Virologica Sinica 39 (2024) 228–234

Contents lists available at ScienceDirect

Virologica Sinica

journal homepage: www.keaipublishing.com/en/journals/virologica-sinica
www.virosin.org

Research Article

The infection kinetics and transmission potential of two Guaico Culex viruses in *Culex quinquefasciatus* mosquitoes

Ru-Yi Chen [a,b,1], Teng Zhao [b,1], Jing-Jing Guo [b,1], Feng Zhu [b,c,1], Na-Na Zhang [b], Xiao-Feng Li [b], Hai-Tao Liu [b], Fei Wang [a,*], Yong-Qiang Deng [b,*], Cheng-Feng Qin [b,*]

[a] College of Bioscience and Bioengineering, Jiangxi Agricultural University, Nanchang, 330045, China
[b] State Key Laboratory of Pathogen and Biosecurity, Beijing Institute of Microbiology and Epidemiology, AMMS, Beijing, 100071, China
[c] School of Life Sciences, Southwest Forestry University, Kunming, 650224, China

ARTICLE INFO

Keywords:
Guaico Culex virus (GCXV)
Jingmenvirus
Culex quinquefasciatus
Vertical transmission

ABSTRACT

Guaico Culex virus (GCXV) is a newly identified segmented Jingmenvirus from *Culex* spp. mosquitoes in Central and South America. The genome of GCXV is composed of four or five single-stranded positive RNA segments. However, the infection kinetics and transmission capability of GCXV in mosquitoes remain unknown. In this study, we used reverse genetics to rescue two GCXVs (4S and 5S) that contained four and five RNA segments, respectively, in C6/36 cells. Further *in vitro* characterization revealed that the two GCXVs exhibited comparable replication kinetics, protein expression and viral titers. Importantly, GCXV RNAs were detected in the bodies, salivary glands, midguts and ovaries of *Culex quinquefasciatus* at 4–10 days after oral infection. In addition, two GCXVs can colonize *Cx. quinquefasciatus* eggs, resulting in positive rates of 15%–35% for the second gonotrophic cycle. In conclusion, our results demonstrated that GCXVs with four or five RNA segments can be detected in *Cx. quinquefasciatus* eggs during the first and second gonotrophic cycles after oral infection.

1. Introduction

Jingmenviruses are characterized by a single-stranded positive RNA genome composed of four or five segments. Notably, two of these segments are genetically related to unsegmented flaviviruses, such as dengue virus (DENV), West Nile virus (WNV), Japanese encephalitis virus (JEV), and yellow fever virus (YFV) (Qin et al., 2014). In recent years, several jingmenviruses have been identified in ticks, mosquitoes, and other arthropods. These include Jingmen tick virus (JMTV) (Qin et al., 2014), Mogiana tick virus (MGTV) (Pascoal et al., 2019), Alongshan virus (ALSV) (Wang et al., 2019), Guaico Culex virus (GCXV) (Ladner et al., 2016), Mole Culex virus (MoCV) (Pauvolid-Correa et al., 2016), Wuhan flea virus (WHFV), Wuhan cricket virus (WHCV), Wuhan aphid virus 1 and 2 (WHAV1 and WHAV2), and Shuangao insect virus 7 (SAIV7) (Shi et al., 2016). Importantly, tick-borne jingmenviruses, including JMTV and ALSV, have been detected in human samples obtained from individuals bitten by ticks in Kosovo and China, underscoring their significant importance in public health (Emmerich et al., 2018; Jia et al., 2019; Wang et al., 2019; Zhang et al., 2023).

GCXV, which was originally isolated from *Culex* spp. mosquitoes in Central and South America between 2008 and 2013, has a genome composed of four or five distinct segments, with an overall size of approximately 12 kb (Ladner et al., 2016). Among these genome segments, three are monocistronic, while the remaining two contain three open reading frames (ORFs), each flanked by highly structured untranslated regions (UTRs). Upon translation, the ORFs encode two nonstructural proteins (NSPs), NSP1 and NSP2, as well as seven structural proteins (VP1 to VP7) (Ladner et al., 2016). Specifically, segment 1 encodes NSP1, which is similar to the flaviviral NS5 protein, while segment 2 encodes NSP2, which is related to the flaviviral NS3 protein (Ladner et al., 2016). NS5 and NS3 are conserved within the *Flavivirus* genus (Duan et al., 2017; Yao et al., 2019). Additionally, NSP2 displays RNA helicase and chaperone activities (Zhang et al., 2021). Segments 3 and 4 each encode three ORFs, most likely corresponding to structural proteins (VP1 to VP6). Finally, segment 5 codes for VP7 (Ladner et al., 2016).

To date, there are limited available data on the infection kinetics and transmission potential of jingmenviruses. In contrast to JMTV, GCXV

* Corresponding authors.
E-mail addresses: wangfei179@163.com (F. Wang), dengyq1977@126.com (Y.-Q. Deng), qincf@bmi.ac.cn (C.-F. Qin).
[1] Ru-Yi Chen, Teng Zhao, Jing-Jing Guo, and Feng Zhu contributed equally to this work and shared the first authorship.

https://doi.org/10.1016/j.virs.2024.03.002
Received 26 August 2023; Accepted 5 March 2024
Available online 8 March 2024

effectively replicated in three mosquito-derived cell lines (Aag2, *Culex tarsalis*, and C6/36) (Ladner et al., 2016; Pauvolid-Correa et al., 2016). However, it failed to replicate in cell lines derived from ticks, sandflies, or vertebrates, suggesting its specificity to mosquitoes (Ladner et al., 2016). MoCV, another jingmenvirus, has been isolated from three pools of *Culex* spp. mosquitoes and has been shown to cause cytopathic effects (CPEs) in C6/36 cells (Pauvolid-Correa et al., 2016). Interestingly, a previous study indicated that all isolates of GCXV possessed four segments, while Segment 5 was dispensable for virus replication (Ladner et al., 2016).

Regarding the infection kinetics and transmission of GCXV in mosquitoes, Ladner et al. demonstrated the detection of viral RNA in female adult *Aedes albopictus* and *Culex quinquefasciatus* mosquitoes through intrathoracic inoculation without causing substantial mortality (Ladner et al., 2016). Unfortunately, viral RNA was not detected in the larval progeny, which suggested a low occurrence or absence of vertical transmission. However, intrathoracic inoculations do not accurately reflect natural infection since mosquitoes do not feed on blood during this process (Huang et al., 2020). Oral infection via blood meals is considered to be more representative of a more natural infection route. Additionally, vertical transmission is also believed to be the primary mechanism for the maintenance of the virus in mosquito populations (Nasar et al., 2014; Sanisuriwong et al., 2021). In particular, *Cx. quinquefasciatus* is one of the most common mosquito vectors worldwide and is known to transmit various viral infectious diseases, including West Nile encephalitis (Nuss et al., 2018). Therefore, further investigations are needed to determine the infectivity and transmission potential of GCXV in *Cx. quinquefasciatus*.

Here, we first rescued and identified four-segment GCXV 4S and five-segment GCXV 5S via a reverse genetics system. Then, we systematically characterized the growth characteristics and protein expression of the rescued viruses in C6/36 cells by using RT-qPCR, immunofluorescence and median tissue culture infectious dose ($TCID_{50}$) assays. Furthermore, we investigated the infectivity and vertical transmission of the two GCXVs in *Cx. quinquefasciatus* mosquitoes after oral infection. Our findings provide crucial evidence supporting the need for heightened surveillance of GCXV in mosquito vectors worldwide.

2. Materials and methods

2.1. Cell lines

Ae. albopictus C6/36 cells were cultured at 30 °C in RPMI-1640 (Thermo Fisher Scientific, USA) supplemented with 10% FBS (Thermo Fisher Scientific) and 1% Pen/Strep (Thermo Fisher Scientific).

2.2. Rescue by reverse genetics

GCXV-LO35 (GenBank accession no: KM461666, KM461667, KM461668, KM461669, and KM461670) was rescued by reverse genetics in C6/36 cells as described previously (Ladner et al., 2016). The five-segment genome of GCXV-LO35, which included the T7 RNA polymerase promoter at the 5′ end, was synthesized by Sangon Biotech. The RNAs were produced by in vitro transcription using the RiboMAX™ Large Scale RNA Production Systems-T7 (Promega, USA) according to the kit's manual. RNA from segments 1 to 5 or segments 1 to 4 was mixed in equimolar amounts, the mixtures were transfected into C6/36 cells with Lipofectamine 3000 (Thermo Fisher Scientific), and the cells were monitored for changes in morphology. Cell viability was assessed, and the supernatants were collected when observing the typical CPE.

2.3. Cell viability assay

C6/36 cells were examined by light microscopy for the development of a CPE each day after transfection. At 72 hours post transfection (hpi), cell viability was determined using a CellTiter 96 nonradioactive cell proliferation assay (Promega) according to the manufacturer's protocol. The percentage of CPE was calculated according to the following formula: value of infected cells/value of uninfected cells.

2.4. RNA extraction and reverse transcription-quantitative PCR (RT-qPCR)

Viral RNA was extracted from the supernatant of inoculated C6/36 cell cultures using the PureLink RNA Mini Kit (Thermo Fisher Scientific) according to the kit protocol. The number of viral RNA copies was determined by primers and probes specific for segments 1 to 5 of GCXV (Supplementary Table S1). In accordance with the manufacturer's protocol, 18 μL reactions were performed with the One Step PrimeScript™ RT-PCR Kit (TaKaRa, Japan) with 2 μL of RNA template, after which RT-qPCR was performed using the LightCycler® 480 Real-Time PCR System. RNA levels were determined by reference to standard curves generated from segmented cDNA (Supplementary Fig. 1). The efficiency of this RT-qPCR system was evaluated by using uninfected C6/36 cells and uninfected mosquitoes as negative controls, and Ct values less than 35 were used.

2.5. Immunofluorescence assay

An immunofluorescence assay was performed to detect viral protein expression. C6/36 cells were harvested in 24-well plates at 7×10^5 cells per well, cultured at 30 °C with CO_2 overnight and subsequently infected with viruses at 100 $TCID_{50}$. At 24, 48, and 72 hours post infection, the supernatants were removed, and the cells were fixed with cold methanol/acetone (7:3) at −20 °C. The cells were incubated with a rabbit polyclonal antibody against the VP4 protein of GCXV Segment 4 (1:1000; synthesized by ABclonal, Wuhan, China) at 37 °C for 1 h and washed three times with PBS. Then, the cells were incubated with secondary antibody (Alexa Fluor 488-conjugated goat anti-rabbit IgG; 1: 1000; Abcam, UK) at 37 °C for 1 h. For nucleus detection, the cells were incubated with 4′,6-diamidino-2-phenylindole (DAPI; 1:5000; Sigma, USA). Fluorescence was observed and imaged on a fluorescence microscope (Zeiss, Germany).

2.6. $TCID_{50}$ assay

The viruses were titrated by the $TCID_{50}$ method. Briefly, the virus samples were serially diluted 10-fold with RPMI-1640 containing 2% FBS, and 100 μL of each diluent was added to 96-well plates covered with C6/36 cells. The titration plates were incubated for 4 days at 30 °C, and the titer obtained was determined using Reed and Muench's guidelines according to the number of cytopathic wells.

2.7. Growth curves

Growth curves of 4S and 5S in C6/36 cells were generated in a 48-well plate. C6/36 cells were seeded at 2×10^5 cells per well, cultured at 30 °C overnight and subsequently infected with viruses at 100 $TCID_{50}$. Cell supernatants were collected at 0, 6, 24, 48 and 72 hours post infection. RNA copies/mL of each segment were detected by RT-qPCR, and viral titers were quantitated by $TCID_{50}$ assay in C6/36 cells.

2.8. Oral infection of mosquitoes

Cx. quinquefasciatus (Haikou strain) were reared in the laboratory long-term. Female mosquitoes were starved 12 h prior to infection 3–5 days after eclosion. Mosquitoes were given an infectious blood meal consisting of 1:1 mouse blood and a GCXV virus suspension. Oral infections with 4S or 5S were performed with a viral titer of 3×10^5 $TCID_{50}$/mL, and the viruses were back-titrated via a $TCID_{50}$ assay to ensure similar doses were used. After 30 min of blood feeding, the mosquitoes were cold-anesthetized, and fully engorged females were transferred to 300 mL plastic cups, maintained at 29 ± 1 °C and 75 ± 5%

relative humidity (RH) with a 14 h/10 h light:dark (L:D) cycle, and provided with 8% sugar water. At different times post infection, the mosquitoes were cold anesthetized for salivation and dissection. The experiments were conducted according to standard procedures in a Biosafety Level 2 laboratory.

2.9. Mosquito processing

To determine the infectivity of 4S and 5S in *Cx. quinquefasciatus*, female mosquitoes were collected at 1, 4, 7 and 14 days post exposure (dpe). To prevent cross contamination of the virus between the midgut, salivary gland, and ovary of each mosquito, these organs were carefully dissected using fresh dissecting needles, and the organs were iteratively rinsed in phosphate-buffered saline (PBS) three times. The midgut, salivary gland, and ovary tissues of each mosquito were individually transferred to 1.5 mL microcentrifuge tubes containing 1 mL of TRIzol (TaKaRa). The midgut was collected from these mosquitoes as a proxy for infection, saliva was collected as a proxy for horizontal transmission, and ovaries were used as a proxy for vertical transmission. All dissection needles were dipped in 75% ethanol, burned, and cleaned before reuse.

2.10. Vertical transmission

To determine the vertical transmission ability of 4S and 5S from mothers to offspring in mosquitoes, the eggs of the first gonotrophic cycle and the second gonotrophic cycle were collected. The gonotrophic cycle was defined as "a complete round of ovarian development in mosquito from the time when the blood meal was taken to the time when the fully developed eggs were laid". To determine the vertical transmission ability of 4S and 5S from mothers to offspring in mosquitoes, 20 pools of eggs (30 eggs per pool) from the first gonotrophic cycle (blood with virus, 10–12 days post eclosion) and the second gonotrophic cycle (blood without virus, 14–16 days post eclosion) were collected. Then, twenty pools of eggs (30 eggs per pool) were collected. The pools were transferred to 1.5 mL microtubes containing 1 mL of TRIzol and stored at −80 °C before virus detection. These organs or eggs were then homogenized using 2-mm zirconia grinding balls (Servicebio, Beijing, China) in a freezer grinder (Xinzhi, Ningbo, China) for 3 cycles of 120 s at 60 Hz and 4 °C. The homogenates were clarified by centrifugation and stored at −80 °C until further processing. RNA was extracted from the homogenates according to the TRIzol Reagent manufacturer's protocol. All dissecting needles were dipped in 75% ethanol, burned, and cleaned before reuse. The infection rate and extent of virus replication in the midgut, salivary gland and ovary tissues of the mosquitoes were subsequently analyzed. The infection rate of each tissue was calculated by dividing the number of viruses-positivity samples by the total number of viruses tested.

2.11. Viral genome quantitation of mosquito samples

The viral RNA copy number of the mosquito samples was determined by GCXV segment 2-specific primers and probes (Supplementary Table S1) as described in Section 2.4. We subsequently selected a housekeeping gene (*RPL8*) to calculate the relative gene expression in mosquito eggs using the One Step TB Green® PrimeScript™ PLUS RT-PCR Kit (TaKaRa). The relative RNA level was calculated with the 2-ΔΔCT method as described previously (Schmittgen and Livak, 2008). The primers and probes used for this analysis are shown in Supplementary Table S1. Finally, the average value was calculated for the positive samples.

2.12. Statistical analysis

All the data were analyzed using GraphPad Prism 8.4.0 software (USA). The data are presented as the means ± SDs or as described in the corresponding legends. For the statistical analysis of other results, statistical evaluation was performed by Student's unpaired *t*-test, one-way ANOVA and two-way ANOVA.

3. Results

3.1. Rescue and identification of two GCXVs in C6/36 cells

Two GCXVs consisting of four segments (referred to as 4S) and five segments (referred to as 5S) (Fig. 1A) were synthesized, transcribed into RNA by *in vitro* transcription and successfully recovered as previously described (Ladner et al., 2016). Moreover, cytopathic effects were observed in the transfected C6/36 cells at 72 hours post transfection and were primarily characterized by cell shrinkage, rounding and detachment (Fig. 1B). Viability was assessed using a CellTiter 96 nonradioactive cell proliferation assay, and the results showed that the viability of the transfected cells was reduced by approximately 30%–40% compared to that of the mock-transfected cells (Fig. 1C). These data demonstrated that the two GCXVs were successfully rescued from the infectious clones.

3.2. Characterization of two GCXVs in C6/36 cells

To further reveal the replication characteristics of the two GCXVs, we determined the viral RNA concentration, viral titers and protein expression of 4S and 5S in C6/36 cells by using RT-qPCR, $TCID_{50}$, and immunofluorescence assays, respectively. The RT-qPCR results indicated that the two viruses exhibited similar replication kinetics in C6/36 cells. The number of RNA copies in individual segments, except for the S5 segment of 4S, significantly increased approximately 10^4–10^5 fold at 24 hpi, after which the peak value eventually reached approximately 10^9–10^{11} RNA copies/mL at 72 hpi. However, the results also indicated that, compared with 5S, LO35 4S had higher RNA loads in certain segments (S1, S3, and S4 segment) at different time points (Fig. 2A). Consistently, the $TCID_{50}$ assay further indicated that the two viruses rapidly proliferated in C6/36 cells at 24 hpi, and the peak viral titer reached approximately 3.16×10^4 $TCID_{50}$/mL at 72 hpi (Fig. 2B). In addition, the immunofluorescence results showed that GCXV VP4 protein-specific proteins were expressed in two GCXV-infected C6/36 cells in a time-dependent manner (Fig. 2C). These findings indicated that rescued GCXVs with and without segment 5 were capable of effective replication and expression in C6/36 cells.

3.3. Oral infectivity of two GCXVs in Cx. quinquefasciatus

To observe and compare the oral infectivity of two GCXVs in *Cx. quinquefasciatus* mosquitoes, a mixture of two GCXVs in mouse blood (50% vol/vol) was used to feed female *Cx. quinquefasciatus* via a membrane blood-feeding system; the process of mosquito infection is presented in Fig. 3A. The results are shown in Fig. 3B–C. First, 4S viral RNA in mosquitoes showed a decreasing trend from 1 to 7 days post exposure (dpe) and a substantial increase at 10 dpe when mosquitoes were fed infectious blood meals, whereas 5S showed a nonsignificant trend at 1–7 dpe and a decreasing trend at 14 dpe. In addition, the viral loads in the midgut, salivary gland and ovary tissues of infected mosquitoes at 1, 4, 7, and 14 dpe did not significantly change. The percentage of 4S-positive cells in the midgut decreased from 65% at 1 dpe to 5%–10% at 4–14 dpe, and the average viral load in the positive samples was 3.20×10^5 RNA copies/mL. In particular, the percentage of 5S-positive cells in the midgut decreased from 80% at 1 dpe to 0%–30% at 4–10 dpe, with average values of 1.15×10^4 RNA copies/mL at 4 dpe. However, 4S viral loads in the salivary glands and ovaries were low or undetectable from 1 to 14 dpe. The percentage of positive individuals in the salivary glands was low, ranging from 0% at 1 dpe to 0%–5% at 4–14 dpe, and values ranged from 3.83×10^3 to 1.53×10^4 copies/mL. In addition, viral RNA was detected in the ovaries at 4 dpi, and the infection rate ranged from 15% to 25%, with a mean value of 6.78×10^4 RNA copies/mL. Similarly, the percentage of 5S-positive salivary glands and ovaries was relatively

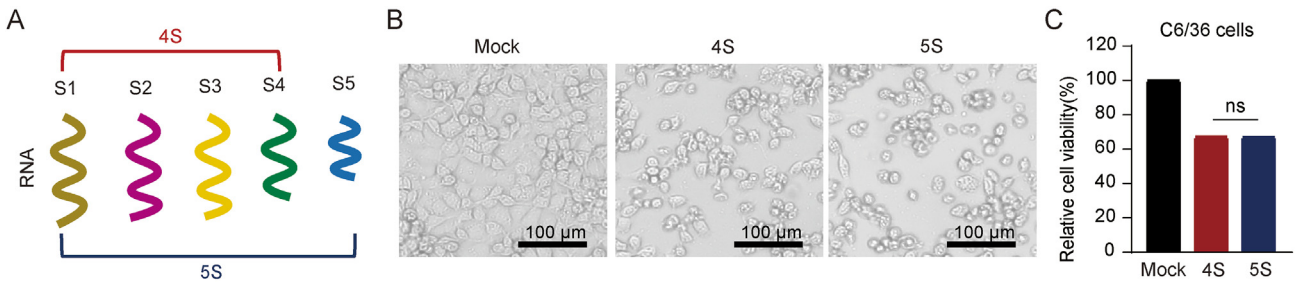

Fig. 1. Genome organization and rescue of two GCXVs. **A** Genome diagram of 4S and 5S. **B** Visible CPE of C6/36 cells at 72 hours post transfection. Scale bar: 100 μm. **C** Cell viability was evaluated by a CellTiter 96 nonradioactive cell proliferation assay at 72 hours post-infection. Each assay was performed in triplicate. The data were analyzed by one-way ANOVA. (ns, $P > 0.05$).

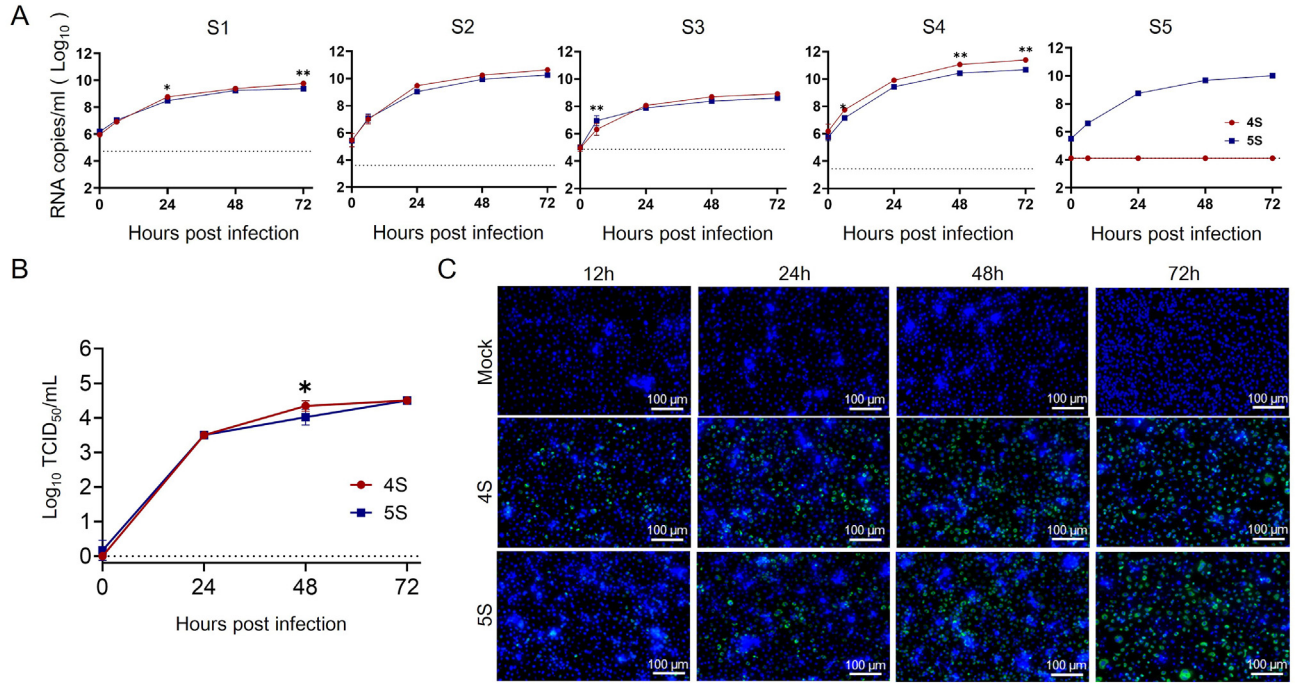

Fig. 2. The replication kinetics of two GCXVs in C6/36 cells. **A** RNA copies/mL of each of the five genome segments of 4S and 5S. Briefly, C6/36 cells were infected with the indicated viruses at a 100 $TCID_{50}$. At 24, 48, and 72 hours post-infection (hpi), the cell supernatants were harvested, and the number of RNA copies/mL of each segment was determined by RT-qPCR. The data represent the means ± SDs of triplicate measurements from a representative experiment. The data were analyzed by Student's unpaired *t*-test (*, $P < 0.05$; **, $P < 0.01$). **B** Replication kinetics of 4S and 5S. The infected supernatants were collected at 24, 48, and 72 hpi, and infectious virus particles were detected by $TCID_{50}$ assay using C6/36 cells. The detection limit is indicated by black dashed lines (Ct = 35). The error bars represent the SDs (n = 3). Statistics on viral titers were performed by two-way ANOVA. (*, $P < 0.05$). **C** Protein expression of 4S and 5S. C6/36 cells were infected with the indicated viruses at a 100 $TCID_{50}$. Uninfected group is designed as negative control. The infected cells were fixed at 12, 24, 48 and 72 hpi, and viral VP4 protein (green) expression was detected via an immunofluorescence assay. Then, the nuclei were stained with 4′,6-diamidino-2-phenylindole (DAPI) (blue). Scale bar: 100 μm.

low, and the percentage of infection positive individuals always remained at 0%–5%, with values of 7.57×10^3 and 9.93×10^3 RNA copies/mL in the salivary glands and the ovary, respectively. Additionally, we also attempted to detect these mosquito samples by $TCID_{50}$ assays, but we failed to detect the infectious virus. Thus, our results showed that these two GCXVs have limited potential for infection in *Cx. quinquefasciatus*, although they can cross the midgut barrier.

3.4. The vertical transmission potential of GCXV in Cx. quinquefasciatus

To observe and compare the vertical transmission potential of the two GCXVs in mosquitoes, we collected and hatched eggs from the first gonotrophic cycle and the second gonotrophic cycle, after which the viral RNA levels were determined by RT-qPCR (Fig. 4A). As shown in Fig. 4B, 9 of the 20 tested pools were positive for two GCXVs in the first gonotrophic cycle, and the average RNA levels (%) reached only 686.37 and 317.61, respectively. In the second gonotrophic cycle, 35% (7/20) and 15% (3/20) of the eggs were positive for both LO35 4S and LO35 5S, with average RNA levels (%) of 1.62×10^4 and 160.55, respectively. These data showed that GCXV RNAs could be detected in eggs from the first gonotrophic cycle and the second gonotrophic cycle after oral infection.

4. Discussion

In this study, we conducted a comprehensive investigation of the biological activity, infection kinetics and vertical transmission potential of two GCXVs (LO35 4S and LO35 5S). Our results showed that two GCXVs were successfully rescued and exhibited comparable replication kinetics, protein expression, and viral titers in C6/36 cells (Figs. 1 and 2). Moreover, our results further demonstrated that the S1 to S4 segments are essential for GCXV infection, which is consistent with Ladner's results (Ladner et al., 2016).

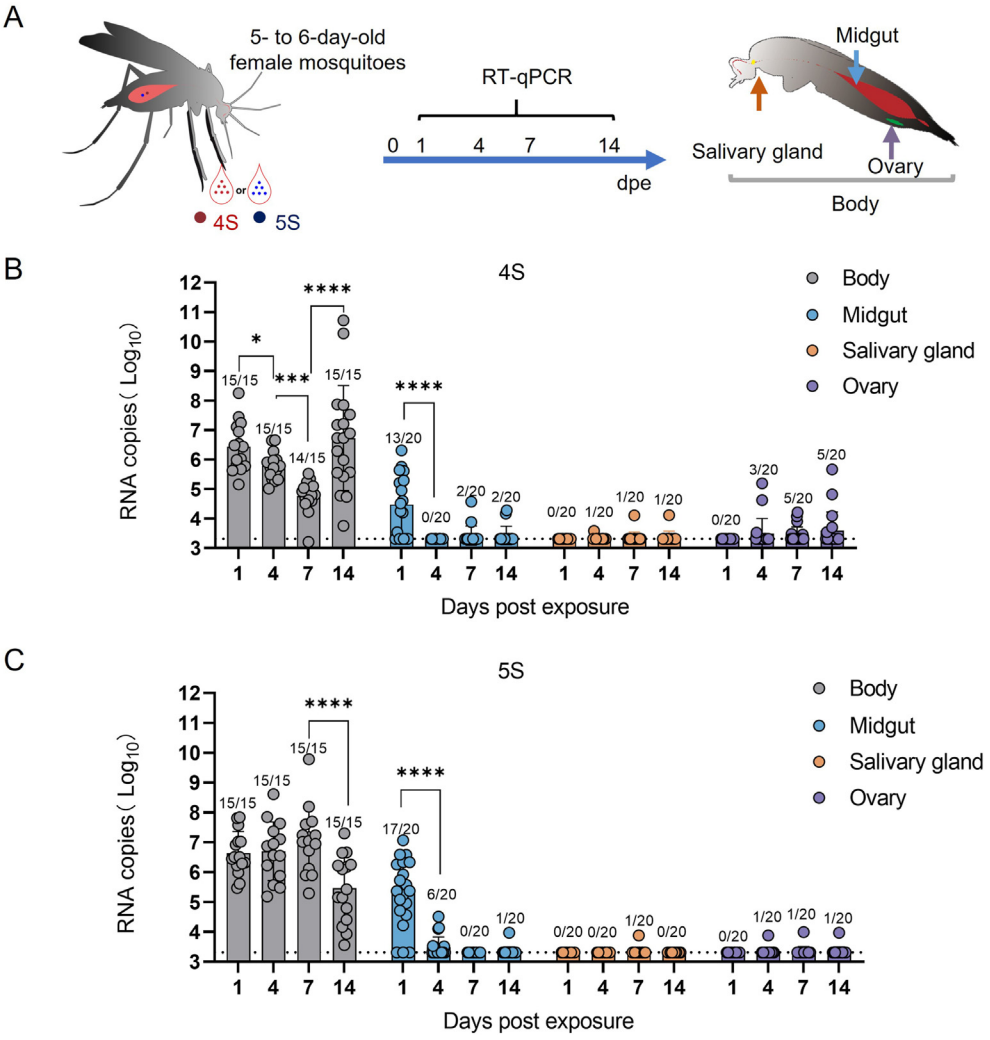

Fig. 3. Oral infectivity of two GCXVs in *Cx. quinquefasciatus*. **A** Schematic diagram of the experimental design. Briefly, mosquitoes were blood meal fed 3.2×10^5 $TCID_{50}$/mL 4S or 5S virus. Then, the mosquitoes were collected at 1, 4, 7, and 14 days post exposure, and the viral loads in the bodies, midguts, salivary glands and ovaries were detected by RT-qPCR. **B, C** The viral loads in mosquito bodies, midguts, salivary glands and ovaries at the indicated times were measured by RT-qPCR. The detection limit is indicated by black dashed lines (Ct = 35). Dot plot showing the mean viral titers and SDs of the viruses. Statistical analyses on viral titers were performed by two-way ANOVA. (*, $P < 0.05$; ***, $P < 0.001$; ****, $P < 0.0001$).

Moreover, our results showed that two GCXVs were capable of replicating in *Cx. quinquefasciatus* after oral infection via blood meals, albeit with low viral loads in the midguts, salivary glands, and ovaries of mosquitoes (Fig. 3). Previously, GCXV was demonstrated to replicate in female adult *Aedes albopictus* and *Cx. quinquefasciatus* through intrathoracic injection but not through vertical transmission (Ladner et al., 2016). Generally, the midgut plays a crucial role in vector competence, as it determines whether the virus will be transmitted by infecting the midgut following an infectious blood meal and subsequently replicating and disseminating throughout the mosquito (Franz et al., 2015). For instance, Kuchinsky et al. demonstrated that Usutu virus (USUV) could notably elevate viral titers and infection rates in body homogenates compared to those in legs, wings and saliva, while only a minor proportion of mosquitoes (1.7%) were capable of disseminating and transmitting the virus (Kuchinsky et al., 2022). Similarly, Cai et al. reported a decrease in the percentage of Tahyna virus-positive individuals, with no virus detected in the saliva of *Cx. pipiens pallens* (Cai et al., 2023). However, the strength of our observation is that our sample size was limited by the small sample size.

Importantly, our results showed that two GCXVs can colonize eggs, resulting in positive rates of 15%–35% for the second gonotrophic cycle (Fig. 4), which is inconsistent with Ladner's result. For tick-borne jingmenviruses, vertical transmission is a mode for maintenance in vector populations. For instance, Kobayashi et al. reported that JMTV was present in unfed host-questing *Amblyomma testudinarium* larvae in Japan (Kobayashi et al., 2021). Remarkably, JMTV can also be transmitted from infected adult female ticks to its progeny in nature, as shown by the presence of Viral RNA in 16 pools of 167 (Wu et al., 2023). However, for mosquito-borne viruses, many factors can influence infection and transmission efficiency, such as colonization and field population, as well as the dose of the infectious blood meal and blood meal presentation (Kauffman and Kramer, 2017). For instance, Zika virus transmission has been observed among *Ae. aegypti* mosquito populations from three different parts of the Americas, the results of which are significantly variable (Roundy et al., 2017). WNV-specific RNA was detected in 2 out of 28 pools of *Cx. quinquefasciatus* only experienced the first gonotrophic cycle (Goddard et al., 2003). In addition, vertical transmission of JEV to the F1 adult stage was demonstrated in *Cx. tritaeniorhynchus*, *Cx. annulus*, *Cx. quinquefasciatus*, and *Armigeres subalbatus*. However, transmission to the F1 larval stage in *Cx. pipiens*, *Aedes vexans*, *Ae. alcasidi*, and *A. flavus*. Moreover, vertical transmission rates also vary (12%–100%) (Rosen et al., 1989). Notably, our results showed that GCXV RNAs could persist

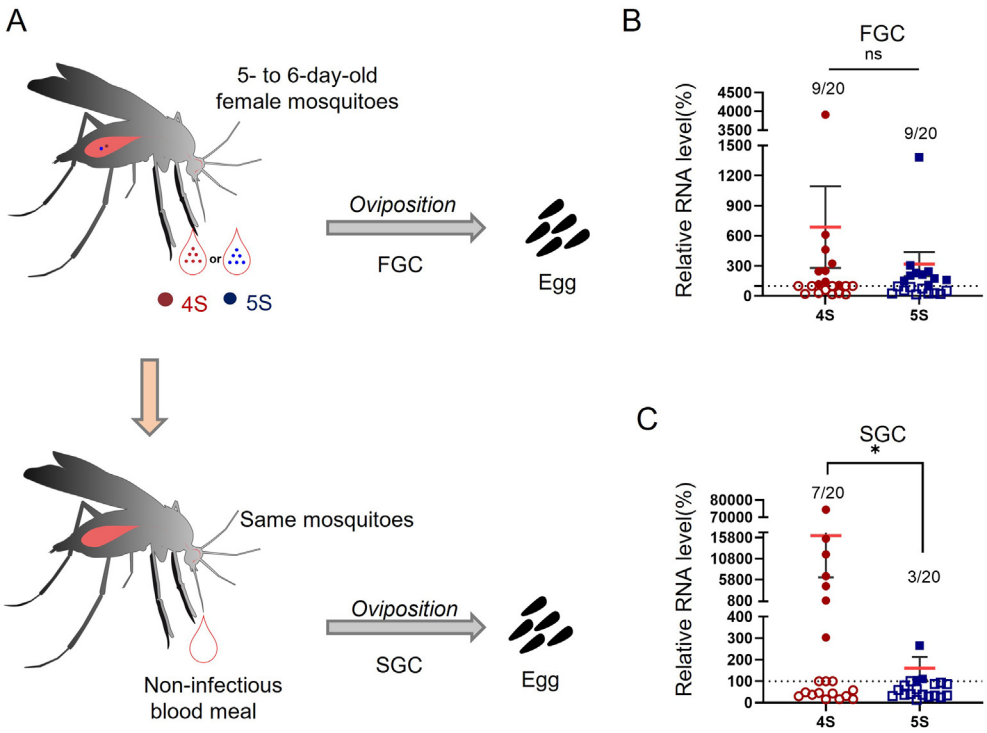

Fig. 4. Vertical transmission of two GCXVs in *Cx. quinquefasciatus*. **A** Schematic diagram. Briefly, pools of eggs (30 eggs per pool) from the first gonotrophic cycle and the second gonotrophic cycle were collected. Vertical transmission ability was measured by the same methods for the first and second gonotrophic cycles. **B, C** Viral loads were normalized against those of *Cx. quinquefasciatus* actin (RLP8) by RT-qPCR in FGC and SGC plants (100% of the mosquito pools were uninfected). The number of infected mosquitoes relative to the total number of mosquitoes is shown at the top of each column. The limit of detection is indicated by dashed lines. Each dot represents a mosquito. Specially, the solid squares/circles represent positive samples, and hollow squares/circles represent negative samples. The mean value and SEM bar are plotted for positive samples. The Mann–Whitney *U* test was performed for statistical analysis (ns, $P > 0.05$; *, $P < 0.05$).

for prolonged periods in mosquito eggs, although the infectious virus was not detected, which suggested that two GCXVs have vertical transmission potential in *Cx. quinquefasciatus* mosquitoes. In fact, a previous study also indicated that Chikungunya virus RNA was detectable in pooled eggs and adult progeny from the second gonotrophic cycle by PCR, but no samples had detectable infectious viruses according to plaque assays (Wong et al., 2016). Therefore, the number of mosquito species need to be increased to further assess the infectivity and transmission potential of the two GCXVs via a more sensitive detection assay.

5. Conclusions

In summary, our findings clearly indicate that GCXVs with four or five RNA segments could be detected in *Cx. quinquefasciatus* eggs during the first and second gonotrophic cycles after oral infection, which demonstrate that two GCXVs have limited infectivity and dissemination in *Cx. quinquefasciatus* mosquitoes.

Data availability

All the data generated during the current study are included in the manuscript.

Ethics statement

All procedures involving infectious viruses were conducted in a Biosafety Level 2 laboratory (BSL-2), and all mosquito infections were conducted in an Arthropod Containment Levels-2 Laboratory with strict practices, procedures, containment equipment, and facility requirements, e.g., multiple layers of physical isolation to prevent mosquito escape at the Beijing Institute of Microbiology and Epidemiology.

Author contributions

Cheng-Feng Qin: conceptualization, project administration, writing-review and editing, supervision. Yong-Qiang Deng: conceptualization, formal analysis, project administration, supervision, methodology, writing-original draft, conceptualization. Fei Wang: conceptualization, supervision. Ru-Yi Chen: methodology, software, visualization investigation, data curation, writing-original draft. Teng Zhao: data curation. Jing-Jing Guo: data curation. Feng Zhu: methodology. Na-Na Zhang: data curation. Xiao-Feng Li: methodology. Hai-Tao Liu: methodology. All the authors have read and approved the contents of the manuscript.

Conflict of interest

Prof. Cheng-Feng Qin is an Editorial Board member for *Virologica Sinica* and was not involved in the editorial review or the decision to publish this article. All the authors declare no competing interests.

Acknowledgements

This work was supported by the National Key Research and Development Project (2023YFC2305901), and J.J.G. was supported by the China Postdoctoral Science Fund (No. 2022M713868).

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.virs.2024.03.002.

References

Cai, T., Liu, R., Jiang, Y., Jia, N., Jian, X., Cheng, X., Song, F., Guo, X., Zhao, T., 2023. Vector competence evaluation of mosquitoes for Tahyna virus PJ01 strain, a new Orthobunyavirus in China. Front. Microbiol. 14, 1159835.

Duan, W., Song, H., Wang, H., Chai, Y., Su, C., Qi, J., Shi, Y., Gao, G.F., 2017. The crystal structure of Zika virus NS5 reveals conserved drug targets. EMBO J. 36, 919–933.

Emmerich, P., Jakupi, X., von Possel, R., Berisha, L., Halili, B., Gunther, S., Cadar, D., Ahmeti, S., Schmidt-Chanasit, J., 2018. Viral metagenomics, genetic and evolutionary characteristics of Crimean-Congo hemorrhagic fever orthonairovirus in humans, Kosovo. Infect. Genet. Evol. 65, 6–11.

Franz, A.W., Kantor, A.M., Passarelli, A.L., Clem, R.J., 2015. Tissue barriers to arbovirus infection in mosquitoes. Viruses 7, 3741–3767.

Goddard, L.B., Roth, A.E., Reisen, W.K., Scott, T.W., 2003. Vertical transmission of West Nile Virus by three California Culex (Diptera: Culicidae) species. J. Med. Entomol. 40, 743–746.

Huang, Y.S., Vanlandingham, D.L., Bilyeu, A.N., Sharp, H.M., Hettenbach, S.M., Higgs, S., 2020. SARS-CoV-2 failure to infect or replicate in mosquitoes: an extreme challenge. Sci. Rep. 10, 11915.

Jia, N., Liu, H.B., Ni, X.B., Bell-Sakyi, L., Zheng, Y.C., Song, J.L., Li, J., Jiang, B.G., Wang, Q., Sun, Y., Wei, R., Yuan, T.T., Xia, L.Y., Chu, Y.L., Wei, W., Li, L.F., Ye, J.L., Lv, Q.Y., Cui, X.M., Guan, Y., Tong, Y.G., Jiang, J.F., Lam, T.T., Cao, W.C., 2019. Emergence of human infection with Jingmen tick virus in China: a retrospective study. EBioMedicine 43, 317–324.

Kauffman, E.B., Kramer, L.D., 2017. Zika virus mosquito vectors: competence, biology, and vector control. J. Infect. Dis. 216, S976–S990.

Kobayashi, D., Kuwata, R., Kimura, T., Shimoda, H., Fujita, R., Faizah, A.N., Kai, I., Matsumura, R., Kuroda, Y., Watanabe, S., Kuniyoshi, S., Yamauchi, T., Watanabe, M., Higa, Y., Hayashi, T., Shinomiya, H., Maeda, K., Kasai, S., Sawabe, K., Isawa, H., 2021. Detection of jingmenviruses in Japan with evidence of vertical transmission in ticks. Viruses 13, 2547.

Kuchinsky, S.C., Marano, J., Hawks, S.A., Loessberg, E., Honaker, C.F., Siegel, P.B., Lahondere, C., LeRoith, T., Weger-Lucarelli, J., Duggal, N.K., 2022. North American house sparrows are competent for Usutu virus transmission. mSphere 7, e0029522.

Ladner, J.T., Wiley, M.R., Beitzel, B., Auguste, A.J., Dupuis 2nd, A.P., Lindquist, M.E., Sibley, S.D., Kota, K.P., Fetterer, D., Eastwood, G., Kimmel, D., Prieto, K., Guzman, H., Aliota, M.T., Reyes, D., Brueggemann, E.E., St John, L., Hyeroba, D., Lauck, M., Friedrich, T.C., O'Connor, D.H., Gestole, M.C., Cazares, L.H., Popov, V.L., Castro-Llanos, F., Kochel, T.J., Kenny, T., White, B., Ward, M.D., Loaiza, J.R., Goldberg, T.L., Weaver, S.C., Kramer, L.D., Tesh, R.B., Palacios, G., 2016. A multicomponent animal virus isolated from mosquitoes. Cell Host Microbe 20, 357–367.

Nasar, F., Haddow, A.D., Tesh, R.B., Weaver, S.C., 2014. Eilat virus displays a narrow mosquito vector range. Parasit. Vectors 7, 595.

Nuss, A.B., Brown, M.R., Murty, U.S., Gulia-Nuss, M., 2018. Insulin receptor knockdown blocks filarial parasite development and alters egg production in the southern house mosquito, Cx. quinquefasciatus. PLoS Negl. Trop. Dis. 12, e0006413.

Pascoal, J.O., Siqueira, S.M., Maia, R.D.C., Juan Szabo, M.P., Yokosawa, J., 2019. Detection and molecular characterization of Mogiana tick virus (MGTV) in Rhipicephalus microplus collected from cattle in a savannah area, Uberlandia, Brazil. Ticks Tick Borne Dis. 10, 162–165.

Pauvolid-Correa, A., Solberg, O., Couto-Lima, D., Nogueira, R.M., Langevin, S., Komar, N., 2016. Novel viruses isolated from mosquitoes in Pantanal, Brazil. Genome Announc. 4, e01195-16.

Qin, X.C., Shi, M., Tian, J.H., Lin, X.D., Gao, D.Y., He, J.R., Wang, J.B., Li, C.X., Kang, Y.J., Yu, B., Zhou, D.J., Xu, J., Plyusnin, A., Holmes, E.C., Zhang, Y.Z., 2014. A tick-borne segmented RNA virus contains genome segments derived from unsegmented viral ancestors. Proc. Natl. Acad. Sci. U. S. A. 111, 6744–6749.

Rosen, L., Lien, J.C., Shroyer, D.A., Baker, R.H., Lu, L.C., 1989. Experimental vertical transmission of Japanese encephalitis virus by Culex tritaeniorhynchus and other mosquitoes. Am. J. Trop. Med. Hyg. 40, 548–556.

Roundy, C.M., Azar, S.R., Rossi, S.L., Huang, J.H., Leal, G., Yun, R., Fernandez-Salas, I., Vitek, C.J., Paploski, I.A., Kitron, U., Ribeiro, G.S., Hanley, K.A., Weaver, S.C., Vasilakis, N., 2017. Variation in Aedes aegypti mosquito competence for Zika virus transmission. Emerg. Infect. Dis. 23, 625–632.

Sanisuriwong, J., Yurayart, N., Thontiravong, A., Tiawsirisup, S., 2021. Vector competence of Culex tritaeniorhynchus and Cx. quinquefasciatus (Diptera: Culicidae) for duck Tembusu virus transmission. Acta Trop. 214, 105785.

Schmittgen, T.D., Livak, K.J., 2008. Analyzing real-time PCR data by the comparative C(T) method. Nat. Protoc. 3, 1101–1108.

Shi, M., Lin, X.D., Vasilakis, N., Tian, J.H., Li, C.X., Chen, L.J., Eastwood, G., Diao, X.N., Chen, M.H., Chen, X., Qin, X.C., Widen, S.G., Wood, T.G., Tesh, R.B., Xu, J., Holmes, E.C., Zhang, Y.Z., 2016. Divergent viruses discovered in arthropods and vertebrates revise the evolutionary history of the Flaviviridae and related viruses. J. Virol. 90, 659–669.

Wang, Z.D., Wang, B., Wei, F., Han, S.Z., Zhang, L., Yang, Z.T., Yan, Y., Lv, X.L., Li, L., Wang, S.C., Song, M.X., Zhang, H.J., Huang, S.J., Chen, J., Huang, F.Q., Li, S., Liu, H.H., Hong, J., Jin, Y.L., Wang, W., Zhou, J.Y., Liu, Q., 2019. A new segmented virus associated with human febrile illness in China. N. Engl. J. Med. 380, 2116–2125.

Wong, H.V., Vythilingam, I., Sulaiman, W.Y., Lulla, A., Merits, A., Chan, Y.F., Sam, I.C., 2016. Detection of persistent Chikungunya virus RNA but not infectious virus in experimental vertical transmission in Aedes aegypti from Malaysia. Am. J. Trop. Med. Hyg. 94, 182–186.

Wu, Z., Chen, J., Zhang, L., Zhang, Y., Liu, L., Niu, G., 2023. Molecular evidence for potential transovarial transmission of Jingmen tick virus in Haemaphysalis longicornis fed on cattle from Yunnan Province, China. J. Med. Virol. 95, e28357.

Yao, Y., Huo, T., Lin, Y.L., Nie, S., Wu, F., Hua, Y., Wu, J., Kneubehl, A.R., Vogt, M.B., Rico-Hesse, R., Song, Y., 2019. Discovery, X-ray crystallography and antiviral activity of allosteric inhibitors of Flavivirus NS2B-NS3 protease. J. Am. Chem. Soc. 141, 6832–6836.

Zhang, J., Zheng, Y.C., Chu, Y.L., Cui, X.M., Wei, R., Bian, C., Liu, H.B., Yao, N.N., Jiang, R.R., Huo, Q.B., Yuan, T.T., Li, J., Zhao, L., Li, L.F., Wang, Q., Wei, W., Zhu, J.G., Chen, M.C., Gao, Y., Wang, F., Ye, J.L., Song, J.L., Jiang, J.F., Lam, T.T., Ni, X.B., Jia, N., 2023. Skin infectome of patients with a tick bite history. Front. Cell. Infect. Microbiol. 13, 1113992.

Zhang, X.Y., Shu, T., Wang, X., Xu, J., Qiu, Y., Zhou, X., 2021. Guaico Culex virus NSP2 has RNA helicase and chaperoning activities. J. Gen. Virol. 102. https://doi.org/10.1099/jgv.0.001589.