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Evaluation of the pathogenicity of culex flavivirus and quang binh virus in mice

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Abstract

As knowledge of Insect-Specific Viruses (ISVs) remains limited, this study aimed to investigate whether Culex Flavivirus (CxFV) and Quang Binh virus (QBV) which were isolated from mosquitoes collected in an Giang province, affect the health of mice following infection. Six- to eight-week-old BALB/c mice were infected with QBV or CxFV. For each virus, mice were divided into three groups (n=5) receiving different inoculation doses (0.2 mL, 0.15 mL, or 0.1 mL per mouse), along with a control group administered DMEM. Each treatment was replicated three times, and animals were observed for 28 days. Body weight was recorded before inoculation and weekly thereafter (days 7, 14, and 28 post inoculation). One mouse from each group was necropsied weekly to assess gross pathology. Throughout the experiment, all infected mice maintained normal feeding and activity, with no clinical symptoms such as lethargy, anorexia, or coat deterioration. Weight data showed a steady increase in both infected and control groups, with the most rapid gain in the first week and gradual increases until day 28. Necropsy revealed no gross abnormalities in the brain or internal organs of infected mice compared with controls. Significance, and The absence of clinical signs and pathological lesions indicates that QBV and CxFV did not affect mammalian health under the tested conditions. These findings support the classification of ISVs as non-pathogenic to mammals.

Keywords: Culex flavivirus, Quang binh virus, BALB/c mice, Viral pathogenesis

Introduction

The *Flavivirus* genus within the family *Flaviviridae* are positive sense single-stranded RNA viruses primarily transmitted between blood-sucking arthropods, which are commonly found in vector mosquitoes, and vertebrates and their natural cycle of transmission depends on replication in both of these (Carvalho and Long, 2021; Tang *et al*., 2023). Members of the genus *Flavivirus* are comprises pathogenic vertebrate-infecting flaviviruses and insect-specific flaviviruses. This genus is also split into four groups as (i) mosquito-borne flaviviruses, (ii) tick-borne flaviviruses, (iii) Insect-Specific Flaviviruses (ISFs), and (iv) no known vector flaviviruses (Colmant *et al*., 2021; Daidoji *et al.,* 2021). Some of them are responsible for several important mosquito-borne diseases of humans and animals, they can caused human diseases which diverse clinical manifestations, ranging from mild or asymptomatic fever to hemorrhagic diseases or encephalitis (Apte-Sengupta, 2014; Carvalho and Long, 2021). These viruses include Dengue, Japanese encephalitis, Murray Valley encephalitis, yellow fever, West Nile fever, Zika, Chikungunya diseases (Apte-Sengupta, 2014; Rudolph *et al.,* 2014). In animals, mosquito-borne flaviviruses as West Nile fever virus, Isutu virus, Japanese encephalitis virus, Turkey meningoencephalomyelitis virus, Wesselsbron virus and Tembusu virus can cause neurologic deficits or disorders, pituitary encephalitis, myocarditis or valvular degeneration, subcutaneous hemorrhage, fetal rigidity, congenital malformations or reduced strength or sometimes asymptomatic (Hammami and Hassine, 2020; Byas and Ebel, 2020; Agliani *et al.,* 2023).

In recent year, various insect-specific flaviviruses, which exclusively infect mosquitoes, have been on the rise (Öhlund *et al.,* 2019; Calzolari *et al.,* 2016). Since 1991, the presence of ISFs has been documented worldwide (Tang *et al.,* 2023). These ISFs identified as infecting mosquito hosts as *Culex, Aedes, Anopheles* and *Mansonia* species. They include Quang Binh Virus (QBV), Calbertado Virus (CLBOV)

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and Culex Flavivirus (CxFV), Nakiwogo Virus (NAKV), Nounane Virus (NOUV), Lammi virus (LAMV), Chaoyang Virus (CHAOV), Barkedji Virus (BJV), Nanay Virus (NANV) (Crabtree *et al.,* 2009; Cook *et al.,* 2009; Hoshino *et al.,* 2007; Bolling *et al.,* 2011; Junglen *et al.,* 2009; Huhtamo *et al.,* 2012; Lee *et al.,* 2013; Evangelista *et al.,* 2013). Of thoses, Quang Binh Virus (QBV) was initially isolated from *Culex tritaeniorhynchus* in Quang Binh province, Vietnam, in 2002 (Crabtree *et al.,* 2009). Subsequent studies identified QBV in An Giang province, Vietnam in 2024 and various provinces and cities in China (Tang *et al*., 2023; Zuo *et al.,* 2014; Tran *et al.,* 2024). This virus has also been isolated from other mosquito species such as *Anopheles sinensis*, *Aedes aegypti, Culex pipiens* and *Culex gelidus* (Tang *et al.,* 2023; Tran *et al.,* 2024). Culex flavivirus, was first isolated from *Culex pipiens* and other *Culex* mosquitoes in Japan in 2007 (Hoshino *et al.,* 2007). This virus also widely infects various mosquito species as *Culex tritaeniorhynchus*, *Culex quinquefasciatus, Anopheles sinensis* in many parts of the world (Liang et al., 2021; Jam et al., 2025). The increasing number of ISVs discovered and characterized demonstrates that this virus group is highly diverse, spread worldwide, and infects a wide range of mosquito species. However, knowledge of ISVs is still limited, and some aspects need to be clarified. Therefore, the purpose of this study was to investigate whether culex flavivirus and Quang Binh virus affect the health of mice when they are infected with these viruses.

Ethical considerations and declaration of conflicr of interest

The mouse experiment was approved and permitted by the Scientific and Academic Council - An Giang University (Decision No. 143/QĐ-ĐHAG). The mouse breeding and experimental procedures were carried out based on international guidelines and regulations on laboratory animal welfare. Mice were kept under controlled settings with proper temperature, light, food and water access. Novocain and zoletil anesthetics were used prior to any invasive procedure to reduce discomfort. Post-experiment care was strictly monitored, and humane endpoints were used to prevent unnecessary distress. The researches declare no financial, institutional, or personal conflicts of interest. The study was conducted with no other purpose but to advance scientific knowledge concerning insect-specific flaviviruses in vertebrate model systems.

Methods

Cells

Mosquito cell lines, C6/36 which is derived from *Aedes albopictus* was used for virus propagation. This cell line was cultured in Dulbecco's Modified Eagle Medium (DMEM; Gibco™, Carlsbad, CA, USA) containing 10% heat-inactivated fetal calf serum (FCS; Hyclone Laboratories, Logan, UT, USA), 100 U/mL of penicillin, and 100 µg/mL of streptomycin and was maintained at 28^0C in 5% CO_2.

Viruses and virus propagation

Two insect-specific flaviviruses including QBV and CxFV were isolated from mosquitoes in An Giang province, Vietnam. C6/36 cells were grown in petri disk using DMEM with 10% FCS, 100 U/mL of penicillin, and 100 µg/mL of streptomycin. The growth medium was decanted and washed two times with sterile Phosphate Buffer Saline (PBS). Cells were inocualated with 250µL isolated viruses with an adsorption period of one hour at 28^0C and shaking every 15 min. The inoculum was removed and the cells were washed two times with sterile PBS. Then 10mL maintenance medium with containing 2% FBS was added and incubated at 28^0C in 5% CO_2 for six days. The cell culture supernatants were harvested, clarified by centrifugation (21,500×g for 5 min at 4^0C) to remove debris, then passed through sterile 0.45-µm filters, aliquoted and stored at −80^0C. The supernatant was tested by RT-PCR to assess virus propagation.

Plaque assay

Vero cells were seeded at a density of $5x10^5$cells in two ml of DMEM containing 10% FBS per well in 6-well plates, then incubated at 37^0C with 5% CO_2 for 24 h. Virus solutions were diluted (10^{-1} to 10^{-6}) and 200 µL of each virus dilution was inoculated onto Vero cells after washing once with DMEM and incubated at 37^0C with 5% CO_2 for 60 min. The cells were washed twice with DMEM and overlaid with 0.8% agarose (Seaplaque GTG Agarose, Lonza, Rockland, ME USA) in DMEM containing 6.7% FCS.

Plates were then incubated at 37^{0}C with 5% CO_2 until plaques appeared. Cells were fixed with 10% buffered formaldehyde for one hour at room temperature, then the agarose layer was removed, and the cells were stained with crystal violet (Wako Pure Chemical Industries, Osaka, Japan). Plaques were counted and virus concentrations were calculated as plaque-forming units (PFU) per mL.

RT-PCR (Reverse transcription PCR)

Each culture supernatant from viruses propagation were extracted RNA by using a QIAamp viral RNA mini kit (QIAGEN, Inc, Valencia, CA, USA) according to the manufacturer's recommended protocol. To detect viral genes, RT-PCR was conducted using the OneStep RT-PCR kit (QIAGEN) with flavivirus primer sets (Table 1). RT-PCR were performed at 50^{0}C for 30 min (reverse transcription), 95^{0}C for 15 min, followed by 35 cycles of 94^{0}C for 30 sec, 55^{0}C for 30 sec, and 72^{0}C for 1 min (amplification), and final extension at 72^{0}C for 10 min. 2% agarose gels were used to separate PCR products by electrophoresis, and intensive bands were purified by using the MinElute gel extraction kit (QIAGEN).

Table 1. Primer pairs used in the study (Target *Flavivirus*)

Name	Polarity	Sequence	Target gene	Size (bp)	Reference
MAMD	Forward	5′AACATGATGGGRAARAGRGARAA-3′	NS5	260	Tran et al., 2024
cFD2	Reverse	5′GTGTCCCAGCCGGCGGTGTCATCAGC-3′			

Mouse experiments

A total of 120 BALB/c mice at the age of 6-8 weeks were obtained from an authorized breeding farm and adapted to steady conditions for three days before the experiment. Housing cages were purchased at commercial stores with a metal mesh top and plastic bottom. Each cage contained a running wheel, food tray, and water bottle suitable for mice. Bedding consisted of clean, dry absorbent material supplemented with activated charcoal to reduce odor accumulation, and washed daily for hygiene. Food and water were given and libitum every day. The diet consisted of a mix of fed ingredients like bread, rice, corn, rice flakes, green vegetables, root crops... Mice were fed twice a day, a bigger quantity late in the afternoon and during the night, usually complemented with other vegetables, root vegetables, and sometimes fruits. The environment where the animals were kept was at 29 ± 1^{0}C temperature, with a light-dark cycle of 14 hours of light and 10 hours of darkness.

For each virus strain, mice were randomly divided into three experimental groups of five. Each group was received different inoculation doses per mouse (as 0.2 mL, 0.15 mL, and 0.1 mL), and a control group that received no viral infection but was administered DMEM solution at the respective doses. The experiment was conducted in three replicates for each virus, each experimental group consisted of 15 biological replicates (individual mice), and the entire experiment was conducted in three independent biological repeats under identical conditions. Observations were recorded for up to 28 days' post-inoculation. The body weight of each mouse was measured before inoculation, then measured weekly on days 7, 14, and 28 after inoculation. During each period, a mouse from every treatment group was randomly removed for necropsy to evaluate any changes in pathology.

Data collection

Data that was gathered included the mortality rates were noted daily during the 28 days observation period to note any fatal results linked to viral exposure. Body weights were measured systematically at predetermined intervals days 0, 7, 14, and 28 to determine any effects on growth and overall health status. Besides quantitative observations, qualitative observations of feeding behavior, water intake, and other clinical signs like lethargy, grooming, posture, and mobility were also observed daily to identify any subtle or gross signs of illness or distress. Additionally, necropsy findings for every randomly chosen mouse at specified time points were meticulously recorded to reveal and describe any pathologic alterations in internal organs. Gross pathology was complemented by

histopathology, where achievable, to derive detailed knowledge of effects at the tissue level for the viruses. All the data generated were systematically organized and examined to establish correlation among viral dose, clinical picture, and pathology outcome.

Evaluation method and statistics

The data were stored using microsoft excel and analyzed using minitab statistical. Data were expressed as mean ± Standard Deviation (SD) or mean ± 95% Confidence Interval (CI) as appropriate. To evaluate changes in body weight over time, A One-Way Analysis of Variance (ANOVA) was performed with "day post infection (dpi)" as the main factor (day 0pi, day 7pi, day 14pi, and day 28pi). When a significant F-value was obtained, Tukey's Honest Significant Difference (HSD) post hoc test was applied to determine pairwise differences among time points. The results were reported with corresponding F-values, Degrees Of Freedom (Df), and adjusted P-values.

Results

In this study, two mosquito-specific viruses namely CxFV and QBV which were isolated in An Giang province (Tran et al., 2024) were used to infect BALB/c mice to evaluate the pathogenicity of these two viruses in vertebrates. To obtain sufficient viral quantity of these viruses for mouse infection experiments, both CxFV and QBV were propagated in C6/36 mosquito cells and the viral supernatant was harvested after six days of incubation. The supernatant harvested from each virus infected cell cultures was used as the viral inoculum for experimental infection. The CPE of the propagation process were observed every day. The results from the CPE expression at six day post infection were showed in the Figure 1. The CPE expression of these two viruses was weak, and they could not form plaques on cells. Therefore, viral replication was not carried out by plaque assay, it was validated by reverse transcription polymerase chain reaction (RT-PCR) (Figure 2), positive results of RT-PCR confirmed the replication of the viruses in the infected cell cultures.

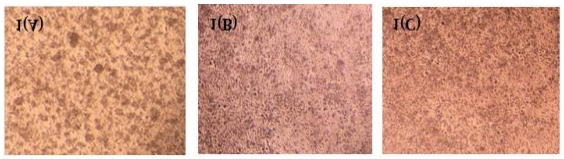

Figure 1. CPE characterized by cell growth after 6 days' propagation in C6/36 cells. Fig. 1(A) C6/36 mock infected cells, Fig. 1(B) C6/36 cells infected with Culex flavivirus, Fig. 1(C) C6/36 cells infected with Quang Binh virus

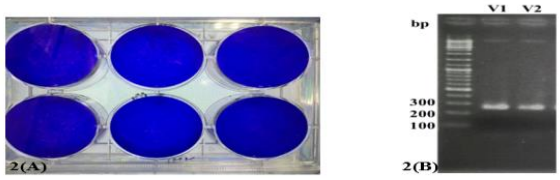

Figure 2. Negative by plaque assay for virus quantification (2A), positive results of RT-PCR confirmed the replication on C6/36 cells (2B). V1: Culex flavivirus, V2: Quang Binh virus

To evaluate the pathogenicity of these viruses in mice, a total of 120 BALB/c mice were divided into four groups, three of which were infected with varying doses as 0.2 mL, 0.15mL and 0.1mL and one for the control. The experiment were monitored over a 28-day period. Besides providing adequate feed and water for their needs and cleaning their cages, the health status of the mice was monitored daily during the experiment. Their eating, resting, physical activity and behavior were observed every day. In addition, the weight of the mice was also monitored periodically on days 0, 7, 14 and 28 after infection. Following the infection, the mice were observed daily for any abnormal conditions and mortality, while their weights were regularly monitored, and necropsies were performed at 7, 14, and 28-day post-infection. The mortality rate in mice was also recorded in Table 2. The mortality among the mice throughout the experimental duration was extremely low. The only one mortality occurred within the control group of the culex flavivirus, and there was no mortality across all the other treatment groups. Mortality within the control group resulted from competition for food during the initial acclimatization period. In particular, the dead mice at arrival were smaller than their same-cage counterparts and presumably were outcompeted for feeding. This disadvantage would have also predisposed them to

attack by same-cage mice.

Table 2. Mortality rate of mice over the experimental period

Virus	Dose (mL/mouse)	Number of Mice	Total Deaths	Mortality Rate (%)
Culex Flavivirus	0.2	15	0	0
	0.15	15	0	0
	0.1	15	0	0
	Control Group	15	1	6.67
Quang Binh virus	0.2	15	0	0
	0.15	15	0	0
	0.1	15	0	0
	Control Group	15	0	0

In addition, during the experiment mice maintained normal feeding behavior and physical activity, and no clinical symptoms such as lethargy, coat deterioration, anorexia or abnormalities in both control and infected mice were recorded. Statistical analysis revealed the body weight of mice in all experimental groups increase on day 7, day 14, and day 28 with the most fluctuation between 7 and 28 dpi (Figure 3). The interval plot illustrates the average body weight gain of mice over the course of the experiment, measured at 0, 7, 14, and 28 days post-infection (dpi). At baseline (0 dpi), the mean body weight was approximately 29.5 g, after which a marked increase was observed by day 7, reaching around 34 g. From day 7 to day 14, the weight continued to rise, albeit at a slower rate, averaging about 35 g, and by day 28, the mice attained the highest mean body weight of approximately 35.5–36 g. The 95% confidence intervals indicate reduced variability in weight measurements as the experiment progressed. Overall, the data demonstrate a consistent upward trend in body weight throughout the study, with the most pronounced gain occurring during the first week post-infection, followed by a gradual increase until day 28.

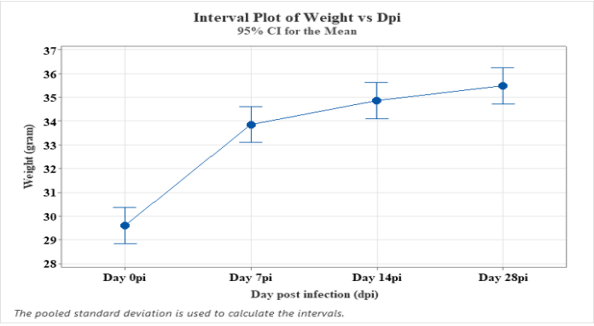

Figure 3. Average body weight gain of mice during experiment

Data represent mean ± 95% Confidence Interval (CI) of mouse body weight at different time points post infection (dpi). One-way ANOVA revealed a significant difference in body weight among the four time points ($F_{(}3,92_{)} = 48.11$, $P < 0.001$), body weight increased significantly from Day 0pi to Day 7, 14 and 28 pi ($P < 0.001$). A modest but significant increase was also observed between day 7pi and day 28pi ($P = 0.017$), while differences between day 7pi and day 14pi or between day 14pi and day 28pi were not statistically significant ($P > 0.05$). Overall, mice exhibited a gradual and significant increase in average body weight throughout the 28-day experimental period.

According to Auguste et al. (2021), vertebate flaviviruses, particularly the Japanese encephalitis virus (JEV), replicated in various sites as brain and abdominal organs, including the spleen, liver, kidney, and others. Consequently, in this research the necropsies at 7, 14, 28 day post-infection were conducted to evaluate the pathological lesions in brain and internal organs of experimental mice. The necropsy results of experimental mice from different treatment groups of both CxFV and QBV revealed no observable pathological lesions in internal organs or brain tissues (Figure 4). Necropsy of mice at 7, 14, and 28 dpi revealed no visible pathological lesions in any organs, including the brain, liver, spleen, and kidneys. Organs appeared normal in size, color, and texture compared with those of control mice. This finding indicates that neither Quang Binh virus nor culex flavivirus produced any observable manifestations of disease in the experimental mice, suggesting their non-pathogenic nature in this vertebrate model.

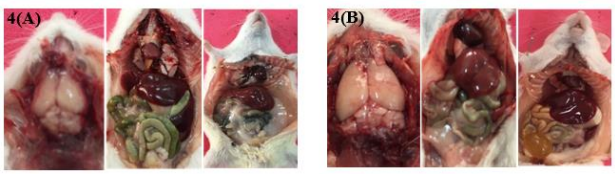

Figure 4. The necropsy results in internal organs or brain

tissues of mice. The necropsy of control mice was shown in (4A) and the necropsy of infected mice is (4B)

Discussion

On the infected cells with these two viruses, slow cell growth and syncytium formation of various sizes were observed, however they exhibited weak CPE when spreading infection into C6/36 cells. The weak of CPE expression of Quang Binh virus and Culex flavivirus may be due to the fact that in mosquito cells, these viruses are adapted to replicate without activating pathways that cause mosquito cell destruction, thus reducing the likelihood of causing visible CPE (Brackney *et al.,* 2010; Blitvich and Firth, 2015). In addition, the extent of CPE expression may vary depending on the virus strain and its transmission history, with previous transmissions of ISFs often lacking CPE, while subsequent transmissions may begin to exhibit morphological changes such as syncytia or vacuoles (Blitvich and Firth, 2015).

Regardless of the different doses administered, the mice infected with these two viruses exhibited no signs of illness or impact on their weight gain. Weight gain also differed in relation to viral inoculation dose with the least weight gain noted in mice which received the highest dose but this difference was not statistically significant. The lesser weight gain in the high-dose group is because they had lower body weight upon entry. Mice infected at different doses also showed different weight gains throughout the experimental period. Of which, mice infected with the highest dose (0.2 mL) having the lowest average weight gain, while 0.1mL infected mice exposed the highest average weight gain, however the difference between these groups was not statistically significant. These results also corroborate that neither CxFV nor QBV had any remarkable effect on the growth or health status of BALB/c mice, reaffirming their status as insect-specific flaviviruses without any vertebrate host pathogenicity. The findings of this study illustrated that the average body weight of mice in all experimental groups gradually rose from the first day of inoculation to 28 days post inoculation.

The experiment results showed CxFV and QBV did not produce any detectable effects on the health of the mice. As noted by Junglen *et al.* (2017) and Öhlund *et al.* (2019), the infectivity of Insect-Specific Flaviviruses (ISF) in vertebrates is limited at multiple stages of the cellular infection cycle, which includes adhesion, entry, RNA replication, as well as virus assembly and release. Colmant *et al.* (2021) suggested that vertebrate cells are protected from infection by vertebrate flaviviruses. This protection is due to the Zinc Antiviral Protein (ZAP) present in animal cells. By binding to CG dinucleotides in viral RNA, this protein inhibits viral replication. Furthermore, the ISF genome is rich in CG dinucleotides. Therefore, since CxFV and QBV are unable to replicate in mouse cells, they do not induce disease or clinical symptoms in mice. This elucidates why, following infection, mice remain healthy and do not succumb to illness after exposure to these two viruses. We acknowledge that histological analysis was not performed in this study due to the limited viral titers available and biosafety constraints. However, the necropsy observations, together with the complete absence of clinical symptoms and mortality, provide strong functional evidence supporting the non-pathogenic nature of both QBV and CxFV in BALB/c mice. Future studies incorporating detailed histopathology and viral load quantification in tissues will be necessary to further confirm and strengthen these conclusions. In addition, insect-specific flavivirus are known to co-circulate with pathogenic flaviviruses in mosquito populations and may influence vector competence by modulating replication or transmission of co-infecting arboviruses (Junglen *et al*., 2017; Öhlund *et al.,* 2019). Therefore, understanding the replication dynamics and host range of these insect-specific viruses may contribute to novel biological control strategies or improved arbovirus surveillance in endemic areas.

Conclusion

Our results demonstrate that CxFV and QBV are non-pathogenic in mice under the tested conditions. While no evidence of vertebrate infectivity was found, future molecular and ecological studies are necessary to fully exclude any potential for cross-species interactions. Future studies should extend the range of investigation by using mammalian hosts or non-human primates to understand the vertebrate host replication of CxFV and QBV. Moreover, an analysis of

the host immune response should be done to elucidate the interactions between insect-specific flaviviruses and vertebrate hosts.

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