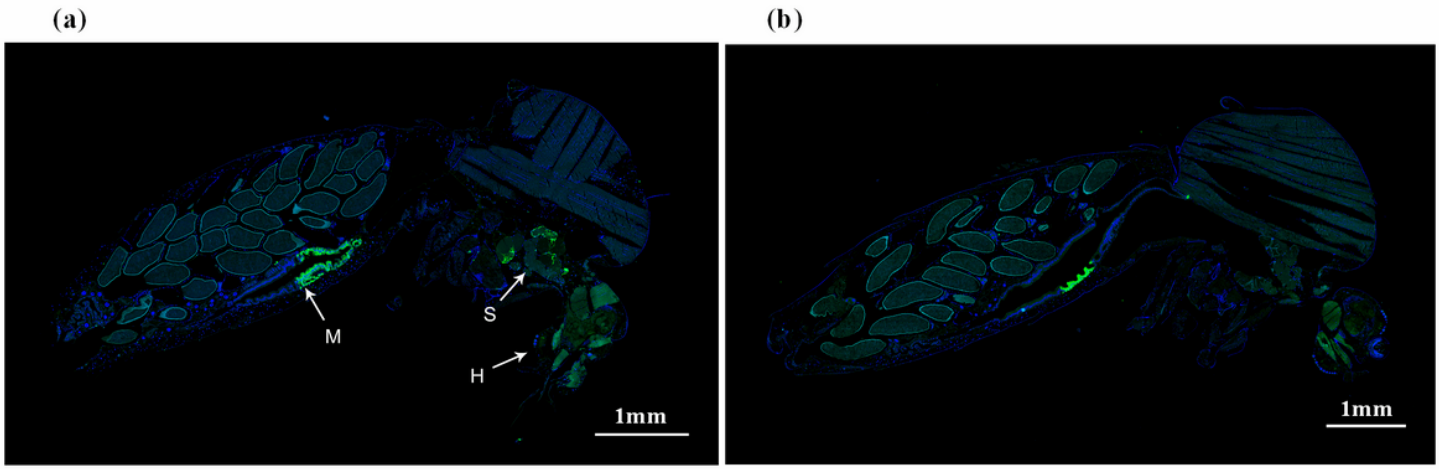

Figure 2

Midsagittal sections showing tissue distribution of DENV QML16 and QML22 strain infection within orally inoculated Aedes aegypti. Mosquitoes were examined for the distribution of DENV infection by IFA analysis of paraffin sections using an anti-flavivirus Envelope protein monoclonal antibody and Alexa-fluor 488 conjugated secondary antibody for DENV (green) and DAPI staining for DNA (blue). A. Example of whole body staining of mosquitoes infected with DENV QML16 at 14DPI showing staining in head (H), midgut (M); and salivary glands (S). B. Example of whole body staining of mosquitoes infected with DENV QML22 at 14 DPI showing infection limited to the midgut. No staining was observed beyond midguts for mosquitoes inoculated with QML22. Scale bars = 1 mm.

Supplementary Files

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- Abstract.jpg