Australia *Ae. aegypti* mosquitoes are susceptible to a highly divergent and sylvatic dengue virus type 2 strain infection but are unlikely to transmit

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Short report

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Abstract

Background: Humans are the primary hosts of the dengue virus; However, sylvatic cycles of transmission can occur among non-human primates and human encroachment to forested regions can be a source of emergence of new strains. We reported the isolation of a highly divergent and sylvatic DENV-2 strain (QML22) from a dengue fever patient returning Australia from Borneo. The objective of the present study was to evaluate the vector competence of Australian Ae. aegypti mosquitoes for this virus.

Methods: Four-day old mosquitoes from two strains of Ae. aegypti from Queensland, Australia, were feed sheep blood meal containing 108 50% cell culture infectious dose per ml (CCID50/ml) of either QML22 or an Australian epidemic DENV serotype 2 strain (QML16) isolated from a dengue fever patient 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, within mosquitoes 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 Ae.aegypti species are susceptible to infection by the sylvatic and highly divergent DENV-2 virus QML22. However, our results indicate that replication of QML22 is attenuated relative to the contemporary strain QML16 and/or 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 virus (DENV) has two ecologically and evolutionary discrete transmission cycles: sylvatic and urban endemic/epidemic [1]. The sylvatic cycle casts non-human primates as the host and several arboreal Aedes mosquito species as the transmission vectors [2, 3]. Alternatively, the urban endemic/epidemic cycle sees humans as the host and the peri-domestic Ae. aegypti mosquito as the principal vector. These two kinds of DENV cycles are evolutionary 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. Sylvatic DENV1-4 strains from Malaysia and DENV-2 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] Whether sylvatic DENV strains can overcome adaptive barriers to infect peri-domestic Ae. 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 over a decade [1, 9–11]. Previous studies testing the ability of sylvatic DENV strains to infect Ae. aegypti have produced a confusing picture in which the susceptibility of Ae. aegypti to infection with sylvatic DENV-2 has ranged from refractory to almost 100% susceptibility [12–15]. Noticeably, none of the viruses studied were recovered from patients (i.e. they were not known to be able to infect humans). Instead, viruses were 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 these 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 reported the isolation of a sylvatic strain of DENV-2, QML22, from a patient returning to Australia from Borneo [5]. The complete genome of QML22 is clearly divergent from Asian and West African lineages of sylvatic DENV-2. It has been reported that DENV susceptibility for Ae. aegypti varied geographically in Australia [18–20]. Here we determined the vector competence of two lines of Ae. aegypti responsible for urban transmission in Australia for this sylvatic strain of DENV by oral infection of mosquitoes with virus and analysis of infection within mosquito bodies, legs and wings and saliva.

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, USA). The DENV-2 strain QML16 was isolated from a dengue fever patient in Australia and 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 to a titer around of 10^8 CCID$_{50}$/ml and the cell culture supernatant was stored at -80 °C for further use.

Ae. aegypti colonies were established from collections in Townsville and Innisfail, north Queensland (QLD), Australia, and maintained within the Australian Defence Force Malaria and Infectious Disease Institute and QIMR Berghofer Medical Research Institute insectaries, respectively. Larvae were reared at a density of 200 larvae in 3 L of reverse osmosis water in plastic trays (48 × 40 × 7 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% sugar solution on cotton wool pledgets which was withheld two day prior to virus feeding. Prior to feeding, mosquitoes (4 day-old) were deprived of sucrose solution for 24 h.

Membrane feeding

Approximately one hundred 3–5 day old mosquitoes were placed into 750 ml containers with gauze covering the opening. DENV-2 strains QML16 and QML22 strains were mixed with defibrinated sheep blood to a titre of 10^8 $CCID_{50}$/ml in C6/36 cells. 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 the unfed 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) set at 28 °C, 75% relative humidity and 12:12 h day:night ligiht schedule with 30 min dawn:dusk periods.

In vitro transmission assays

At 7, 10 and 14 dpi, female mosquitoes were anesthetized using CO_2 and ice; legs and wings were removed. In vitro transmission assays were performed as previously described [22, 23]. For each mosquito, the proboscis was placed in a capillary tube containing 20 µl of a 1:1 solution of 50% sucrose and FBS. After 30 min, the contents were expelled into 0.25 ml MD (MD, 2% FBS in RPMI 1640, 50 µg/ml penicillin/streptomycin, 50 µg/ml gentamycin, 2.5 µg/ml Amphotericin B and 10 mM HEPES).

Virus titre determination

Legs and wings samples and body samples from individual mosquitoes were placed into 2 ml screw cap vial with 1 ml MD with 4–5 zirconium silica beads. The samples were homogenized by shaking tubes for 1 min 30 s in a chilled block using a MiniBeadbeater-96 sample homogenizer (Biospec Products, Bartlesville, OK, USA) followed by centrifugation (twice at 17,000 × g, 10 min, 4 °C)

Virus stocks and virus in mosquito samples were titrated using a Cell Culture Enzyme-linked Immunosorbant Assay (CCELISA) modified on the method of Broom et al [24]. Briefly, virus stocks and samples were ten-fold serially diluted and inoculated onto a monolayer of C6/36 cells grown in RPMI 1640 supplemented with L-glutamine, 5% heat inactivated FBS, 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-dengue monoclonal antibody hybridoma supernatants (4G2[25], 6B-6C1:3H5[26], at a ratio of 1:1:1) as the primary antisera, Horseradish peroxidase (HRP-) conjugated goat anti-mouse polyclonal antibody (DAKO, Carpinteria, CA, USA) (1:2000 in PBS-Tween) as the secondary antisera and 3,3',5,5'-Tetramethylbenzidine (TMB) Liquid Substrate System for Membranes (Sigma-Aldrich, St. Louis, MO, USA) as the substrate. Wells with cell monolayers that stained blue were scored as positive for infection. The $CCID_{50}$ was determined from titration endpoints as described everywhere [27] and expressed as the $CCID_{50}$/ml in C6/36 cells.

Infection rate was defined as the proportion of mosquitoes with DENV positive bodies/total number of engorged mosquitoes. Dissemination and transmission rates were defined as the proportions of infected

mosquitoes with positive legs/wings and salivary secretions/ the total number of engorged mosquitoes. We compared the virus titers and proportions using Mann-Whitney U-test and Chi square test.

Mosquito immunohistochemistry

Histological analysis of DENV infection within mosquitoes using indirect immunofluorescence assay (IFA), microscopy was performed based on the methods of described in our previous publication [22]. Briefly, mosquitoes with legs and wings removed were fixed in 4% paraformaldehyde/0.5% Triton X for 12 hr, dehydrated in xylol then a graded ethanol series, embedded in paraffin and 3−4 μM sections affixed to slides using standard histological procedures. 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 in PBS + 0.025% Tween 20 (PBST) twice for one minute each wash. Non-specific antibody binding was inhibited by incubating the sections in Biocare Medical Background Sniper + 2% BSA for 30 min. Excess Sniper/BSA was removed from the sections and the sections were incubated with undiluted 4G2 hybridoma supernatant for 2 hr at room temp Sections were washed three times with PBST. Alexa Fluor donkey anti-mouse AF488 diluted 1:300 in PBS was applied for 30 minutes. Sections were washed three times with PBST before being counterstained with DAPI for 10 min, washed several times with PBS before being mounted.

Results

Fewer mosquitoes of Townsville Ae. Aegypti colony became infected when fed QML22 (38.7%, n = 75) virus containing blood meals compared to that of QML16 containing blood meal fed (75%, n = 75) (Fig. 1a and b. Chi square test, p < 0.0001), however body infection rates remained stable between 7 and 14 DPI time points for both virus strain.

Despite the lower body infection rates among mosquitoes fed on the QML22 strain, body titres of infected mosquitoes were not significantly different to mosquitoes infected with the QML16 strain (Fig. 1b) (Mann Whitney, p > 0.05).

DENV-2 QML22 virus was first detected from mosquito legs and wings at 10 DPI, in contrast to the QML16 strain which infected legs and wings by as early as 7 DPI. Furthermore, QML22 disseminated to legs and wings in fewer mosquitoes that QML16 and virus grew to lower titres than the QML16 strain at 7 and 10 DPI (Fig. 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, Fig. 1b).

No live virus was detected in saliva expectorates of mosquitoes fed on blood meals containing the QML22 strain at 7, 10 or 14 days post feeding. This was in contrast to mosquitoes fed on blood meals containing DENV2 QML16, which yielded 16% (4/25) and 28% (7/25) virus positive saliva samples at day 10 and 14 DPI, respectively. The virus titres of these samples reached a maximum of 1250 CCID50/mosquito.

We repeated this procedure on a second line of Ae. aegypti established from mosquitoes collected 250 km away (Innisfail) several years after the Townsville line were established. Mosquitoes were fed on similar concentrations of QML16 and QML22 to those the Townsville strain. The infection rate for the QML22 virus within Innisfail Ae. aegypti was lower (16%, n = 25 at 14 DPI) than that of the Townsville strain, while the infection rates of QML16 in both Ae. aegypti lines were similar (72%, n = 25 for Innisfail and 75%, n = 75 for Townsville, p < 0.001, Fig. 1a&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, Fig. 1b). This suggests that the Innisfail Ae. aegypti mosquitoes were less susceptible to infection with QML22 than those from Townsville. Low numbers of infected mosquitoes from Innisfail prevented statistical analysis.

Histological analysis of mosquitoes infected with dengue QML16 and QML22 supported the above results. Paraffin embedded sections were stained by indirect immunofluorescence employing anti-DENV envelope protein monoclonal antibody (4G2) and Alexa-fluor488-labelled anti-mouse immunoglobulin (green) [22] (Fig. 2). Disseminated virus infection was observed in 88% (n = 25) mosquitoes ≥ 10 d post feeding on dengue QML16 and infection was observed in 50% of salivary glands (n = 6). In contrast, no virus dissemination had occurred beyond the midgut in any mosquitoes examined histologically at ≥ 10 d post feeding DENV2 QML22 (n = 13).

Discussion

The importation of a pathogenic, transmissible and highly divergent DENV-2 genotype into Australia with a human population largely susceptible to DENV outbreaks, plus the endemic of the primary Dengue transmission vector Aedes aegypti in north Queensland[28], could have significant public health implications. Here we demonstrated that strains of Ae. aegypti from two population centres in north Queensland, Australia; Townsville and Innisfail, are susceptible and able to transmit a contemporary epidemic DENV-2 strain but they are much less susceptible to a highly divergent and sylvatic strain, DENV-2 QML22, and are potentially unable to transmit it.

Variable vector competence of Aedes aegypti populations from around the world for sylvatic DENV has been reported. A sylvatic strain of DENV-2 isolated from a mosquito in Senegal in 1965 was shown to infect 50–91% of Ae. aegypti among eight different Senegalese Ae. 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]. Our infection rates more closely resemble those achieved when Ae. aegypti collected from Galveston, United States and from Bolivia became infected when fed on blood meals containing 10^8 to $10^{9.5}$ $TCID_{50}$/ml of strains of sylvatic DENV-2 isolated from a mosquito in Burkina Faso, West Africa, and a sentinel monkey in Malaysia [29]. Variable vector susceptibility (0–26% infection rate) was also observed in another study testing the vector competence of six Senegalese Ae. aegypti populations after feeding a 10^{6-7} PFU/ml virus of a sylvatic strain DENV-2 isolated from Senegal in 1999[12]. Noticeably, all these studies used dissemination to distal body tissues to determine the virus

transmission potential based on an assumption that these mosquitos were capable of transmitting DENV if the virus had disseminated from midgut into the hemocoel [15, 16]. In our experiment, we observed the dissemination of the QML22 strain into legs and wings but could not recover live virus from saliva at any time points. Our data also suggests that the vector competence of Australian Ae. aegypti mosquitoes for this sylvatic strain varies depending on the geographical origin of mosquito populations, even though they showed similar susceptibility for a contemporary epidemic DENV-2 QML16 strain. While it is possible that strains of Ae. aegypti from localities outside Australia may not have as stringent barriers to the dissemination and transmission of this highly divergent strain of DENV-2, it is unlikely that the barriers would be completely absent. Investigations to determine the mechanism underpinning the resistance of Ae. aegypti to infection with this sylvatic strain of DENV-2 are likely to be complex, given the enormous differences between the nucleotide and amino acid sequences of it and other strains of DENV-2 for which Ae. aegypti is known to be able to be a competent vector [5].

In order to transmit the virus to an uninfected human, DENV must counteract the mosquito innate immune system to replicate and disseminate through the mosquito before infecting saliva[30]. Several physiological 'barriers' to this dissemination have been hypothesised, including midgut infection and escape barriers (MIB and MEB) and salivary gland infection and escape bariers (SGIB and SGEB). Earlier studies have indicated that the MIB is a major determinant of VC for Dengue virus [31, 32]. The lower body infection rate of QML22 (Fig. 1a) suggests 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 potentially less compatible to that of the QML16 strain. When the MIB was overcome, the QML22 strain reached body similar virus titers to the QML16 strain (Fig. 1b). However, lower dissemination rates and virus titers in legs and wings indicate that replication way be attenuated in comparison to the QML16 strain. Although the QML22 strain reached the same titre in legs and wings as QML16 at 14DPI (Fig. 1b), no live virus was detected in mosquito saliva by both CCELISA and immune-histological methods. This may indicate the presence of SGIB/SGEB preventing transmission. The relative importance of attenuated virus replication or physiological infection barriers remains to be determined.

The marked differences between VC of this highly divergent/primitive strain of DENV 2 (QML22) and a mainstream strain (QML16) suggest further studies with Ae. albopictus and arboreal strains of Ae. are also warranted, if colonies can be established, to determine whether other strains of Ae. aegypti also are poor vectors of QML22 or whether there is a gradient of competencies from arboreal to urban mosquitoes.

Conclusions

We demonstrated that both Ae. aegypti species from Townsville and Innisfail North Queensland are highly susceptible and able to transmit a contemporary epidemic DENV-2 strain but are much less susceptible to a highly divergent and sylvatic DENV-2 QML22 and potentially unable to transmit it. Our findings support a conclusion that sylvatic DENV is unlikely to enter urban human –mosquito-human transmission cycle to cause secondary human infection in Australia[33].

Abbreviations

CCELISA: Cell Culture Enzyme-linked Immunosorbant Assay; VC: vector competence (VC); DENV: Dengue virus; ATCC: American Type Culture Collection; QLD: Queensland; HRP: Horseradish peroxidase; CCID50: 50% cell culture infectious dose; IFA: indirect immunofluorescence assay; PBST: PBS+0.025% Tween 20; DPI: Days post infection; FCS: Foetal bovine serum. 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
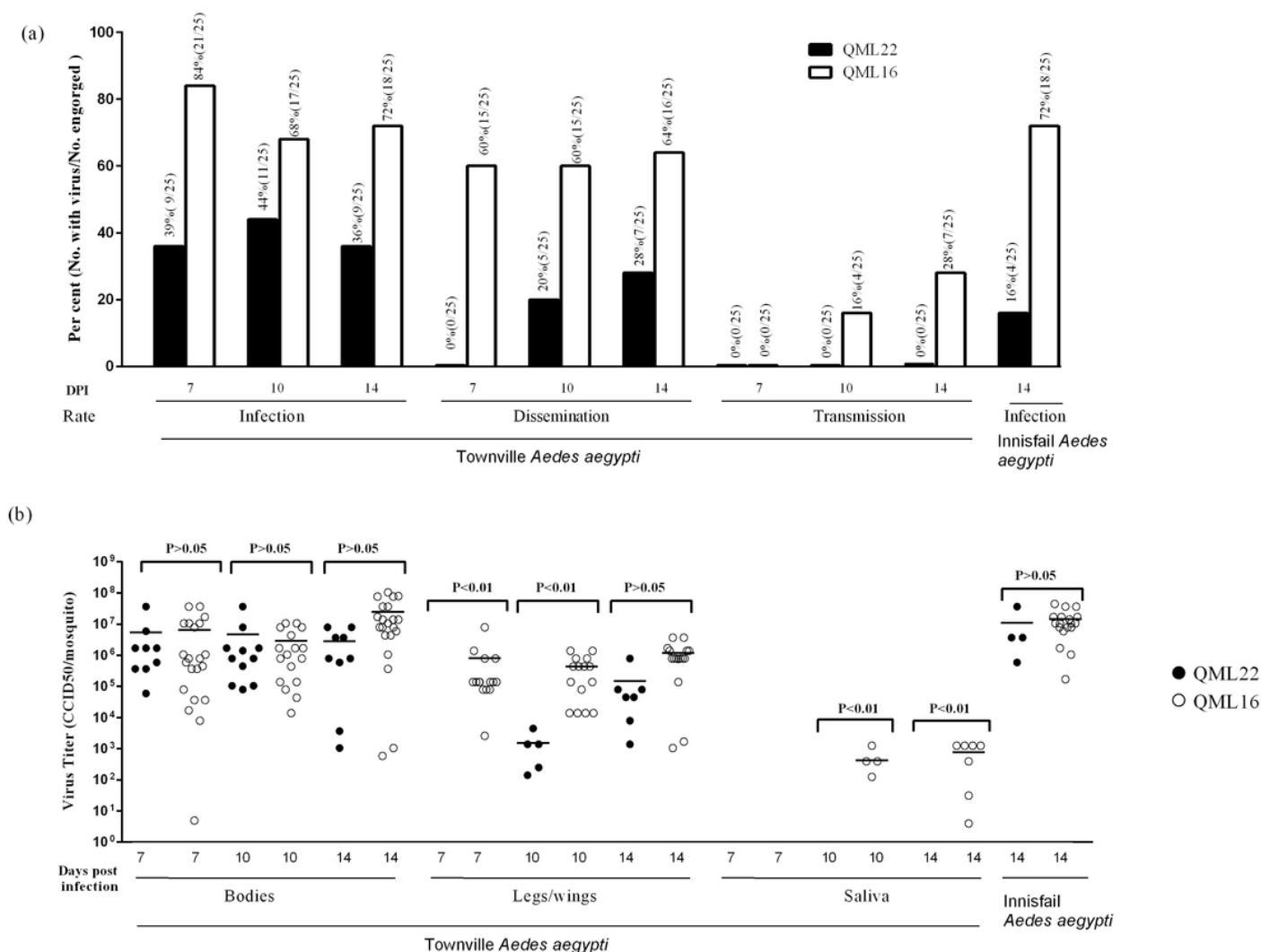
Figure 1

Infection rate, dissemination and transmission potential of DENV-2 QML16 (open symbols) and DENV-2 QML22 (closed symbols) in Ae. 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.
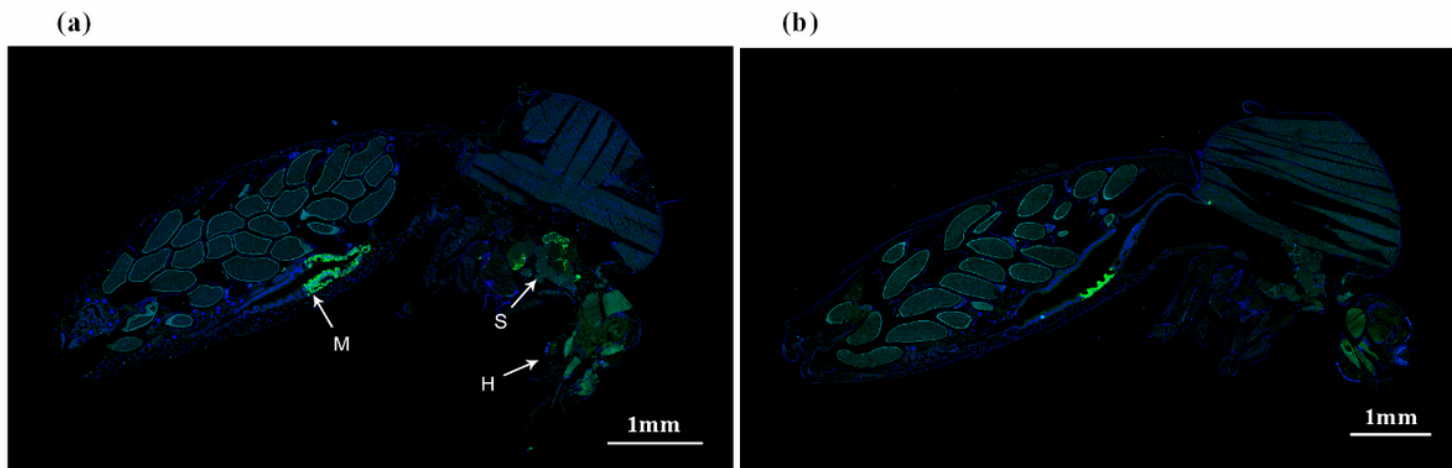
Figure 2

Midsagittal sections showing tissue distribution of dengue virus QML16 and QML22 strain infection within orally inoculated Aedes aegypti. Mosquitoes were examined for the distribution of DENV infection by immunofluorescence 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 showing staining in head (H), midgut (M); and salivary glands (S). B. Example of whole body staining of mosquitoes infected with DENV QML22 showing infection limited to the midgut. No staining was observed beyond midguts for mosquitoes inoculated with QML22. Scale bars = 1 mm.