VIROLOGICA SINICA

Electronic Supplementary Material

Severe Acute Respiratory Syndrome Coronavirus 2 Does Not Replicate in Aedes Mosquito Cells nor Present in Field-Caught Mosquitoes from Wuhan

Han Xia[1,2] • Evans Atoni[1,2] • Lu Zhao[1,2] • Nanjie Ren[1,2] • Doudou Huang[1] • Rongjuan Pei[1,2] • Zhen Chen[1] • Jin Xiong[1] • Raphael Nyaruaba[1,2] • Shuqi Xiao[1] • Bo Zhang[1,2] • Zhiming Yuan[1,2]✉

1. Key Laboratory of Special Pathogens and Biosafety, Wuhan Institute of Virology, Center for Biosafety Mega-Science, Chinese Academy of Sciences, Wuhan 430071, China
2. University of Chinese Academy of Sciences, Beijing 100049, China

Supporting information to DOI: 10.1007/s12250-020-00251-0

SUPPLEMENTARY METHODS

Virus and Cell lines

A clinical isolate of SARS-CoV-2 (nCoV-2019BetaCoV/Wuhan/WIV04/2019) was propagated in Vero E6 cells, and the viral titer determined through plaque assay. Thereafter, a working stock of 1.0×10^7 plaque-forming units/mL (PFU/mL) was generated. *Aedes albopictus* cells (C6/36 cells) were cultivated at 28 °C in Roswell Park Memorial Institute (RPMI) 1640 medium (Gibco, USA) containing 10% FBS, *Aedes aegypti* cells (Aag2 cells) were in Schneider's Drosophila media (Gibco, USA) with 5% FBS , while *Spodoptera frugiperda* cells (Sf9 cells) were cultivated in Sf-900 medium (Gibco, USA). Vertebrate cells, African monkey kidney cells (Vero-E6 cells, (ATCC, no. 1586) were grown at 37°C with 5% CO_2 atmosphere in Dulbecco's minimal essential medium (DMEM) (4.5 g/liter D-glucose) (Gibco, USA) containing 10% FBS. All the medium were supplemented with 1% penicillin/streptomycin. All the infection experiments were performed in a biosafety level-3 (BLS-3) laboratory.

Growth Curves of SARS-CoV-2 in Cultured Cells

Vero E6, C6/36, Aag2 and Sf9 cells were grown in 12-well plates with 4×10^5 cells per well, then infected with SARS-CoV-2 at the multiplicity of infection (MOI) of 0.0001, 0.01, and 0.1, respectively. After 1 h of incubation, the virus inoculum was discarded, and 2 mL medium with 2% FBS was added to each well. 250 µL supernatant was collected daily (days 0–7) from each well and immediately replenished with another equal volume of fresh medium. Viral RNA copies of the collected samples were detected using qRT-PCR.

This experiment was repeated three times.
At 7 p.i., the supernatant harvested from C6/36, Aag2, and Sf9 cells was reinoculated back into Vero E6 cells.

Mosquito collection and preparation

From April to May, 2020, field mosquitoes were trapped by Maxttrac Trap (Guangzhou Changsheng Chemical Co., Ltd., China) in four sites in the region of Wuhan. The sampling sites include a residential area close to Huanan seafood market, East Lake region, the First People's Hospital of Jiangxia District, Huoshenshan Hospital, and the pig farm in Huangpi District. The mosquitoes were transported to the laboratory on time, taxonomically classified, and thereafter pooled (10 to 20 mosquitoes per pool) based on their collection sites and respective species.

The pooled mosquito samples were triturated by a Tgrinder electric tissue grinder OSE-Y30 (TIANGEN,China) on ice using sterile pestles with 400 μL of Roswell Park Memorial Institute (RPMI) medium. Mosquito homogenates were clarified by centrifugation at 12,000×rpm (4 °C for 15 min) and the supernatants were collected and stored at –80 °C.

RNA extraction and qRT-PCR

Two hundred supernatant samples obtained from cultured cells and mosquito homogenate were collected for total RNA extraction using QIAamp 96 virus QIAcube HT kit and QIAcube HT instrument (Qiagen, USA), as per the manufacturer's guidelines.

SARS-CoV-2 specific quantitative real-time reverse transcription PCR (qRT-PCR) was performed using primers (RBD-qF1: CAATGGTTTAACAGGCACAGG; RBD-qR1: CTCAAGTGTCTGTGGATCACG) and probe (RBD-probe1(FAM): ACAGCATCAGTAGTGTCAGCAATGTCTC) targeting the RBD region. The reaction was performed on a CFX96 Touch Real-time PCR Detection System (Bio-Rad, USA) using a PrimeScript RT-PCR Kit (Takara, China), in accordance with the manufacturer's instructions. R (version 3.5.1) was utilized in plotting the virus growth curves and statistical analysis.

Immunofluorescence Assay

To detect expression of viral proteins, the cells infected with SARS-CoV-2 were fixed with 4% paraformaldehyde for 36 hours. Thereafter, the cells were washed three times in phosphate buffer saline (PBS), and then blocked with 1% bovine serum albumin (BSA) at room temperature for 1 hour. Next, the cells were incubated with the primary antibody target NP protein for SARS-CoV-2 ([1:1000 dilution], kindly provided by Prof. Zhengli Shi) for 1 hour. Subsequently, the cells were washed again three times in PBS, and incubated with the secondary antibody (Goat Anti-Rabbit IgG H&L (Cy3 ®) [1:200 dilution]) (Abicam, USA) for 1 hour. The nuclei were stained with Hoechst 33342 dye (Beyotime, China), and the images were taken by fluorescence microscopy.

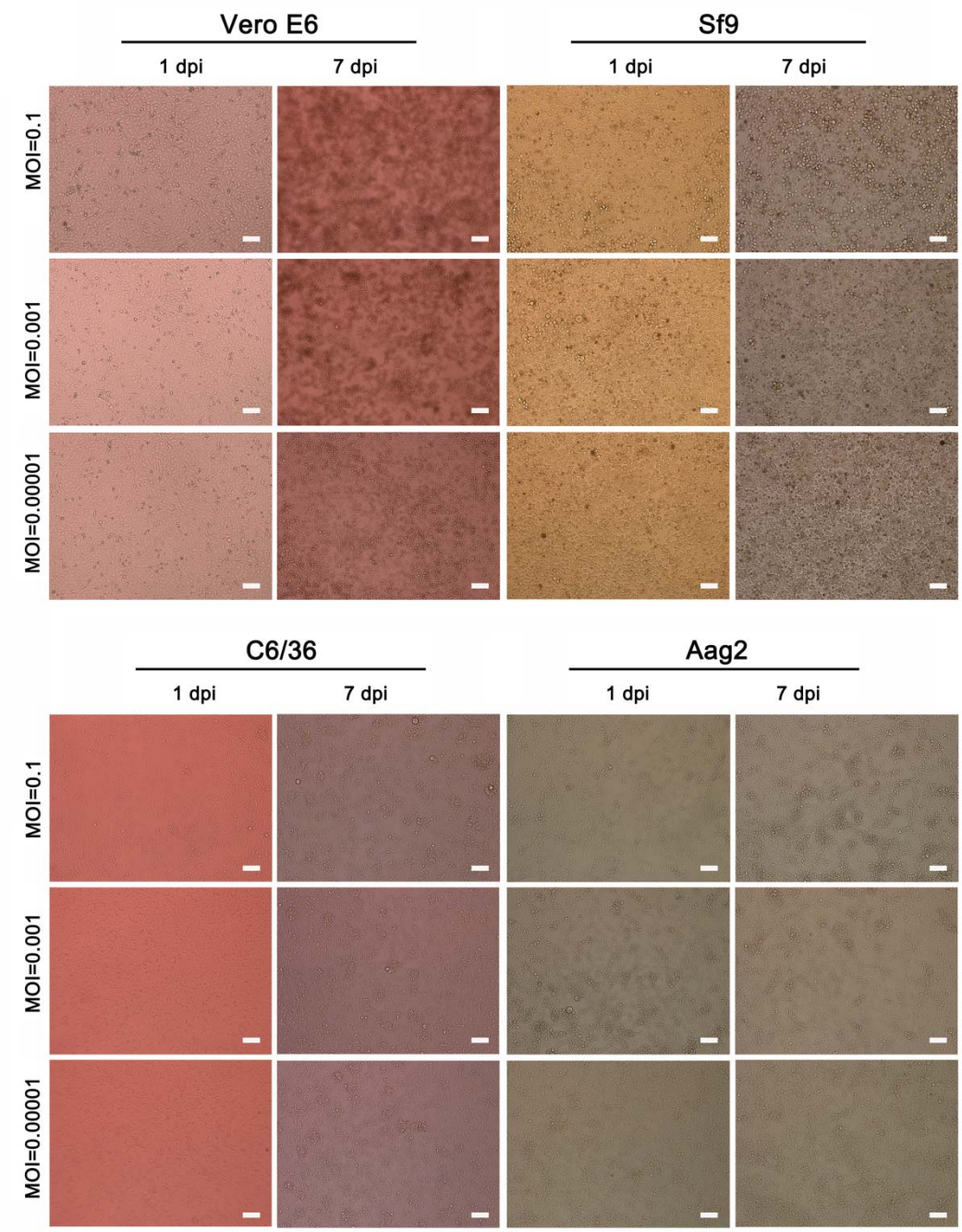

Fig. S1. Cytopathic effect detection in SARS-CoV-2 infected VeroE6, C6/36, Aag2, and Sf9 cells using low, mid and high MOI. Bars, 100 μm.

Table S1. Detailed mosquito sampling sites information in Wuhan.

Location	Collection time	GPS Coordinates	Mosquito-species	Mosquito number		
The First Poeple's Hospital (Jiangxia District)	2020.4.28	30°22′23″N, 114°18′52″E	*Culex*.spp	♀15/♂15	30	104
	2020.4.29		*Culex*.spp	♀1/♂3	4	
	2020.4.30		*Culex*.spp	♀7/♂1	8	
	2020.5.12		*Culex*.spp	♀21/♂10	31	
	2020.5.15		*Culex*.spp	♀20/♂11	31	
Residential area close to Huanan seafood market (Jianghan District)	2020.4.28	30°37′13″N, 114°15′25″E	*Culex*.spp	♀11/♂10	21	119
	2020.4.29		*Culex*.spp	♀9/♂18	27	
	2020.5.12		*Culex*.spp	♀5/♂23	28	
	2020.5.15		*Culex*.spp	♀15/♂28	43	
Huoshenshan Hospital (Caidian District)	2020.4.28	30°31′42″N, 114°4′50″E	*Culex*.spp	♀16/♂35	51	235
	2020.4.29		*Culex*.spp	♀11/♂51	62	
	2020.5.12		*Culex*.spp	♀13/♂27	40	
	2020.5.15		*Culex*.spp	♀58/♂24	82	
East Lake (Wuchang District)	2020.4.28	30°32′13″N, 114°22′52″E	*Culex*.spp	♀2/♂4	6	73
	2020.4.29		*Culex*.spp	♀7/♂4	11	
	2020.5.12		*Culex*.spp	♀21/♂27	48	
	2020.5.15		*Culex*.spp	♀7/♂1	8	
Pig Farm at Caizha (Huangpi District)	2020.5.16-18	30°52′52″N, 114°22′30″E	*Culex*.spp	353♀	353	353
			Anopheles.spp	281♀	281	281