



Identification and genetic analysis of a totivirus isolated from the *Culex tritaeniorhynchus* in northern China

Fan Li^{1,2} · Jiang Du³ · Zhiqiang Wu³ · Weijia Zhang^{2,4} · Shihong Fu^{1,2} · Jingdong Song¹ · Qianying Wang^{1,2} · Ying He^{1,2} · Wenwen Lei^{1,2} · Songtao Xu^{1,2} · Aiqiang Xu⁵ · Li Zhao⁴ · Guodong Liang^{1,2} · Huanyu Wang^{1,2}

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Abstract

Totiviridae, a viral family of double-stranded RNA (dsRNA) viruses, contain a single dsRNA genome 4.6–7.0 kb in length. *Totiviridae* were initially only known to infect fungi and other eukaryotes as well as plants, but an increase in totiviruses has been detected in insects, mosquitoes, and bats. Here, we describe the isolation and characterization of a strain belonging to the family *Totiviridae* isolated from *Culex tritaeniorhynchus* in Kenli, China, in 2016. We isolated a totivirus from field-collected mosquitoes in China by cell culture in *Aedes albopictus* C6/36 cells, identified the virus by morphological observation and complete genome sequencing, and characterized it by phylogenetic analysis. Transmission electron microscopy identified icosahedral, non-enveloped virus particles with a mean diameter of 35–40 nm. The genome was 7612 bp in length, including two open reading frames (ORFs). ORF1 (5058 nt) encodes the capsid protein, while ORF2 (2216 nt) encodes the viral RNA-dependent RNA polymerase (RdRp). Nucleotide and amino acid homology analysis of isolate showed higher levels of sequence identity with isolate CTV_NJ2 (China, 2010) with 94.87% nucleic acid identity and 97.32% amino acid identity. The isolate was designated *C. tritaeniorhynchus totivirus* KL (CTV-KL). This is the first identification of a totivirus in a *C. tritaeniorhynchus* in northern China. Analysis of the virus's morphology, characteristic and genome organization will further enrich our understanding of the molecular and biological characteristics of dsRNA *Totiviridae* viruses.

Keywords Totivirus · *Totiviridae* · dsRNA virus · *Culex tritaeniorhynchus* · Genetic analysis · Virus identification

Abbreviations

ORFs	Open reading frames
RdRp	RNA-dependent RNA polymerase
CTV	<i>Culex tritaeniorhynchus totivirus</i>
CPEs	Cytopathic effects
dsDNA	Double-stranded DNA
ssDNA	Single-stranded DNA
dsRNA	Double-stranded RNA
ssRNA+	Positive single-stranded RNA

ssRNA–	Negative single-stranded RNA
ssRNA-RT	Single-stranded retro-transcribing RNA
dsDNA-RT	Double-stranded retro-transcribing DNA

Introduction

Based on the Baltimore classification, viruses are classified into seven groups: double-stranded DNA (dsDNA), single-stranded DNA (ssDNA), double-stranded RNA (dsRNA), positive single-stranded RNA (ssRNA+), negative single-stranded RNA (ssRNA–), single-stranded retro-transcribing RNA (ssRNA-RT), and double-stranded retro-transcribing DNA (dsDNA-RT) (Baltimore 1971). The latest release of the International Committee on Taxonomy of Viruses (ICTV) recognized 256 species of dsRNA viruses, which accounts for less than 4.6% of total established viral species. Due to their rarity, there is an inadequate appreciation of the diversity of dsRNA viruses (King et al. 2018).

Totiviridae, a viral family of dsRNA viruses, consist of a single dsRNA that is 4.6–7.0 kb in length with two open

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✉ Huanyu Wang
wanghy@ivdc.chinacdc.cn

Extended author information available on the last page of the article

reading frames (ORFs) encoding a capsid protein (CP) and an RNA-dependent RNA polymerase (RdRp) (de Lima et al. 2019; Ghabrial 2008). Viral particles of *Totiviridae* are approximately 40 nm in diameter with icosahedral symmetry. The *Totiviridae* family consists of five genera: *Totivirus*, *Victorivirus*, *Giardiavirus*, *Leishmanivirus*, and *Trichomonasvirus* (Ghabrial and Nibert 2009; Goodman et al. 2011). With the availability of more advanced detection techniques, viruses from a wider range of host organisms are being discovered at an increasing pace. *Totiviridae* was previously known to infect fungi and other eukaryotes as well as plants; however, recently a handful of related but yet unassigned totivirus-like viruses have been isolated from insects, mosquitoes, and bats (Huang et al. 2018; Yang et al. 2012; Zhai et al. 2010). The identification of these viruses will contribute to our understanding of the diversity and evolution of dsRNA viruses.

Mosquitoes are vectors of parasites and viruses that cause diseases of immense importance to public health. Mosquitoes are the major vector of *Flaviviridae* (ssRNA+) and *Togaviridae* (ssRNA+) viruses, with *Reoviridae* (dsRNA) and *Birnaviridae* (dsRNA) being reported recently (Kuwata et al. 2015; Shapiro et al. 2005; Strauss and Strauss 1994; van Cleef et al. 2014). In particular, mosquito-borne viruses are of increasing concern as arboviral diseases emerge and re-emerge in conjunction with global warming, which facilitates the geographic expansion of mosquito species (Kobayashi et al. 2002). Here, we report the isolation of a strain belonging to the family *Totiviridae* from mosquitoes collected in the Shandong Province of northern China during a 2016 arbovirus survey.

Materials and methods

Mosquito collection

Kenli is a district in the city of Dongying, located in the northern Shandong province of eastern China near the mouth of the Yellow River. The arbovirus survey was conducted on September 7, 2016 in the residential regions of the village of Dongsui, a small river estuary town in the Kenli District (37°38'31.94" N, 118° 51' 41.69" E; altitude, 2 m). Mosquitoes were collected from a pigsty at night using ultraviolet light traps (12 V; 300 mA; Wuhan Lucky Star Environmental Protection Tech Co., Ltd., Hubei, China). Traps were set from 7:00 p.m. to 7:00 a.m., which spanned the period from sunset to sunrise. The captured mosquitoes were frozen at $-20\text{ }^{\circ}\text{C}$ for at least 40 min and identified according to morphological keys from Lu (1997a, b). Only female mosquitoes were selected for further study. Approximately 100 mosquitoes of the same species were pooled in

cryogenic vials and stored in liquid nitrogen until further use (Li et al. 2010).

Virus isolation

Mosquitoes were removed from liquid nitrogen and immediately homogenized and centrifuged as previously reported (Sun et al. 2009). The supernatants were inoculated in a monolayer of *Aedes albopictus* mosquito C6/36, baby hamster kidney BHK-21, or African green monkey kidney cells in 24-well plates and incubated at $28\text{ }^{\circ}\text{C}$, $37\text{ }^{\circ}\text{C}$ and $37\text{ }^{\circ}\text{C}$, respectively. After incubation for 24 h, the cells were observed for cytopathic effects (CPEs) every 8 h for 6–7 days. A specimen was regarded as a positive isolate if it caused CPEs in three successive cell passages. Infected cell supernatants were stored at $-80\text{ }^{\circ}\text{C}$ until further analysis (Lu et al. 2009).

Virus electron microscopy

For negative staining, 1 ml of cell culture supernatant was ultracentrifuged at $100,000g$ for 10 min and the pellet was re-suspended in 50 μl infected cell supernatant. The virus was then absorbed onto Formvar and carbon-coated grids for 1 min and stained with 1% (w/v) phosphotungstic acid (pH 6.8) for 1 min. Grids were air-dried and observed under a Tecnai 12 transmission electron microscope (FEI, Eindhoven, Netherlands).

For ultrathin sections, cell pellets were fixed in a solution of 2% formaldehyde and 2.5% glutaraldehyde, post-fixed in 1% osmium tetroxide, dehydrated in an ethanol gradient, embedded in epoxy resin-812, and polymerized at $60\text{ }^{\circ}\text{C}$ for 24 h. Ultrathin sections (80 nm) were obtained from the resin blocks, mounted on copper grids, and stained with uranyl acetate and lead citrate. Finally, the ultrathin sections were observed using transmission electron microscopy.

Extraction of nucleic acids and deep sequencing

Viral RNA was extracted from infected culture supernatants with positive CPEs using the QIAamp Viral RNA Mini Kit (Qiagen, Valencia, CA, USA). Complementary DNA (cDNA) synthesis was performed using Ready-to-Go RT-PCR Beads (Amersham Biosciences Co., Piscataway, NJ, USA). The library was prepared using an NEBNext Ultra RNA Library Prep Kit for Illumina (New England Biolabs, Hitchin, UK). The library was quantified on a Qubit 3.0 fluorometer (Thermo Fisher) and Agilent Bioanalyzer 2100 (Agilent). Paired-end sequencing [2×150 base pairs (bp)] was performed on an Illumina MiSeq v2 platform. Sequencing reads were filtered using previously described criteria to obtain valid data (Yang et al. 2011). For analysis, reads were filtered based on their length and mean quality values (Q30).

Sequence analysis

Contigs were prepared by de novo assembly and subjected to BLASTx alignment (E value $< 10^{-5}$) against the non-redundant (NR) National Center for Biotechnology Information (NCBI) protein databases (<https://www.ncbi.nlm.nih.gov>). Mapping of sequence reads against the target genomes was done using the Bowtie 2 alignment algorithm in usegalaxy (<https://usegalaxy.org/>). RNA ligase-mediated (RLM)-RACE PCR amplification was used to confirm the 5'- and 3'-terminal sequences. ORFs were identified using GENE-TYX-MAC or EnzymeX version 3.3.3 (<https://nucleobyte.com/enzymex/index.html>). Sequence similarities were calculated using the BLASTx program available from NCBI. The complete nucleotide sequence of the virus reported in this study has been submitted to the GenBank database under accession number MN614415.

Phylogenetic analysis

Multiple alignments were performed with MAFFT version 7 (<https://www.ebi.ac.uk/Tools/>) using previously described parameters (Kato and Toh 2008). The aligned matrix data were confirmed manually. Phylogenetic tree construction was based on a neighbor-joining (NJ) method. The statistical significance of the resulting tree was evaluated using a bootstrap test with 1,000 replicates. The phylogenetic tree (mid-point rooted) was visualized and refined with FigTree version 1.4.2 software (<https://tree.bio.ed.ac.uk/software/>).

Results

Virus isolation

On September 7, 2016, a total of 3000 mosquitoes consist of two species, *Culex tritaeniorhynchus* (2300, 76.7%) and *Anopheles sinensis* (700, 23.3%), were collected in Kenli District, Shandong Province, China.

The virus isolated from *C. tritaeniorhynchus* initially caused CPEs in *A. albopictus* C6/36 cells beginning at 72 h after inoculation and blind passage. The main characteristic of the CPEs was cell shedding, shrinking, and floating with eventual detachment from the growth surface (Fig. 1). No CPEs were observed in BHK-21 and Vero cells during three blind passages. Virus particles were enriched by ultracentrifugation from infected culture supernatants and stained negatively. The negative stained virus particles were non-enveloped and approximately 40 nm in diameter (Fig. 2a). Virus particles and virus inclusion bodies were observed in the cytoplasm on ultrathin sections (Fig. 2b). No such particles were observed in mock-infected controls (data not shown). For archiving purposes, the isolate was designated CTV_KL.

Sequence analysis of the CTV_KL genome

To identify these viruses, the viral RNAs were subjected to next-generation sequencing and RACE analyses. The assembled genome of CTV_KL was 7612 bp with two ORFs (ORF1 and ORF2). The first ORF (ORF1) spans nucleotides 7–5061 (5055 nt in length) and encodes a 1685 aa protein, while the second ORF (ORF2) spans nucleotides 5319–7532 (2217 nt in length) and encodes 738 aa. The 5' and 3' untranslated regions of CTV_KL were 6 and 77 nt long, respectively. BLASTx analysis of the contigs obtained by de novo assembly showed that they matched most closely with the unassigned species of *Totiviridae*. While the CTV_KL ORFs only showed a 3–8% aa similarity to the classified *Totiviridae* viruses, it shared a 94.87% nt and 97.32% aa identity with the unclassified *C. tritaeniorhynchus* totivirus isolate CTV_NJ2 (KX456218) and 76.82% nt and 84.16% aa identity with totivirus-like virus ToV-TJ (JN391187) (Table 1).

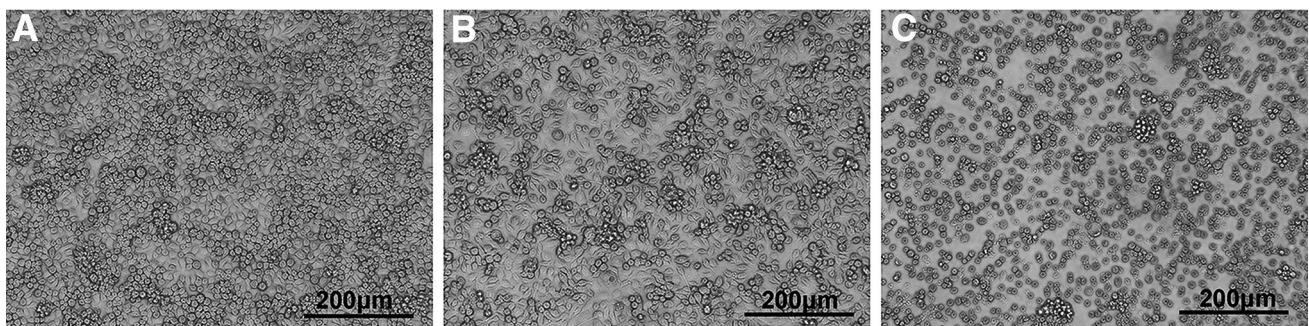


Fig. 1 Cytopathic effects (CPEs) caused by CTV-KL in C6/36 cells. **a** Phase-contrast micrographs of control (mock-infected) C6/36 cells. **b** CTV-KL-infected cells 72 h post-infection. **c** CTV-KL-infected cells 120 h post-infection. Scale bars indicate 200 μ m

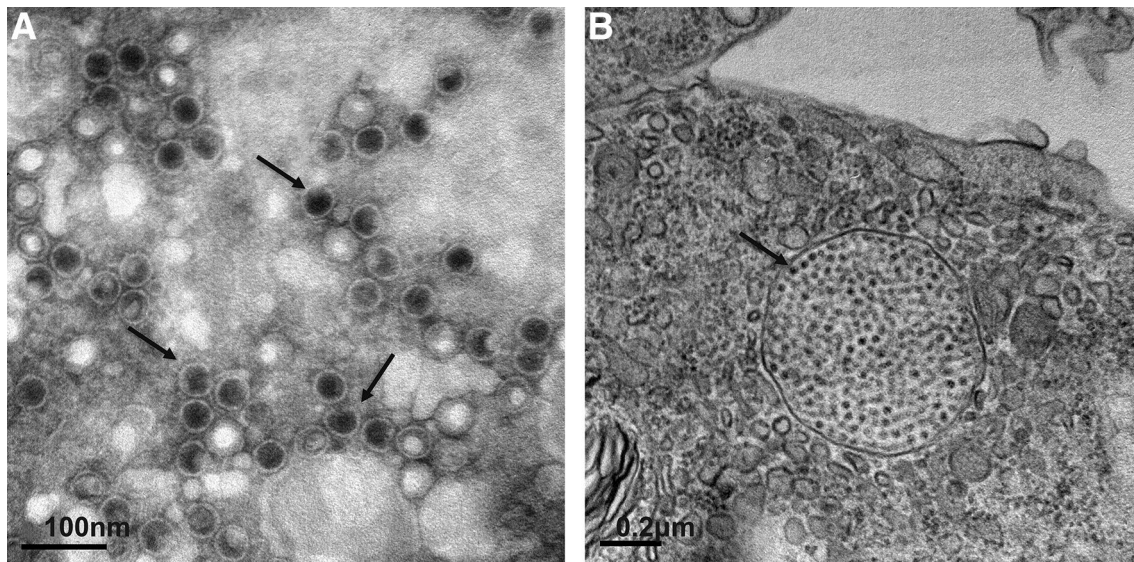


Fig. 2 Negative-contrast electron micrographs of CTV-KL particles. **a** Arrows indicate CTV-KL particles in infected cell culture supernatant. Scale bars indicate 100 nm. **b** Ultrathin sections show large,

electron-dense viral inclusion body (arrow) within the cytoplasm composed of a large number of CTV-KL in infected C6/36 cells. Scale bars indicate 0.2 μm

Table 1 Sequence Identities between CTV-KL and other related arthropod-infecting toti-like viruses

Strain	CTV-NJ2	OMV-AK4	Tov-TJ	CTV-NJ3	DTV	ASV
CTV-KL						
NA ^a	0.94	0.76	0.76	0.65	0.64	0.67
CP ^b	0.97	0.84	0.84	0.47	0.55	0.45
RDRP ^c	0.99	0.87	0.87	0.60	0.64	0.49

^aNucleotide sequence identities of whole genome

^bAmino acid sequence identities of capsid protein region

^cAmino acid sequence identities of RDRP region

Phylogenetic analysis of CTV_KL

To study the evolutionary relationship of CTV_KL with other viruses in the family *Totiviridae*, RdRP amino acid sequences with $\geq 35\%$ similarity with CTV_KL were downloaded from GenBank and used to construct a phylogenetic tree (Fig. 3). CTV_KL was classified into the clade of arthropod-infecting toti-like viruses. The phylogenetic analysis showed that CTV_KL was closely related to the unclassified *Totiviridae* *C. tritaeniorhynchus* totivirus, Omono River virus, and Penaeid shrimp infectious myonecrosis virus. However, CTV_KL formed a distinct cluster with Wuhan insect virus (KX882989), Wenzhou toti-like virus (KX882995), and Golden shiner totivirus (NC_030295).

Discussion

We identified and characterized a new strain of *Totiviridae* virus designated CTV-KL, isolated from *Culex* mosquitoes in northern China. The CTV-KL particle was non-enveloped, isometric, and approximately 40 nm in diameter. The genome was composed of a single 7.6 kb dsRNA containing two ORFs. Genetic and phylogenetic analyses suggested that CTV-KL is most closely related to unassigned members of the family *Totiviridae*. In the NJ phylogenetic tree, CTV-KL fell in a high confidence branch with CTV_NJ2, ToV-TJ, and OMRV-AK4, and formed a distinct cluster with Golden shiner totivirus (NC_030295), Wuhan insect virus (KX882989), and Wenzhou toti-like virus (KX882995). *C.*

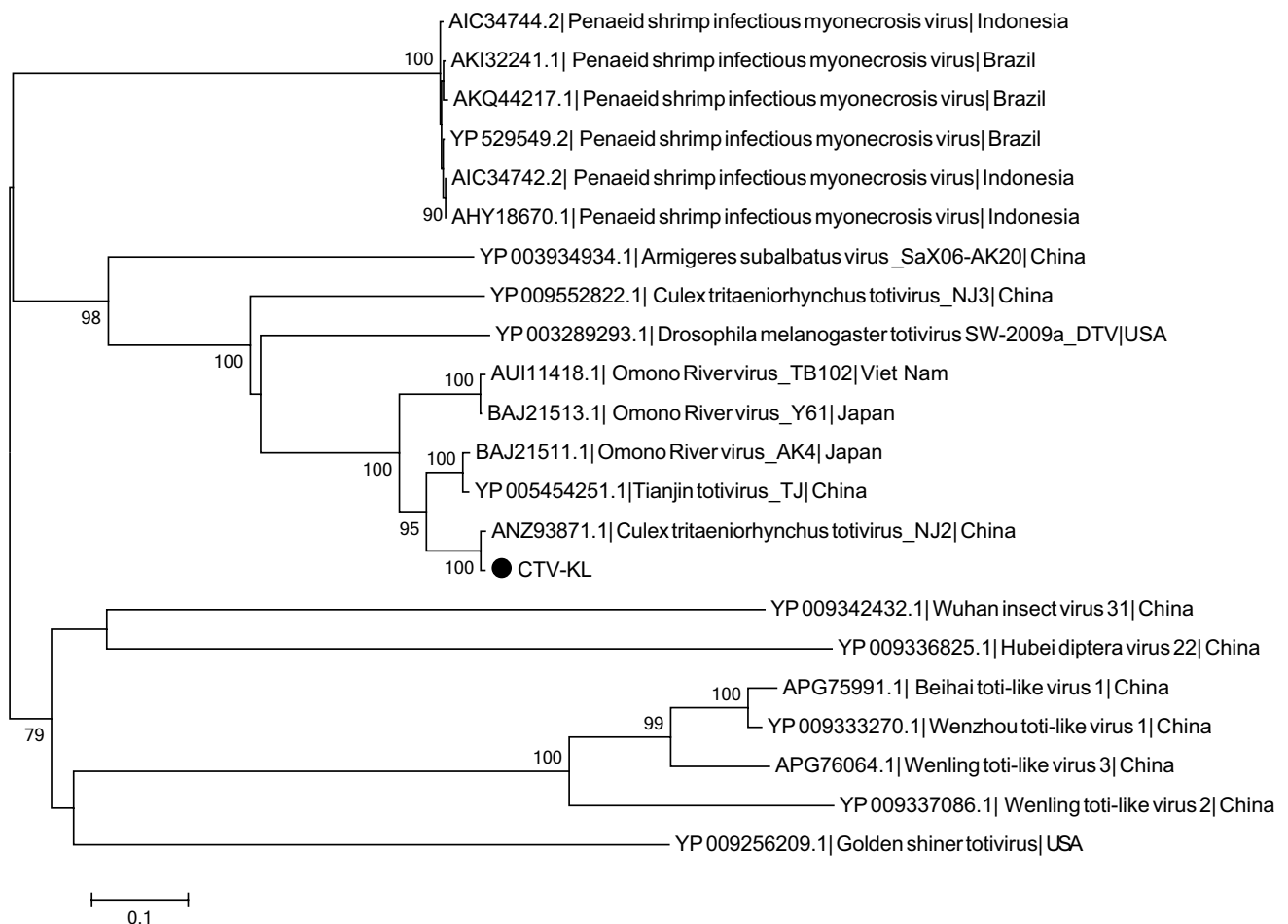


Fig. 3 Neighbor-joining tree inferred from molecular phylogenetic analysis based on the amino acid sequences of the RDRP segments. The newly discovered CTV-KL strain is labeled with a solid circle.

Numbers associated with branches indicate the percentage of 1000 bootstrap replicates that support the existence of these branches. Branches with < 60% bootstrap support have been collapsed

tritaeniorhynchus totivirus (CTV_NJ2) was isolated in Nanjing, China, in 2016, ToV-TJ was isolated from bats in Tianjin, China in 2010, and three OMRV viruses were isolated in Vietnam and Japan from three different *Culex* species (Isawa et al. 2011). In addition, CTV_KL also fell into a cluster with unassigned *Totiviridae* infectious myonecrosis virus (IMNV) isolated from the skeletal muscle of Penaeid shrimp (*Litopenaeus vannamei*) in Brazil in 2003, *Drosophila* totivirus (DTV) found in S2-GMR *Drosophila* cells, and *Armigeres subalbatus* totivirus (AsTV) from *Armigeres subalbatus* (Poulos et al. 2006; Tang et al. 2005; Wu et al. 2010; Zhai et al. 2010).

Previously, viruses in *Totiviridae* were only associated with latent infections of their fungal or protozoan hosts until the isolation of IMNV from Penaeid shrimp (*L. vannamei*) in northeastern Brazil in 2005 and DTV from *Drosophila melanogaster* in the USA (Poulos et al. 2006; Wu et al. 2010; de Lima et al. 2019). With the rapidly evolving application of next-generation sequencing technology, more members

of this family are being discovered in a wider range of host organisms than ever before (Kondo et al. 2016; Read et al. 2019). IMNV, AsTV, and DTV have recently been proposed as members of a novel *Totiviridae* genus, “Artivirus” (derived from “arthropod totivirus”) (Zhai et al. 2010). The widespread geographic distribution and broad host range in arthropods suggests that these totivirus-like viruses may be common worldwide (Fig. 2). The potential biological significance of these viruses and their vector hosts needs to be further investigated.

Mosquitoes are vectors of parasites and viruses that cause diseases of immense importance to public health. In particular, mosquito-borne viruses are of increasing interest and concern because of the recent emergence and re-emergence of arboviral diseases. In addition, ecological changes due to global warming may facilitate an expansion of the distribution of vector mosquitoes. The vector competence of mosquitoes, host competence for viral transmission, and viral pathogenicity are the three factors used

for assessing the susceptibility of vertebrates to mosquito-borne viruses (Hardy et al. 1983). It has been discovered that OMRV establishes latent infection in host mosquitoes, IMNV causes progressive disease in crustacean hosts (Poulos et al. 2006), and DTV persistently infects cultured *Drosophila* cells together with four other RNA viruses (Wu et al. 2010). The CTV-KL genome sequence was 76.3% identical to OMRV-AK4 isolated from mosquitoes in Japan (Isawa et al. 2011), suggesting that CTV_KL may be a pathogen of mosquitoes. The virulence, host range, specificity to mosquitoes of CTV-KL will require further investigation. A detailed analysis of CTV-KL is needed for assessment of its biological properties and potential ecological impacts on host mosquitoes and other viruses.

Conclusions

We have identified and characterized a strain (CTV-KL) of family *Totiviridae* that may be biologically significant to mosquitoes and humans. This is the first identification of a totivirus in a *C. tritaeniorhynchus* in northern China. Several important questions related to its transmission, host maintenance, and the potential impact of infection on the host's survival, evolution warrant further investigation.

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Author contributions FL and HW were major contributors in writing the manuscript. WZ and AX collected the mosquitoes sample. SF, FL, WZ, QW, YH, and WL performed virus isolation. FL sequenced the genome of CTV-KL. JS performed the virus electron microscopy. FL, JD, and ZW did the Bioinformatics analysis. HW, LZ, and GL conceived the study and drafted the manuscript. All authors read and approved the final manuscript.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no competing interests.

Ethics approval and consent to participate Not applicable.

Consent for publication Not applicable.

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Affiliations

Fan Li^{1,2} · Jiang Du³ · Zhiqiang Wu³ · Weijia Zhang^{2,4} · Shihong Fu^{1,2} · Jingdong Song¹ · Qianying Wang^{1,2} · Ying He^{1,2} · Wenwen Lei^{1,2} · Songtao Xu^{1,2} · Aiqiang Xu⁵ · Li Zhao⁴ · Guodong Liang^{1,2} · Huanyu Wang^{1,2}

Fan Li
frances.li99@hotmail.com

Jiang Du
dujiang@ipbcams.ac.cn

Zhiqiang Wu
wuzq2009@ipbcams.ac.cn

Weijia Zhang
zwj_0308@126.com

Shihong Fu
shihongfu@hotmail.com

Jingdong Song
sjdccc@163.com

Qianying Wang
18513675636@163.com

Ying He
annihe@aliyun.com

Wenwen Lei
flyingwenwen@163.com

Songtao Xu
xstz886@sina.com

Aiqiang Xu
aqxuepi@163.com

Li Zhao
dlzhl@sdu.edu.cn

Guodong Liang
gdliang@hotmail.com

¹ NHC Key Laboratory of Biosafety, National Institute for Viral Disease Control and Prevention, Chinese Center for Disease Control and Prevention, Beijing 102206, People's Republic of China

² State Key Laboratory for Infectious Disease Prevention and Control, Chinese Center for Disease Control and Prevention, Beijing 102206, People's Republic of China

³ NHC Key Laboratory of Systems Biology of Pathogens, Institute of Pathogen Biology, Chinese Academy of Medical Sciences, Peking Union Medical College, Beijing 100176, People's Republic of China

⁴ School of Public Health, Shandong University, Jinan 250012, People's Republic of China

⁵ Institute for Immunization Program, Shandong Province Center for Disease Control and Prevention, Jinan 250014, People's Republic of China