

Refractoriness of Indian *Aedes aegypti* to Oral Infection with Yellow Fever Virus 17D Strain

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ABSTRACT

Yellow fever virus (YFV) is the causative agent of yellow fever. It is one of the most important hemorrhagic arboviral infection of global public health significance. It is categorised under category 'C' of potential bioterrorism agent. Effect of geographical variation on vector competence in *Ae. aegypti* has been well documented for several viruses including YFV. In the present study, the vector competence of *Ae. aegypti* mosquitoes collected from Gwalior, India for YFV 17D vaccine strain was evaluated to understand the risk of its transmission. Further the risk associated with transmission of YFV 17D vaccine strain from viremic vaccinees to mosquitoes and subsequently to naive individuals was assessed. *Ae. aegypti* were orally infected with high titer of YFV 17D strain and the infection status was investigated at 7 and 14 day post infection (dpi) using a highly sensitive quantitative RT-PCR assay. None of the *Ae. aegypti* mosquito orally infected with YFV 17D strain was found to be positive for YFV. The infection rate was found to be zero per cent at both 7 dpi and 14 dpi. These results demonstrated the inability of the YFV 17D strain to cause infection or replication in the midgut of *Ae. aegypti*. Due to the highly attenuated replication of this strain in *Ae. aegypti* midgut, there is a minimal risk of its transmission. Further, it is unlikely for a mosquito that feeds on a viremic vaccinee to get infected with this vaccine strain. The risk of transmission of YFV 17D strain by Indian *Ae. aegypti* mosquitoes is negligible. Further vector competence study using epidemic strain of YFV will aid in risk assessment analysis of YFV in India.

Keywords: Yellow fever 17D vaccine strain, *Aedes aegypti*, vector competence, India

1. INTRODUCTION

Yellow fever is an important hemorrhagic arboviral infection reported from many parts of Africa and central South America. It is categorised under category 'C' of potential bioterrorism agent under NIAID list¹. It is also included under the export control list of Australia Group². This is a zoonotic disease, transmitted by both sylvatic and urban cycle. In sylvatic cycle, the transmission occurs between *Aedes* mosquitoes and monkey in the forest, as monkeys are the main reservoirs. In urban areas, *Ae. aegypti* mosquitoes transmit the virus to humans^{3,4}. An estimated 200,000 cases of yellow fever occurs each year worldwide, with the case-fatality rate of ~15 per cent⁵. From 1700 to early 1900, YF outbreaks were recorded, however close study of this disease was done during Spanish-American War and construction of the Panama Canal in late 19th century⁶. YFV is the prototype virus of genus *Flavivirus* in family *Flaviviridae*. It is a small, enveloped virus that contains a single positive strand RNA as its genome, which is ~11 kb in size. It encodes a single ORF that consists of three structural and seven non structural proteins in the following order: 5' -C-prM(M)-E-NS1-NS2A-NS2B-NS3-NS4A-NS4B-NS5-3'⁷.

Most of the YF infections are asymptomatic, but in some cases, there is fever, muscle pain, headache and vomiting are reported. However severe illness involves jaundice, abdominal pain and haemorrhagic manifestations, which may lead to death after 10-14 days of the onset of illness⁸. There is no specific anti-viral therapy against YFV except the YF 17D vaccine. This is a live attenuated formulation, which is safe and affordable and has been in practice since 1940s. During the initial passages, this strain was neurotropic, which lost its pathogenicity at 114th passage for both mice and monkeys⁶. The vaccine provides immunity within a week to 95 per cent of vaccinees. Long lasting protection (>30 yrs) against YFV can be obtained through a single dose of this vaccine with rare side effects⁵. According to CDC, YF vaccination is not recommended in areas, involving low potential for YFV. It should be considered for travellers that are travelling to YFV endemic areas, or when travelling to areas that are heavily exposed with mosquitoes⁹. Government of India recommends the vaccination for Indians travelling to the endemic countries of Africa and South America. The vaccine virus replicate slowly in humans, leading to a risk of transmission from vaccinees to mosquitoes, which can further replicate within the mosquitoes and can be potentially transmitted to naïve individuals. Therefore, there is a need to evaluate the replication and transmission efficiency of the Indian *Ae. aegypti* mosquitoes to transmit YF 17D vaccine. In

view of this, Indian *Ae. aegypti* were orally infected with YF 17D vaccine virus and their potential to allow replication and transmission of YFV was assessed.

2. MATERIALS AND METHODS

2.1 Adaptation and Propagation of YFV 17D

Vaccine Strain

Lyophilised Yellow fever virus vaccine 17 D-204 (STAMARIL[®], Sanofi Pasteur, France) was reconstituted in 0.5 ml of eagles minimum essential medium (EMEM) (Sigma, USA), aliquoted and stored in -80 °C until use. C6/36 cells (clone of *Ae. albopictus* midgut epithelial cells) were obtained from NCCS, Pune was maintained at Virology Division, Defence Research & Development Establishment (DRDE), Gwalior, India. C6/36 cells were seeded in 75 cm² flask to 75–80 per cent confluency. Cells were washed with PBS and infected with 100 µl volume of reconstituted vaccine virus. The virus was allowed to be adsorbed for 2 hrs at 37 °C with intermittent shaking in an incubator without CO₂. Following adsorption, cells were washed with PBS. Fresh EMEM medium containing 2 per cent FBS was added and cells were incubated at 32 °C for 5 days up to the appearance of CPE. Cell control was kept alongside. After visualisation of CPE, cells were freeze-thawed and supernatant was harvested, filtered, aliquoted and stored at -80 °C for further use. The process was repeated twice and the virus at passage level 3 (P3) was used for further study.

2.2 Titration of YFV

Virus was harvested on 5 dpi and titrated through plaque assay in Vero cells (African green monkey kidney cells). For this, Vero cells were seeded in 24 well plate (Greiner Bio-one, Germany). After 24 hrs, medium was gently aspirated and cells were washed with PBS. Serial 10-fold dilutions of virus were prepared in EMEM and then Vero cells were infected with particular virus dilution in triplicate. After 2 hrs of virus adsorption, the medium was aspirated and replaced with overlay medium containing EMEM, 1.25 per cent methylcellulose, 2 per cent FBS and incubated for 5 days. The medium was removed and cells were fixed with chilled methanol. Subsequently, 0.25 per cent crystal violet was added to stain the cell monolayer. Plate was rinsed and dried at room temperature, and plaques were counted. Number of plaque per well was calculated and virus titer in terms of plaque-forming units (PFU/ml) was determined.

2.3 Mosquitoes

Ae. aegypti used in this study were collected from Gwalior district in 2010 and maintained as a colony in Vector Management Division, DRDE. The mosquitoes were kept at a temperature of 28 ± 2 °C with 70–80 per cent relative humidity and 14:10 light:dark photo period. As energy source, 10 per cent sucrose solution (soaked in cotton pads) was provided to adult mosquitoes and larvae were allowed to feed on yeast tablets.

2.4 Oral Infection of YFV 17D Vaccine Strain in

Ae. Aegypti Mosquitoes

Female *Ae. aegypti* (4–5 days old) were used for oral

infection experiments. Prior to infectious blood meal, mosquitoes were starved for 24 hours, to enhance blood feeding. The infectious blood meal was prepared by adding 1 ml of YFV 17D vaccine in 2 ml of washed rabbit RBC's. ATP was added in the blood meal to a final concentration of 5 mM. Mosquitoes were orally infected through Hemotek feeding membrane using circulating water (maintained at 37 °C) as described previously¹⁰ (Fig. 1). After 45 min of feeding, two mosquitoes were collected for titration of imbibed virus. Sixteen *Ae. aegypti* from two independent experiments were processed on 7 and 14 dpi to determine infection rates as described previously¹⁰.

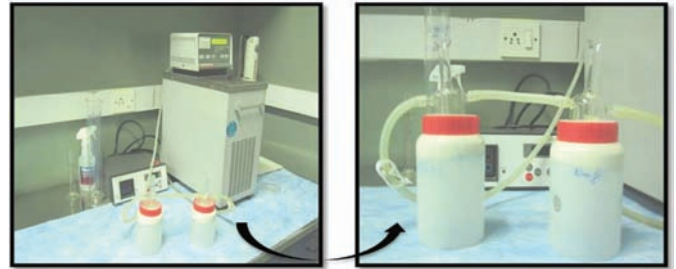


Figure 1. (a) Oral infection set up and (b) Closed view.

2.5 Processing of Individual Mosquito and Viral RNA Extraction

Individual *Ae. aegypti* mosquito was homogenised in 2 ml tubes by adding 300 µl of EMEM (Sigma, USA) and 2 mm stainless steel beads using a Tissue Lyser LT (Qiagen, Germany). The mosquito homogenate was then clarified by centrifugation at 6000 x g for 10 min. 140 µl of supernatant was used for extraction of RNA using QIAamp viral RNA mini kit (Qiagen, Germany). The RNA was finally eluted in 50 µl elution buffer. RNA from mosquito samples was subjected to YFV specific quantitative RT-PCR.

Oral infection and mosquito processing experiments were performed in BSL-3 laboratory.

2.6 Quantitative RT-PCR

The primers for quantitative RT-PCR (qRT-PCR) assay were selected from a previously published report that targets the junction region of 5' NTR and capsid gene¹¹. The sequence of forward primer is YFS: AATCGAGTTGCTAGGCAATA AACAC (genomic position: 29–53) and sequence of reverse primer is YFAS: TCCCTGAGCTTTACGACCAGA (genomic position: 141–121). For detection and quantitation of YFV titer in mosquitoes, qRT-PCR was carried out using SS III Platinum one step qRT-PCR kit (Invitrogen, USA) in Mx3005P system (Stratagene, USA). The reaction was carried out in a 25 µl volume. It comprised of 2X Master mix-12.5 µl, 0.25 µmol each of forward and reverse primers-0.5 µl (YFS and YFAS), enzyme mix (Taq DNA polymerase and reverse transcriptase)-0.25 µl, nuclease free water-9.87 µl and RNA-2.5 µl. The thermal profile consisted of reverse transcription-30 min at 50 °C, polymerase activation-10 min at 95 °C, followed by 40 cycles of PCR at 95 °C-30 s, 54 °C-60 s, and 72 °C-30 s. After amplification, a melting curve analysis was performed with the

melting curve analysis software of Mx3005P according to the manufacturer's instructions. The RNA copies from Ct values were extrapolated using a standard curve reported by Dash¹², *et al.*

2.7 Statistics

Sixteen mosquitoes from two independent experiments were processed on 7 dpi and 14 dpi to determine infection rates. Data is represented as Mean \pm Standard deviation (Microsoft Excel 2010).

3. RESULTS

3.1 Propagation and Titration of YFV

After serial passage of YFV 17D vaccine in C6/36 cells, YFV was produced. On 5 dpi, minor cytopathic effects like granulation, cell clumping were observed. Viral titer was found to be 5×10^4 PFU/ml, 8×10^5 PFU/ml, and 4×10^6 PFU/ml at P1, P2, and P3 respectively as calculated through plaque assay. Cytopathic effect and virus titration of P3 are shown in Fig. 2(a) and 2(b), respectively. The virus at P3 was used for mosquito oral infection.

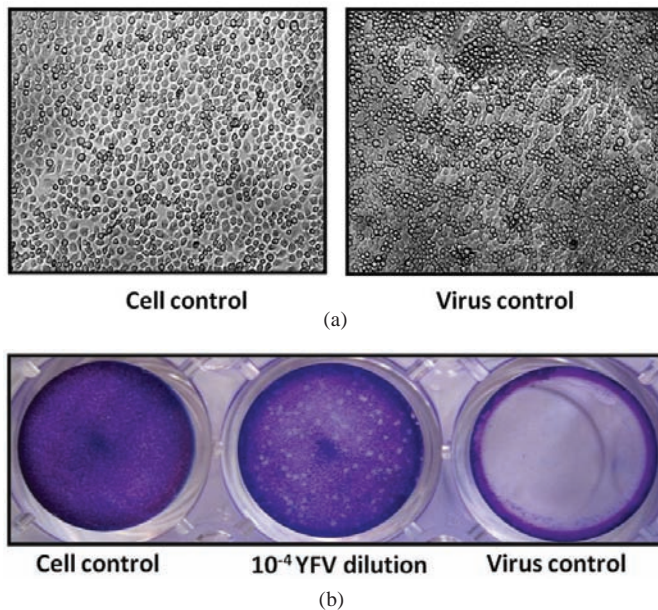


Figure 2. (a) Propagation of YFV 17D in C6/36 cells. Cytopathic effect in C6/36 cells at 5 dpi and (b) Titration of YFV 17D by plaque assay in Vero cells.

3.2 Infection Rates in *Ae. aegypti*

For oral infection experiment, 100 mosquitoes were used, out of which 65 mosquitoes were engorged with blood meal. From these 65 mosquitoes, 32 mosquitoes (stage 3+)¹³ were selected for infectivity analysis at 7 dpi and 14 dpi. Viral RNA titer in blood meal was found to be 4×10^6 RNA copies/ml. Viral RNA titer in '0' day mosquito was found to be 4×10^4 RNA copies/ml in 7 days and 14 days post infected mosquitoes. The infection rate of YFV in these mosquitoes was monitored at 7 dpi and 14 dpi, in which viral RNA copies were not detected in any of the *Ae. aegypti* mosquito, orally infected with YFV vaccine strain, as determined through quantitative RT-PCR assay. Thus the infection rate was found to be 0 per cent at both 7 dpi and 14 dpi (Fig. 3).

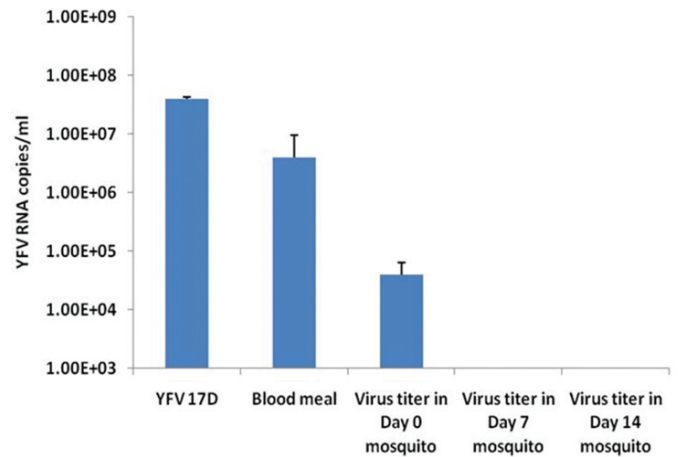


Figure 3. YFV titer in YFV 17D, blood meal, 0 day mosquito and 7, 14 dpi infected mosquitoes are indicated.

4. DISCUSSION

Yellow fever virus is the causal agent of yellow fever disease. It is endemic to tropical regions of Africa and central South America, affecting humans and non-human primates. The expansion in the geographical habitats of *Ae. aegypti*, the major vector of YFV, has led to severe epidemics of fatal hemorrhagic disease. Since 1950s, large outbreaks of YFV occurred in South America involving Peru, Bolivia, Brazil, Colombia, Ecuador. Despite mass vaccination campaigns to prevent these outbreaks, major YFV epidemics continued to occur¹⁴. Resurgence of YFV in South America has been attributed to low or incomplete vaccine coverage in endemic areas, urbanisation and human migration to forests¹⁵.

Absence of YFV in Asia is an enigma to scientific fraternity. Several hypothesis including cross-immunity generated in response to other flaviviruses (Dengue and Japanese encephalitis virus) or due to low vector competence of Asian *Ae. aegypti* mosquitoes for YFV have been advocated. However, none of these have been proven scientifically. Increase in international travel and globalisation enhances the risk of YFV spread to South and South-East Asian countries with tropical climate. Rapid spread of the virus to areas with high vector density and naive population like India and other South-East Asian countries could potentially occur¹⁶. The recent introduction and successful establishment of West Nile virus, Chikungunya virus and Zika virus in naive territories reflects the real threat of arboviral infections.

Several studies have been performed to analyse the risk associated with the transmission of the vaccine strains from viremic vaccinees to mosquitoes and then from mosquitoes to naive individuals. Only 10 per cent oral infection rates were observed in *Ae. aegypti* from Puerto Rico, while 100 per cent infection rates were obtained upon intra-thoracic (IT) inoculation¹⁷. However, IT route does not demonstrate natural infection process as the midgut barriers are bypassed, so this does not reflect the actual scenario.

Besides this, the assessment of vector competence from different geographical strains of mosquitoes for different viral strains is important to estimate the risks associated with potential outbreaks. Vector competence is the susceptibility of a

vector to oral infection and transmission of a pathogen. Several previous reports have demonstrated the vector competence of *Ae. aegypti* from different geographical locations for YFV. Australian population of *Ae. aegypti* was found to be susceptible for African strain of YFV as >70 per cent infectivity and >50 per cent transmission was achieved. However, the infection and transmission rates were lower for South American strains of YFV¹⁸. Also, *Ae. aegypti* population from Santa Cruz and Bolivia showed infectivity and transmissibility for South American YFV strain¹⁹. Other vectors like *Ae. simpsoni*, *Ae. furcifer* are also susceptible to the African YFV suggesting the sylvatic mode of transmission between monkeys²⁰. Other mechanisms of arboviral transmission in nature is vertical transmission i.e. the transmission of virus from infected female mosquito to progenies. This is considered as an alternative survival mechanism for virus in nature. Vertical transmission of YFV by *Ae. aegypti* was demonstrated naturally from field collected male and female mosquitoes²¹. Experimental evidence was provided with *Ae. aegypti* colonies from Dakar and Koungheul that transmitted YFV to male and female progenies with increase in infection rates at later oviposition cycles²².

In the present study, we evaluated the vector competence of YFV 17D vaccine strain for Indian *Ae. aegypti* mosquitoes. Vector competence is greatly affected by oral infectious dose (OID) provided to mosquitoes. It has been observed that, upon increasing viral dose, infectivity increased in mosquitoes²³. Therefore a high dose is provided in these experiments to maximise the chances of infection. Detection and quantification of YFV in YFV 17D vaccine virus was performed using a highly sensitive quantitative RT-PCR assay. The YFV in infected mosquitoes was titrated as RNA copies rather than the infectious virus because of the high sensitivity of real time RT-PCR compared to the conventional plaque assay. The sensitivity of YFV specific quantitative RT-PCR assay employed in this study was found to be high as it has a detection limit of 30 RNA copies/reaction¹². The infection rate of YFV in *Ae. aegypti* mosquitoes was monitored at 7 and 14 dpi. It was observed that none of the *Ae. aegypti* mosquito orally infected with YFV 17D strain was found to be positive for YFV. The infection rate was found to be 0 per cent at both 7 and 14 dpi. Our results are in agreement with a previous report, where infection rate was found to be 0 per cent in orally infected *Ae. aegypti* from Thailand²⁴. These results demonstrated the inability of the YFV 17D strain to cause infection or replication in the midgut epithelial cells of Indian *Ae. aegypti*, representing the negligible risk of YFV spread in India. The inability of 17D strain to establish infection in midgut led to failure in dissemination and further transmission of the YFV. Several factors including genetics of both virus and vector along with midgut microflora might have attributed to this refractoriness²⁵. Further investigations using virulent/epidemic strain of YFV is required to evaluate vector competence in Indian *Ae. aegypti* mosquitoes from different geographical locations.

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In the current study, he conceived, supervised, analysed the data, finalised the results.