Isolation and characterization of the first porcine Getah virus strain HNJZ-S1 from an aborted piglet in China

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Abstract

Severity of porcine reproductive and respiratory syndrome (PRRS) implicated co-infection with other pathogens in pig herd in China, which normally were found Japanese encephalitis virus (JEV), pseudorabies virus (PRV), porcine reproductive and respiratory syndrome virus (PRRSV), porcine circovirus, porcine parvovirus and classical swine fever virus (CSFV). Here we verified a specific fragment which was similar to Getah virus (GETV) by RT-PCR and sequencing from aborted fetuses in Henan province, China. Then we isolated and purified the stain virus. We named it as HNJZ-S1. Furthermore, we characterized HNJZ-S1 by passage and plaque titer, whole genome sequencing, Electron microscopy and animal infection experiments with mice and piglets. The data show that the full-length genome sequence of HNJZ-S1was 11689 bp with homologous 97.4%–99.3% identical to the GETV sequence available in GenBank. Phylogenetic tree analysis showed that HNJZ-S1 was closely related to mosquito isolate 12IH26 from Japan, horse isolates 14-I-605-C1 and 14- I-605-C2, and mosquito isolate HB0234 from China. Animal experiments showed that the isolate HNJZ-S1 caused abortion in pregnant mice and was lethal postnatal day 3 mice and piglets. This study provides important information on the tropism, infection, pathogenicity, environmental microbiology and public health implications of pig derived GETV and will also serve as a source of reliable research materials.

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Running Head: the first porcine Getah virus strain in China

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ABSTRACT

Severity of porcine reproductive and respiratory syndrome (PRRS) implicated co-infection with other pathogens in pig herd in China, which normally were found Japanese encephalitis virus (JEV), pseudorabies virus (PRV), porcine reproductive and respiratory syndrome virus (PRRSV), porcine circovirus, porcine parvovirus and classical swine fever virus (CSFV). Here we verified a specific fragment which was similar to Getah virus (GETV) by RT-PCR and sequencing from aborted fetuses in Henan province, China. Then we isolated and purified the stain virus. We named it as HNJZ-S1. Furthermore, we characterized HNJZ-S1 by passage and plaque titer, whole genome sequencing, Electron microscopy and animal infection experiments

with mice and piglets. The data show that the full-length genome sequence of HNJZ-S1was 11689 bp with homologous 97.4%–99.3% identical to the GETV sequence available in GenBank. Phylogenetic tree analysis showed that HNJZ-S1 was closely related to mosquito isolate 12IH26 from Japan, horse isolates 14-I-605-C1 and 14- I-605-C2, and mosquito isolate HB0234 from China. Animal experiments showed that the isolate HNJZ-S1 caused abortion in pregnant mice and was lethal postnatal day 3 mice and piglets. This study provides important information on the tropism, infection, pathogenicity, environmental microbiology and public health implications of pig derived GETV and will also serve as a source of reliable research materials.

Keywords: Swine, Getah virus, Abortive, Virus isolation, Pathogenicity

The mosquito borne Geta virus (GETV) belongs to the genus *Alphavirus* , family *Otariidae* . The viral genome of GETV is composed of positive single-stranded RNA including a 5'-cap structure, two open reading frames (ORFs) and a 3'-poly(A) tail. The two ORFs encodes the structural (C/capsid, E3, E2, 6K, E1) and non-structural proteins (NSP1 to NSP4) respectively, which are flanked by a 44 nt 26S junction region associated with transcription of an intracellular subgenomic 26S RNA(Strauss et al., 1994). Previous serological studies showed that this virus can infect multiple species, including humans(Li et al., 1992; Marchette et al., 1980), horses, pigs, cattle, monkeys and birds(Li et al., 2007). The symptom of infection includes rash, fever and joint edema in horses(KAMADA et al.,1991; KAMADA et al.,1991), reproductive disorders in pregnant sows and tremors in piglets, deaths only occur in pigs in the fetal stage(Yago et al., 1987; Kawamura et al., 1987).

GETV was well known that the first isolated from *Culex* sp. mosquitoes in Malaysia in 1955 and is now widely distributed across Asia (Ksiazek et al., 1981) (Morita and Igarashi, 1984)(Ksiazek et al., 1981; Morita et al., 1984; Leake et al., 1986; Bryant et al., 2005), Europe and Australia based on viral isolation and/or molecular epidemiological investigations(Fukunaga et al., 2000). In 1964, GETV was first isolated from mosquitoes on Hainan Island in China (Li et al., 1992) and was later detected in mosquitoes in several provinces and cities (Zhai et al., 2008). In addition, recently two high pathogenic swine GETV strains were detected in blue foxes in China (Shi et al., 2019).

In November 2016, during attempt to detect pathogens in aborted fetuses from a pig farm in Henan Province, we found that classical swine fever virus (CSFV), porcine reproductive and respiratory syndrome virus (PRRSV), Japanese encephalitis virus (JEV) and pseudorabies virus (PRV) were negative. Interestingly, a non-specific band was amplified using CSFV primers. The non-specific band was sequenced and analyzed by BLAST search against the GenBank database. It turned out that the amplicon had high homology to GETV. GETV was isolated by cell passage and plaque purification, and was subjected to electron microscopy observation, genome sequencing. Moreover, we determined the pathogenicity of the strain for pregnant and neonatal mice, and the piglets.

Materials and Methods

Sample collection

Sample collection (n=7) was conducted in a pig farm in Henan Province on September 12, 2016. The lungs and spleens of aborted fetuses were frozen and transferred to lab.

Viral screening

The lungs and spleens of aborted fetuses were immerged into precooled phosphate-buffered saline (PBS, PH7.4) at 1:5 and grounded. After three freeze-thaw cycles, the samples were centrifuged at 10,000 g for 10 min, and the supernatant was stored at -70°C prior to RNA extraction or virus isolation.

Total viral RNA was extracted from 300 ul of virus-containing supernatant using Trizol reagent following manufacture instructions (Invitrogen Carlsbad, CA). The reverse transcription reactions were performed using random primers and M-MLV reverse transcriptase (ThermoFisher Scientific China Ltd. Shanghai). CSFV was verified by the specific primers reported previously (Yanting et al., 2009). GETV C gene was amplified by forward primer ACCGAAGAAGCCGAAGAA and reverse primer GCACTCRAGGTCATACTTG de-

signed from the sequences of GETV HB0234 (accession number: EU015062) and 12IH26 (accession number: LC152056) available in GenBank. The amplified products were analyzed by 1.5% agarose gel electrophoresis and gel imaging (Sangon Biotech Co., Ltd., Shanghai).

Viral isolation and purification

The sample supernatant filtered by 0.22 μm filter (Millipore) for viral isolation. Marc-145 cells (stored in our lab) were cultured in 10 cm dishes up to confluency at 37°C in a 5% CO_2 incubator. 1 mL of filtered supernatant per dish was added into monolayer Marc-145 cells and incubated for an hour. Viral solution was discards and washed out with PBS twice. The cells were continued to culture with 2% fetal bovine serum in DMEM and monitored cytopathic effect (CPE) daily. Once CPE were observed, the virus-containing supernatant was collected, subjected to three freeze-thaw cycles at -70°C and room temperature, and then used to inoculate Marc-145 cells for viral passages.

To purify the virus, single plaques were picked and amplified with Marc-145 cells for three generations. And then virus was identified by RT-PCR using GETV C gene primers. The purified GETV were conducted the viral titer assessment at each time point, and the $TCID_{50}$ value was calculated according to the Reed-Muench method.

The whole viral genomic sequencing and phylogenetic analysis

The viral genome was amplified in fragments using specific primers reported previously [16], and the non-coding region was amplified according to the instructions of the 5'Full RACE and 3'Full RACE Core Set Ver.2.0 kits (Takara Biomedical Technology Co., Ltd, Dalian). After each fragment was ligated into the pMD18-T vector, E*scherichia coli* DH5α cells were transformed with recombinant plasmids. All the fragments were sequenced by Sangon Biotech Co., Ltd (Shanghai).

The sequenced fragments were spliced using DNAMAN software. Sequence similarity and phylogenetic analyses were conducted using DNAStar and MEGA4.0. Furthermore, the amino acid sequences of GETV E2 genes were compared and homology was analyzed using MegAlign. Phylogenetic analysis of amino acid sequences derived from the E2 gene were conducted using neighbor-joining methods (1000 bootstrap replicates) in MEGA4.0.

Virial particle investigation by electron microscope

Marc-145 cells were infected with HNJZ-S1 for 36 h. The cells were collected, subjected to three freeze-thaw cycles, and centrifuged at 10,000 g for 5 min. One drop of the supernatant was added to a copper mesh covered with Formvar film. Excess liquid was removed from the edge of the mesh after 3 min, then the mesh was air-dried at room temperature. The mesh was stained with negative dyeing including 2% phosphotungstic acid hydrate (pH 7.2) for 5 min, excess staining solution was removed with filter paper and air-dried at room temperature. The mesh was observed under JEM-1400 transmission electron microscopy (voltage 100 KV) (JEOL Ltd Japan).

Animal infections

Ten gestation Kunming mice at day 10 with GETV negative by oral and anal swab tests, 120 mice at the age of postnatal day 3, and Ten 3-day-old piglets without colostrum were randomly divided into two groups, respectively. One group were lightly anesthetized with isoflurane and intranasally inoculated with 10^6TCID_{50} of virus in 100ul DMEM, while pure DMEM was used for the other group as control. Animal were monitored for signs of infection (body weight loss, hunched posture, ruffled coat, lethargy and dehydration, abortion and death) daily. Dead or dying animal were examined, and Lung and spleen of newborn mice and piglets were dissected for viral assay by RT-PCR.

Data analysis

Differences were considered statistically significant at $P < 0.05$. Statistical tests were performed by GraphPad 7.0.

Results

GETV was detected in aborted fetuses

A nonspecific band was detected in aborted swine fetuses using CSFV-specific RT-PCR primers (**Figure 1A**). The amplicon was purified and sequenced. The sequence was highly homologous (96.45%) to the gene E2 and E3 sequence of *Alphavirus* GETV by BLAST indicating GETV infection. The sample supernatant was used to inoculate Marc-145 cells for viral isolation and purification. GETV was detected at the third and fourth passage of the cells using the GETV specific primers (**Figure1B**).

Verification and Replication of GETV in vitro

The filtered sample supernatant was used to inoculate Marc-145 cells, and obvious CPE was observed after three generations (**figure 2A**). A single plaque clone was obtained for plaque purification. Compared with the control group, cells infected with the isolate became rounded and partly exfoliated about 24-hour post-infection. A large number of cells exfoliated after 48 hours, at which time most of the adherent cells became rounded. Both RT-PCR detection and sequencing results confirmed the isolate as GETV, and it was named HNJZ-S1. Plaque forming unit show round (**Figure 2B**). Monitoring of proliferation showed that the virus titer peaked at $10^{7.25}$ $TCID_{50}$/mL around 36 h post-inoculation Marc-145 cells, and then began to decrease slowly (**figure C**).

The morphology of HNJZ-S1 was similar to *Alphavirus* by electron microscopy

Electron microscopic observation showed the diameter of the virus particles were about 70 nm with spherical shape, capsule, fibril processes (**Figure 3**). It indicated HNJZ-S1 has the typical morphological characteristics of *Alphaviruses* .

The full-length genome sequence of HNJZ-S1

Fragments of the HNJZ-S1 genome were amplified using specific primers along with 3' and 5' RACE kits. The full-length genome sequence was 11689 bp in length with base composition 19.57% T, 26.16% C, 28.08% A and 26.19% G. The genomic composition and the size of the ORFs were basically consistent with GETV strains collected in GenBank. The genome included a 5' untranslated region (UTR), a 3' UTR and two ORFs in between. Seventy-eight nucleotides at the 5' end and 401 nucleotides at the 3' end were UTRs; the 7407 nucleotides following the 5' UTR encoded four non-structural proteins (NSP1, NSP2, NSP3 and NSP4), and the 3759 nucleotides before the 3' UTR encoded five structural proteins (C, E3, E2, 6K and E1). The 44 nucleotides in between the two ORFs were non-coding connecting regions (**Table 1**). The whole sequence was uploaded into the GenBank with accession number KY3638.

Phylogenetic analysis of HNJZ-S1

Whole genome sequence alignment showed that the sequence similarity between HNJZ-S1 and most reference strains available in GenBank was 97.4–99.6%, which the lowest (97.4%) one was Sagiyama strain isolated from Japan in 1956, while the highest one was JL17-08 strain isolated from China (99.6%) (**Table 2**). Phylogenetic tree analysis of nucleotide sequence confirmed above results which HNJZ-S1 was clustered into mosquito isolates such as JL17-08, JL1707 and HB0234 isolated from China (**Figure 4A**). Phylogenetic analysis of amino acid sequences derived from the E2 gene showed that HNJZ-S1 was also closely related to reference strains published in the past 30 years (**Figure 4B**).

GETV HNJZ-S1 has obvious pathogenicity in pregnant mice and neonatal mice the

Five pregnant mice gave the birth of 57 mummified fetuses (**Figure 5A**) and miscarriages (**Figure 5B**) between 3 and 6 days after exposure with HNJZ-S1, as well as 11 normal spring. Meanwhile, 67 normal offspring were born in five litters from the control group (**Figure 5C**). There were no mummified fetuses or miscarriages found in the control group. Thus, composition of average litter size was significantly different between the HNJZ-S1 infection and non-infect groups (**Figure 5D**). GETV was confirmed from aborted fetuses and premature offspring by RT-PCR.

Mice at age of postnatal day 3 were incubated with HNJZ-S1. All the infected group showed severe hindlimb paralysis and slow growth after three days exposure, and there were no survivals beyond 10 days after exposure (**Figure 6A**). There was no obvious abnormality in newborn mice of the control group (**Figure 6B**).

HNJZ-S1 has obvious lethal effect on 3-day-old piglets

Given the pathogenicity for pregnant and neonatal mice, we performed the infection of neonatal day 3 piglets. After 12 hours exposure, the piglets showed red skin in the infection group. Their body temperatures increased to >40°C, with the peck of 40.7°C (**Figure 7A**). Later, the piglets developed yellow dilute feces. All the piglets died between 18- and 32-hours post-infection (**Figure 7B**). There were no disease signs or mortality observed in piglets of the control group.

Discussion

In China, GETV was first identified in Hainan Province in southern China in 1964, and one GETV strain designated M-1 was isolated from wild-caught mosquitoes (Li et al., 1992). Since then, several investigators have demonstrated that GETV is present in mosquitoes in China with a wide distribution (Shi et al., 2019). Serum antibody against GETV can be detected in humans, pigs, horses, cows, sheep, kangaroos, and other animals (Zhang et al., 2016). Before 2016, studies about GETV were limited to serological investigations of the virus in human and animals, while the isolation and identification of the virus were limited only in mosquitoes in China(Shi et al., 2019; Li et al., 2019; Yang et al., 2018). There were few studies focus on pathogenic analysis of GETV on pig. In this study, GETV named HNJZ-S1 was isolated from pig herd for the first time in China. We characterized HNJZ-S1 by passage and plaque titer, whole genome sequencing, Electron microscopy and animal infection experiments with mice and piglet.

Severity of porcine reproductive and respiratory syndrome (PRRS) implicated co-infection with other pathogens in pig herd in China, normally found JEV, PRV, PRRSV, porcine circovirus, porcine parvovirus and CSFV. Therefore, our laboratory often performed multiplex RT-PCR or PCR assay to identify pathogens related to pig reproductive disorder. Recently our laboratory has used a pair of RT-PCR primers to detect the E2 gene of CSFV since 2007. Amplicon was expected to be 276 bp. However, from September 2016, samples have shown a nonspecific band around 1000 bp instead. Curiously we sequenced the fragment. The sequence was identical between 8147bp to 9469bp of the GETV genome. The results of the whole genomic sequence alignment showed that there is no homology between CSFV and GETV. It was an accident that the sequence of the pair primers matched both viral genomes by 96.45%. However, we did not find any one of normal testing pathogens, such as JEV, PRV, PRRSV, porcine circovirus, porcine parvovirus and CSFV by RT-PCR or PCR. We only detected one non-specific band using CSFV primers by RT-PCR. Therefore, abortion in this case may have been caused by something new pathogen. We purified virus using Marc-145 cells by single CPE picking and then perform the whole genomic sequencing. We named this strain HNJZ-S1 and uploaded it into GenBank accession KY3638. The data show the full-length genome sequence of HNJZ-S1was 11689 bp with homologous 97.4%–99.3% identical to the GETV strains available in GenBank (**Table 1**). Phylogenetic analysis showed that HNJZ-S1 was closely related to Japanese mosquito isolate 12IH26, horse isolates 14-I-605-C1 and 14- I-605-C2, and Chinese mosquito isolate HB0234 (**Table 2 and Figure 4**). Alignment of the isolate reported in this study with available GETV full-length genome sequences showed that the genetic conservation of GETV was higher than that of other single-stranded positive sense RNA viruses such as PRRSV and PEDV. The genomes of strains isolated over the past 20 years were highly similar. Although the results of this study showed that HNJZ-S1 was closely related to Chinese mosquito isolates, Japanese and Korean pig and horse isolates, whether mosquitoes can transmit GETV independently among the above three countries is a question worth further study. In addition, other routes of transmission should also be considered, such as air transport of viral vectors, migratory birds and other blood-sucking insects.

Given the clinical findings and HNJZ-S1 verification, we investigated the morphology of viral particle and performed pathogenicity. Our results showed that the GETV HNJZ-S1 strain could cause death of 3-day-old newborn mice (**Figure 5 and 6**) and 3-day-old piglets (**Figure 7**). No obvious lesions were observed in

dying piglets except in the thinner intestinal wall. GETV nucleic acids were detected in the brain, spleen, lung, kidney, bladder, small intestine and rectum of dying piglets, and HNJZ-S1 obtained from piglet brain tissue. Animal experiments and studies reported that GETV mainly infects pregnant and newborn animals (Yage et al., 1987; Yang et al., 2018) coordinating with this study. Therefore, the study of human GETV infection should focus on serological investigations of pregnant women or newborns, rather than all people. This may help researchers to determine whether GETV poses a risk to humans. Furthermore, pig-derived GETV was described and isolated for the first time in China due to a nonspecific amplification event during CSFV RT-PCR detection in our laboratory, which indicated that the original CSFV amplification primers need to be optimized.

Our results confirmed the existence of GETV infection in Chinese pigs and provided valuable data for the studies on GETV pathogenicity, pathogenic mechanism, public health and might opened a new field for the study on GETV infection. China is the world's largest pork producer and consumer and the possibility that human contact with GETV-infected pigs and their products may frequent. Therefore, the epidemiology of pig diseases in China is closely related to human health. This study is not only a warning for public health safety, but also takes a step toward understanding transmission of the virus.

We respect that there are several limitations to our study. Although GETV had lethal effects on newborn piglets as well as obvious pathogenicity in pregnant mice and newborn mice shown in this study, experiments are required to further elucidate the basis for pregnant sow. GETV has been studied for more than 60 years. Compared with other viruses of the same genus, however, much remains unknown regarding the transmission mechanisms and pathogenicity of GETV. A more comprehensive and in-depth understanding of this virus my help us to effectively prevent and control human and animal diseases caused by GETV.

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Conflict of interest statement

The authors declare no conflict of interest.

Ethics Statements

All the sample collections were performed after obtaining verbal approval of farm owner. This study was approved by the Ethics Review Committee of the National Institute for Viral Disease Control and Prevention, Chinese Center for Disease Control and Prevention. All the animal studies were conducted in accordance with the principles of Henan agricultural university Animal Care and Use Committee (IACUC).

Data Availability Statement

All data generated or analyzed during this study are included in the article.

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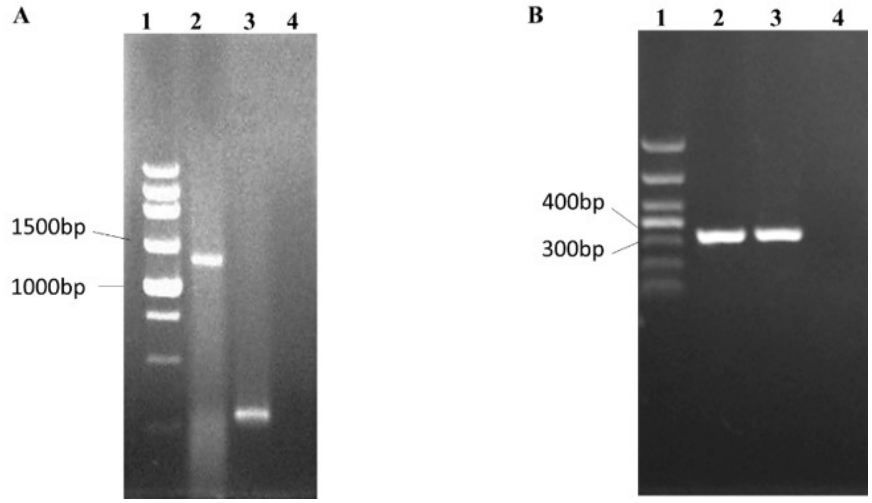

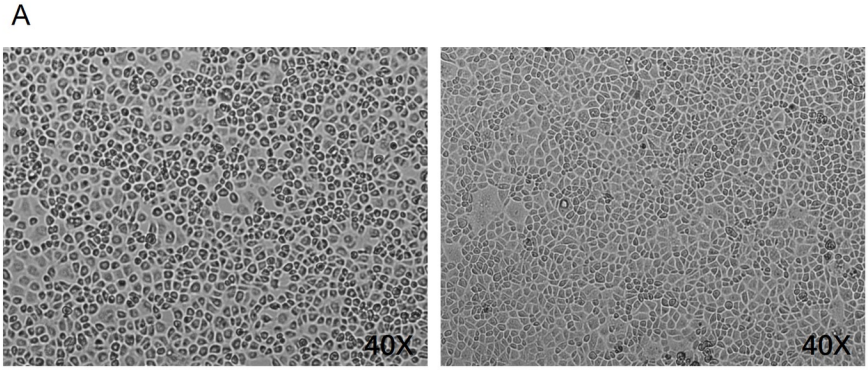

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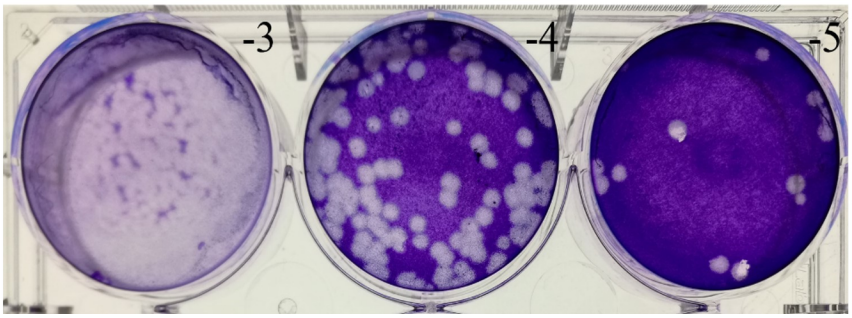

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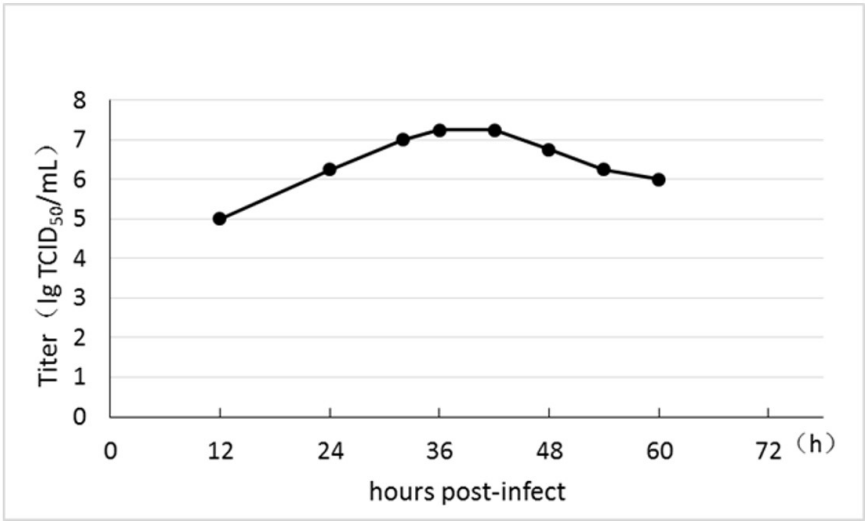

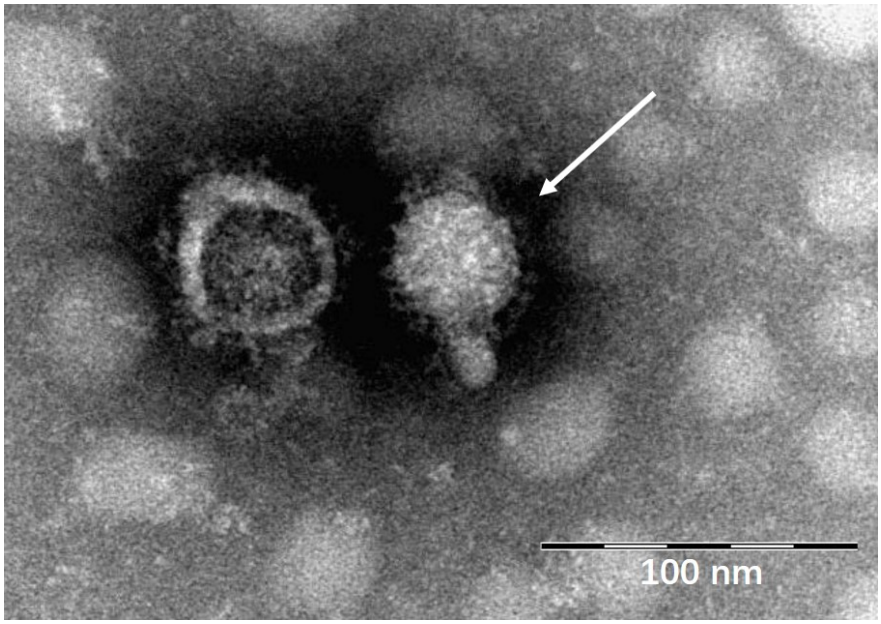

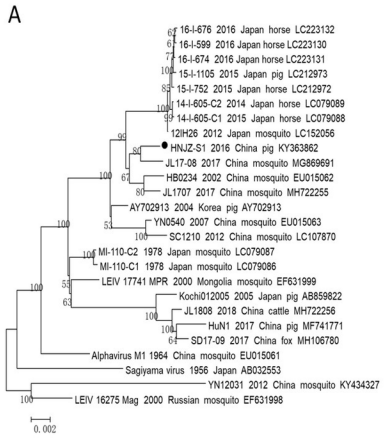

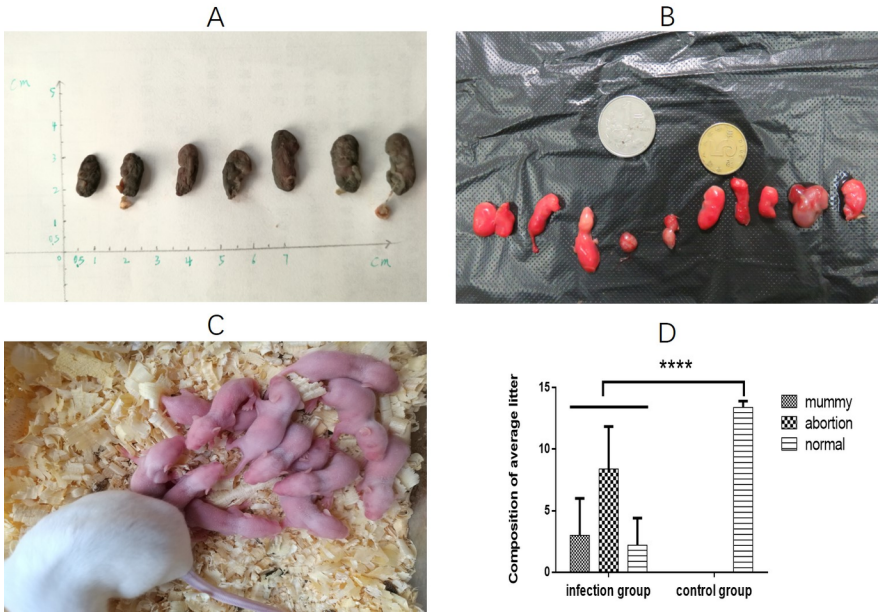

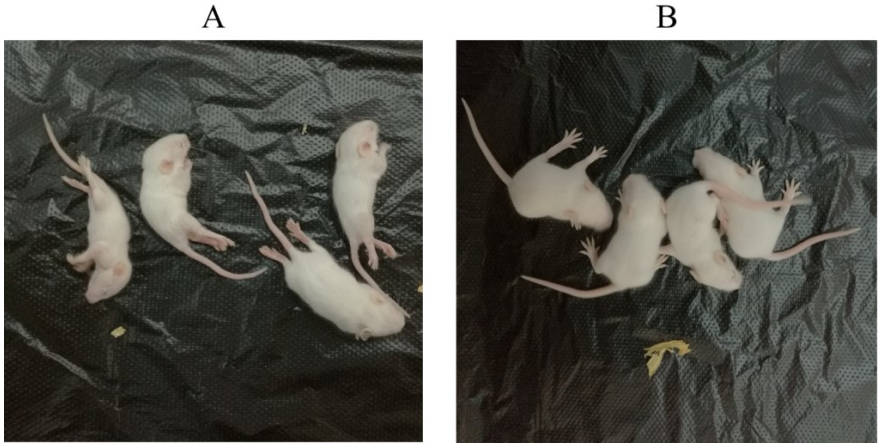

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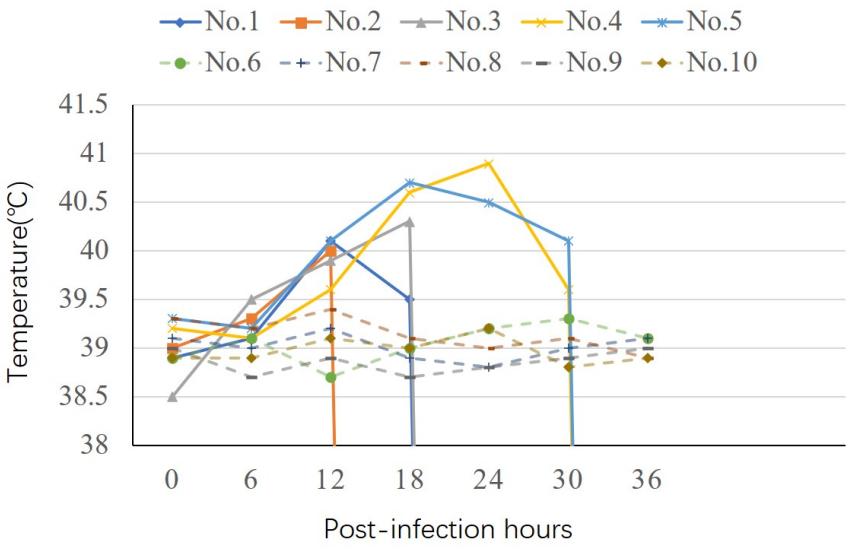
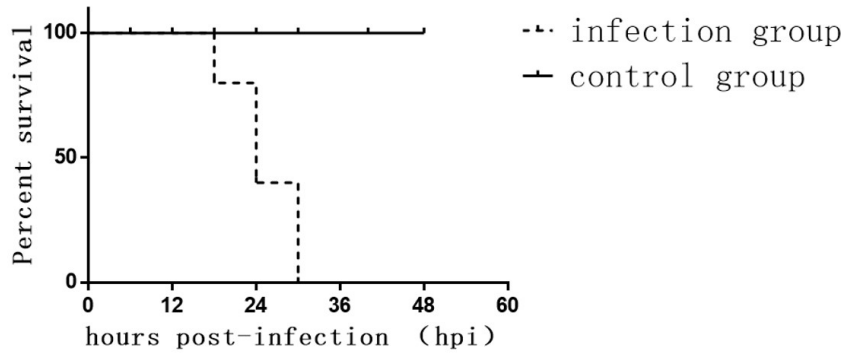


B



Nucleotide	Protein	Location	Length/ nt	Length/aa
5'UTR	/	1-78	78	/
Non-structural protein ORF	NSP1-4	79-7482	7404	2468
Junction	/	7483-7526	44	/
structural protein ORF	C-E3-E2-6K-E1	7527-11288	3762	1254
3'UTR	/	11289-11689	401	/

Percent Identity

	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	
1		97.4	98.2	98.7	98.7	97.7	98.7	99.2	99.3	98.8	97.9	96.3	99.2	99.2	99.2	99.2	99.2	99.1	99.2	99.1	99.4	97.6	99.6	97.8	99.0	97.9	**1**
2	2.7		98.2	98.0	98.0	98.1	98.0	97.5	97.7	97.3	97.3	96.7	97.4	97.3	97.3	97.3	97.3	97.3	97.3	97.3	97.4	97.0	97.3	97.2	97.5	97.3	**2**
3	1.8	1.9		98.9	98.9	98.4	98.9	98.3	98.6	98.2	98.1	97.1	98.2	98.2	98.1	98.1	98.1	98.1	98.1	98.1	98.2	97.8	98.2	98.0	98.3	98.0	**3**
4	1.3	2.0	1.1		99.9	98.4	99.4	98.8	99.1	98.7	98.6	97.0	98.6	98.6	98.6	98.6	98.6	98.5	98.6	98.6	98.7	98.3	98.7	98.5	98.8	98.6	**4**
5	1.3	2.0	1.1	0.1		98.3	99.4	98.8	99.1	98.7	98.6	97.0	98.7	98.6	98.6	98.6	98.6	98.6	98.6	98.6	98.7	98.3	98.7	98.5	98.8	98.6	**5**
6	2.4	1.9	1.6	1.7	1.7		98.4	97.7	98.1	97.6	97.6	97.8	97.7	97.6	97.6	97.6	97.6	97.6	97.6	97.6	97.6	97.3	97.6	97.5	97.8	97.5	**6**
7	1.3	2.0	1.1	0.6	0.6	1.7		98.7	99.1	98.6	98.6	97.0	98.6	98.6	98.6	98.5	98.6	98.5	98.6	98.6	98.7	98.3	98.7	98.5	98.8	98.6	**7**
8	0.8	2.6	1.8	1.3	1.3	2.3	1.3		99.3	98.9	97.9	96.3	99.2	99.1	99.1	99.1	99.1	99.1	99.1	99.1	99.6	97.6	99.2	97.8	99.1	97.9	**8**
9	0.7	2.3	1.4	0.9	0.9	2.0	0.9	0.7		99.2	98.3	96.7	99.2	99.2	99.1	99.1	99.1	99.1	99.1	99.2	98.0	99.2	98.1	99.4	98.2		**9**
10	1.2	2.7	1.9	1.4	1.4	2.4	1.4	1.1	0.8		97.9	96.3	98.8	98.8	98.8	98.7	98.7	98.7	98.7	98.9	97.6	98.9	97.8	99.7	97.8	AY702913 2004 Korea pig AY702913	**10**
11	2.1	2.7	2.0	1.4	1.4	2.5	1.4	2.1	1.8	2.2		96.3	97.9	97.8	97.8	97.8	97.8	97.8	97.8	97.9	99.2	97.9	99.4	98.0	99.6	SC1210 2012 China mosquito LC107870	**11**
12	3.8	3.4	3.0	3.1	3.1	2.3	3.1	3.8	3.4	3.8	3.8		96.3	96.3	96.3	96.2	96.2	96.2	96.2	96.2	96.3	96.1	96.3	96.3	96.4	96.3	Kochi012005 2005 Japan pig AB859822	**12**
13	0.8	2.7	1.8	1.4	1.3	2.4	1.4	0.8	0.8	1.2	2.2	3.8		99.9	99.9	99.9	99.9	99.9	99.9	99.9	99.2	97.6	99.2	97.7	98.9	97.8	YN12031 2012 China mosquito KY434327	**13**
14	0.8	2.7	1.9	1.4	1.4	2.4	1.4	0.9	0.9	1.2	2.2	3.8	0.1		100.0	99.9	99.9	99.9	99.9	99.9	99.1	97.6	99.1	97.7	98.9	97.8	12IH26 2012 Japan mosquito LC152056	**14**
15	0.8	2.7	1.9	1.4	1.4	2.4	1.4	0.9	0.9	1.3	2.2	3.8	0.1	0.0		99.9	99.9	99.9	99.9	99.9	99.1	97.6	99.1	97.7	98.9	97.8	14-I-605-C1 2015 Japan horse LC079088	**15**
16	0.9	2.8	1.9	1.5	1.4	2.5	1.5	0.9	0.9	1.3	2.3	3.9	0.1	0.1	0.1		99.9	99.9	100.0	99.9	99.1	97.5	99.1	97.6	98.9	97.7	14-I-605-C2 2014 Japan horse LC079089	**16**
17	0.8	2.7	1.9	1.4	1.4	2.4	1.4	0.9	0.9	1.3	2.2	3.9	0.1	0.1	0.1	0.1		99.9	99.9	99.9	99.1	97.5	99.1	97.7	98.9	97.7	15-I-1105 2015 Japan pig LC212973	**17**
18	0.9	2.8	1.9	1.5	1.4	2.5	1.5	0.9	0.9	1.3	2.3	3.9	0.1	0.1	0.1	0.1	0.1		100.0	100.0	99.1	97.5	99.1	97.6	98.9	97.7	15-I-752 2015 Japan horse LC212972	**18**
19	0.8	2.8	1.9	1.4	1.4	2.5	1.5	0.9	0.9	1.3	2.3	3.9	0.1	0.1	0.1	0.0	0.1	0.0		100.0	99.1	97.5	99.1	97.6	98.9	97.7	16-I-676 2016 Japan horse LC223132	**19**
20	0.9	2.8	1.9	1.5	1.4	2.5	1.5	0.9	0.9	1.3	2.3	3.9	0.1	0.1	0.1	0.1	0.1	0.0	0.0		99.1	97.5	99.1	97.7	98.9	97.7	16-I-674 2016 Japan horse LC223131	**20**
21	0.6	2.7	1.8	1.3	1.3	2.4	1.4	0.4	0.8	1.1	2.2	3.8	0.8	0.9	0.9	0.9	0.9	0.9	0.9	0.9		97.6	99.5	97.7	99.1	97.8	16-I-599 2016 Japan horse LC223130	**21**
22	2.4	3.0	2.3	1.8	1.8	2.8	1.7	2.4	2.1	2.5	0.8	4.0	2.5	2.5	2.5	2.6	2.5	2.6	2.5	2.5	2.5		97.6	99.6	97.7	99.5	JL1707 2017 China mosquito MH722255	**22**
23	0.4	2.7	1.8	1.3	1.3	2.4	1.4	0.8	0.8	1.1	2.2	3.8	0.8	0.9	0.9	0.9	0.9	0.9	0.9	0.9	0.5	2.5		97.8	99.1	97.8	HuN1 2017 China pig MF741771	**23**
24	2.3	2.8	2.1	1.5	1.5	2.6	1.5	2.2	1.9	2.3	0.6	3.8	2.3	2.4	2.4	2.4	2.4	2.4	2.4	2.4	2.3	0.4	2.3		97.9	99.8	JL17-08 2017 China mosquito MG869691	**24**
25	1.0	2.6	1.7	1.2	1.2	2.3	1.2	0.9	0.6	0.3	2.0	3.7	1.1	1.1	1.1	1.2	1.1	1.2	1.1	1.1	0.9	2.3	0.9	2.1		98.0	SD17-09 2017 China fox MH106780	**25**
26	2.2	2.8	2.0	1.5	1.5	2.5	1.4	2.2	1.8	2.2	0.4	3.8	2.2	2.3	2.3	2.3	2.3	2.3	2.3	2.3	2.2	0.5	2.2	0.2	2.1		YN0540 2007 China mosquito EU015063	**26**
	1	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**12**	**13**	**14**	**15**	**16**	**17**	**18**	**19**	**20**	**21**	**22**	**23**	**24**	**25**	**26**	JL1808 2018 China cattle MH722256	

HNJZ-S1 2016 China pig KY363862

Sagiyama virus 1956 Japan AB032553

Alphavirus M1 1964 China mosquito EU015061

MI-110-C1 1978 Japan mosquito LC079086

MI-110-C2 1978 Japan mosquito LC079087

LEIV_16275_Mag 2000 Russian mosquito EF631998

LEIV_17741_MPR 2000 Mongolia mosquito EF631998

HB0234 2002 China mosquito EU015062