



RESEARCH ARTICLE

Re-isolation of Wuxiang Virus from Wild Sandflies Collected from Yangquan County, China

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Abstract

We previously isolated a new species of the genus *Phlebovirus* from wild sandflies collected from Wuxiang County in central China, which named the Wuxiang virus (WUXV). In this study, we re-isolated the WUXV from wild sandflies collected from two villages in Yangquan County, China in 2019. Four virus isolates that caused cytopathic effects in BHK-21 cells were successfully isolated from sandfly specimens collected from chicken pens and sheep pens. Phylogenetic analyses of the *L*, *M* and *S* gene segments of the viruses revealed that the four virus strains represented the previously isolated WUXV. The minimum infection rate (MIR) of the virus isolated from the sheep pen was 3.21, and the MIR of the virus isolated from the chicken pen was 3.45. The positive rates of Wuxiang virus neutralizing antibodies in serum samples of local healthy people and domestic chickens were 8.7% (4/46) and 100% (4/4), respectively, suggesting that Wuxiang virus can infect human and animal. In view of the fact that Wuxiang virus is infectious to humans and animals and has a relatively wide geographical distribution in China, it is of great public health significance to strengthen the investigation and study on the infection status of Wuxiang virus in humans and animals.

Keywords Sandfly · Phlebovirus · Wuxiang virus (WUXV)

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Introduction

Phleboviruses belong to the *Phenuiviridae* family of the order *Bunyavirales* (Maes *et al.* 2019). In March 2020, the International Committee on Taxonomy of Viruses (ICTV) released the latest *Phenuiviridae* classification guidelines. The *Phenuiviridae* family includes 60 different virus types, such as the sandfly-borne Toscana virus (TOSV), Toros virus (TORV), the mosquito-borne Rift Valley fever virus, and sandfly fever Naples virus. According to the latest ICTV classification guidelines, the tick-borne severe fever with thrombocytopenia syndrome virus (SFTSV) and Heartland virus (HLV) are not included in the *Phlebovirus* genus (Kuhn *et al.* 2020; Walker *et al.* 2020). The number of newly identified Phleboviruses has increased drastically over the past few years, suggesting that Phleboviruses are abundant in nature.

In 2018, we isolated a sandfly-borne Wuxiang virus (WUXV) from a sandfly in China; we termed this isolate SXWX1813-2 virus (Wang *et al.* 2020). SXWX1813-2 virus inoculating in BHK-21 cells led to significant

cytotoxicity. In newborn mice, the virus caused morbidity and death. Whole-genome sequencing of this new WUXV isolate revealed three gene segments (*L* gene, *M* gene, and *S* gene). Molecular genetic evolution analyses of the viral genome indicated that the WUXV differs phylogenetically from other Phleboviruses, forming an independent phylogenetic branch (Wang *et al.* 2020).

The SXWX1813-2 virus is the first Phlebovirus isolated from a sandfly in East Asia (Depaquit *et al.* 2010; Liang *et al.* 2018; Wang *et al.* 2020). However, it remains unclear whether there are sandfly-borne viruses in nature in other regions. In 2019, we isolated viruses from sandflies collected from Yangquan County, and identified four isolates that can cause cytopathic effects (CPE) in BHK-21 cells. Molecular analyses showed that all four isolates belonged to the WUXV. Herein, we report the re-isolation of a new Phlebovirus from a sandfly collected in a natural environment in China. The results also showed that local healthy people and chicken serum samples were positive for neutralizing antibody of Wuxiang virus.

Materials and Methods

Sample Collection

Wild sandfly specimens were collected from chicken, sheep, and cattle pens used to breed blood-sucking insects. Specimens were collected using an insect collector (MM200; Guangzhou Changsheng Chemical Co., Ltd., Guangdong, China). The specimen collectors were placed at each sampling point from 6 PM to 7 AM. The collected insects were placed in a low-temperature refrigerator for 20 min and then kept on ice during morphological identification and isolation of blood-sucking insects. Sandfly specimens were divided based on the collection environment and stored in liquid nitrogen until further testing (Fu *et al.* 2017; Song *et al.* 2017; Wang *et al.* 2020).

Whole blood of local healthy people and chicken were also collected at place where the sandfly specimens were collected, and the sera were obtained after centrifugation at 2000 rpm for 20 min. Sera samples were transported to the laboratory with ice platoon, stored in a low temperature refrigerator for testing.

Cells

The golden hamster kidney cells BHK-21 were maintained in 90% Eagle's medium (made in-house) supplemented with 7% fetal bovine serum (FBS; Invitrogen, Carlsbad, CA, USA), 1% penicillin and streptomycin (100 U/mL), 1% glutamine (30 g/L), and 1% NaHCO₃. The *Aedes albopictus* egg cells C6/36 were cultured in 89% RMPI

1640 (Invitrogen) supplemented with 10% FBS (Invitrogen) and 1% penicillin and streptomycin (100 U/mL). BHK-21 and C6/36 cells were maintained in a 5% CO₂ humidified atmosphere. BHK-21 cells were cultured at 37 °C, whereas C6/36 cells were kept at 28 °C (Fu *et al.* 2017; Song *et al.* 2017; Wang *et al.* 2020).

Virus Isolation

Sandfly specimens were pooled into 23 groups with 50–100 sandflies in each group. Specimens were placed in a glass grinder and homogenized in grinding solution (93% Eagle's medium, 5% penicillin and streptomycin [100 U/mL], 1% glutamine [30 g/L], and 1% NaHCO₃) at 4 °C. Samples were centrifuged (4 °C, 12,000 rpm, 30 min), and 100 µL of the supernatant was inoculated into BHK-21 cell and C6/36 cells seeded at a density of 80% in 24-well plates (Corning Inc., New York, NY, USA). After inoculation, BHK-21 and C6/36 cells were cultured in a 5% CO₂ atmosphere at 37 °C and 28 °C, respectively. CPE was monitored under a microscope every 12 h. When CPE became evident, viral samples were collected and stored at –80 °C until further characterization. Specimens were blindly passaged three times; cells without CPE were discarded (Fu *et al.* 2017; Song *et al.* 2017).

Viral RNA Extraction and cDNA Synthesis

Viral total RNA was extracted using a Viral RNA Mini Kit (QIAamp; Qiagen, Valencia, CA, USA) following the manufacturer's instructions. Total RNA was extracted from sandfly specimens after homogenization, as well as from the supernatant of infected BHK-21 and C6/36 cells. The extracted RNA was immediately placed in a 65 °C water bath for 10 min and then transferred on ice for 2 min. RNA samples (32 µL) were used for first-strand cDNA synthesis (Ready-To-Go You-Prime First-Strand Beads; GE Healthcare, Little Chalfont, UK). The reaction also included 1 µL of random primers (TaKaRa, Shiga, Japan) and was incubated at 37 °C for 1 h. The resulting cDNA was used immediately, or stored at –40 °C until further use (Feng *et al.* 2017; Ren *et al.* 2017; Wang *et al.* 2020).

Gene Amplification and Sequencing

Viral genes were amplified by polymerase chain reaction (PCR), which included cDNA template, GoTaq® Green Master Mix 2 × (Promega, Madison, WI, USA), and 10 µmol/L of each primer (total reaction volume of 25 µL). Amplification products were resolved by 1% agarose gel electrophoresis and subjected to sequencing (Feng *et al.* 2017; Ren *et al.* 2017; Wang *et al.* 2020). The sequences of the primers used in this study were previously described

(Wang *et al.* 2020). We used nine primer pairs for WUXV *M* genes and six for WUXV *S* genes. Four *M* gene amplification primers and two *S* gene amplification primers did not yield amplification products. Therefore, amplification primers were redesigned for genes that could not be amplified. Specifically, we designed five *M* gene amplification primer pairs and two *S* gene amplification primer pairs. We designed eleven *L* gene primer pairs for PCR amplification. By using these primers, we obtained amplification products for all viral genes. The sequencing primers used in this study are listed in Supplementary Table S1.

Nucleotide Sequence Analysis

BLAST analyses were conducted using the viral gene nucleotide sequences. SeqMan software (DNASar, Madison, WI, USA) was used for sequence splicing and quality analyses, and BioEdit (version 7.0; <http://www.mbio.ncsu.edu/BioEdit/bioedit.html>) was used for multiple sequence alignment. System evolution analysis was conducted using MEGA 6.0 software (<https://www.megasoftware.net/>) and the neighbor-joining method (bootstrap value of 1000). MegAlign was used for homology analysis of nucleotide and amino acid sequences (DNASar) (Feng *et al.* 2017, 2019; Song *et al.* 2017; Wang *et al.* 2020). Viral gene sequences were obtained from GenBank (NCBI, Bethesda, ND, USA) for comparison.

Molecular Identification of Sandflies

DNA extracted from pools of sandflies positive for WUXV was used for mitochondrial cytochrome C oxidase I (COI) amplification (Folmer *et al.* 1994) and sequencing. The sequences of the upstream COI primer (LCO1490; ggt-caacaaatcataaagatattgg) and downstream COI primer (HC02198; taaacttcagggtgacacaaaatca) were used for BLAST analyses of GenBank data to determine the sandfly species (Folmer *et al.* 1994; Wang *et al.* 2020).

Minimum Infection Rate (MIR)

The infection rate of a pool of positive specimens was calculated using the following formula: pool infection rate = number of positive specimens ÷ total number of specimens × 100%. Assuming that each positive pool contained only one infected sandfly, we calculated the MIR of 1000 sandflies as follows: number of positive specimen pools (number of infected sandflies) ÷ total number of sandflies × 1000 (Feng *et al.* 2012).

Detection of Neutralizing Antibody

BHK-21 cells were introduced into a 6-well plate and grown into monolayer cells the next day, with a coverage rate of 80%. The viral solution was diluted with serum-free Eagle's solution to a concentration of 200 PFU/0.1 mL. Serum samples were placed in 56 °C water bath for 30 min to inactivate complement. Serum samples were diluted with serum-free Eagle's solution at 1:5. Set back titration of 100 PFU, 50 PFU, 10 PFU. The diluted virus solution was mixed with serum in equal proportion and incubated in 37 °C incubator for 1 h. 0.1 mL of the serum and virus mixture was added to each well, and the virus back titration was added to the wells at the same time for 2 wells per titer, and incubated at 37 °C for 1 h. Then 4 mL methylcellulose was added to cover cells per well, and the cells were cultured in incubator at 37 °C. Cytopathy of cells was observed daily. When the plaques were obvious, crystal violet staining was performed, and plaques were observed and counted. The plaque reduction neutralization test (PRNT50) was used as the standard, when the corresponding plaque number was less than that of the standard, and the dilution ratio of serum samples was considered as the neutralization titer (Cao *et al.* 2016).

Results

Sandfly Sample Collection

Sandfly specimens were collected in June 2019 from sheep, chicken, and cattle pens in two villages in Yangquan County, Shanxi Province. A total of 1535 sandflies were collected from the two villages (1047 and 488 from the first and second villages, respectively). In total, 935 of these sandflies were collected from a sheep pen, 290 from a chicken pen, and 310 from a cattle pen (Table 1).

Virus Isolation

The collected sandflies were pooled into 23 groups before homogenization, and the supernatant of each pool was inoculated into BHK-21 and C6/36 cells after homogenization. SXYQ1916 sandfly specimens were also inoculated into BHK-21 cells, and CPE was observed after 3 days of inoculation. Notably, cells exhibited significant shrinkage and shedding (Fig. 1). Sandfly specimens were inoculated into BHK-21 cells, and continued culture and passaging of cells, then four virus strains were isolated (Table 2). In contrast, the inoculation of sandfly supernatants into C6/36 cells did not lead to CPE. And we obtained nothing after PCR amplification using C6/36 cell supernatants and WUXV-specific primers.

Table 1 Collection sites of sandfly specimens in Yangquan County, Shanxi Province in 2019.

Collection site	Breeding places			Total
	Sheep pen	Chicken pen	Cattle pen	
1	554	183	310	1047
2	381	107	/	488
Total	935	290	310	1535

"/" represents the missing cattle pen.

"1" indicates the first sampling village (east longitude 113°37'12", north latitude 37°46'48"). There were more than 100 chickens in the chicken pen, 170 sheep in the sheep pen, and 18 cattle in the cattle pen. The distance between the three pens was less than 1000 m. "2" indicates the second sampling village (east longitude 113°38'24", north latitude 37°45'). There were 70 sheep in the sheep pen and 10 chickens in the chicken pen. Sheep and chicken pens were in the courtyard of the residents, which had two dogs in the house. The two pens were approximately 2 km apart, and the two villages were about 4 km apart.

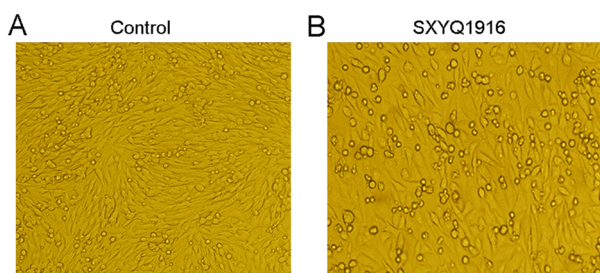


Fig. 1 CPE of the WUXV isolate SXYQ1916 in BHK-21 cells. **A** BHK-21 cells as negative control were cultured for 3 days. **B** BHK-21 cells were also cultured for 3 days after the inoculation of WUXV isolate SXYQ1916. Culture in the presence of the SXYQ1916 virus led to a decrease in the number of adherent BHK-21 cells, and profound cell rounding and detachment. Magnification, $\times 100$.

The MIR of WUXV

By dividing all 1535 sandflies into 23 pools and inoculating them into BHK-21 cells, we obtained four virus isolates that caused CPE in BHK-21 cells. The infection rate of

these viral pools was 17.39% (4/23), and the MIR was 2.61 (4/1535 \times 1000). Among the 14 pools of sandfly specimens collected from the sheep pen, three contained the WUXV. The infection rate of WUXV pools isolated from the sheep pen was 21.43% (3/14), and the MIR was 3.21 (3/935 \times 1000). The infection rate of viral pools isolated from the chicken pen was 20.00% (1/5), and the MIR of the WUXV virus was 3.45 (1/290 \times 1000). In addition, 310 sandflies were collected from the cattle pen and divided into four pools. We could not isolate a virus from any of these viral pools, and we did not detect any of the WUXV genes. The infection rates of the different WUXV isolates are shown in Table 3.

Homology in the Gene Sequences of Virus Isolates

The sequences of the coding regions of the virus WUXV *S* and *M* genes were determined for all four virus isolates. Interestingly, the two genes were of the same length in all four virus isolates. The coding sequence of the *S* gene was 1611 base pairs (bp), whereas the coding sequence of the *M* gene was 4089 bp, similar to the length of the respective genes of the WUXV (SXWX1813-2). The *L* gene of the SXYQ1927-2 virus isolate was amplified, and the nucleotide sequence was determined and analyzed. The length of the nucleotide and the amino acid sequences of the encoding region of the *L* gene are 6273 nt and 2090 aa, respectively. BLAST analyses revealed that the sequences of the *M* gene exhibited similarities of 96.8%–99.5% among the four strains. Similarly, the amino acid sequences of the NS and N proteins encoded by the *S* gene were 98.2%–99.6% similar among the four strains (Supplementary Tables S2–S4). The results of the homology analysis of the newly isolated virus (SXYQ1916) and other Phleboviruses are shown in Table 4. We found 96.7%, 97.1%, and 98.0% sequence similarity in genes *M*, *NS*, and *N* between the newly isolated strain SXYQ1916 and a

Table 2 Isolation of Wuxiang virus in Yangquan County, Shanxi Province in 2019.

Strain number	Collection site	Breeding places	Number of Phlebotomus chinensiss	CPE/ Gene amplification						
				BHK-21 cell	WUXV			C6/36 cell	WUXV	
					L	M	S		M	S
SXYQ1931	1	Sheep pen	60	+	/	MW192757	MW192753	–	–	–
SXYQ1916	2	Sheep pen	49	+	/	MW192754	MW192750	–	–	–
SXYQ1930	2	Sheep pen	74	+	/	MW192756	MW192752	–	–	–
SXYQ1927-2	2	Chicken pen	54	+	MW368897	MW192755	MW192751	–	–	–

Numbers indicate the collection location. Numbers 1 and 2 indicate the first and second sampling villages, respectively; "+" means that the experimental result is positive; "–" means that the experimental result is negative; "/" means that the experiment was not performed.

Table 3 Infection rate of Sandfly-borne virus.

Breeding places	Number of sandflies	Pool	Infection rate of pools (%)	MIR (/1000)
Chicken pen	290	5	20.00 (1/5)	3.45 (1/290)
Sheep pen	935	14	21.43 (3/14)	3.21 (3/935)
Cattle pen	310	4	0.00 (0/4)	0.00 (0/310)

WUXV strain (SXWX1813-2) previously isolated in China (Wang *et al.* 2020); the respective amino acid sequence similarities were 97.6%, 97.7%, and 99.6%. Moreover, comparisons between SXYQ1916 and a TORV strain (213/Turkey/2012) isolated from Turkey sandfly specimens revealed 72.0% nucleotide (75.1% amino acid) sequence similarity in the M segment, 75.4% (85.1%) similarity of the NS protein in the S segment, and 82.2% (96.4%) similarity of the N protein in the S segment (Table 4). These results suggested that the virus isolated from sandflies in Yangquan County is the same with the WUXV strain previously isolated from sandflies in China.

Phylogenetic Analysis of Viral Genes

We performed phylogenetic analysis based on the genetic sequences of the four virus strains isolated from sandfly specimens in Yangquan County in 2019, and those of 60 sandfly viruses reclassified by ICTV in 2020. We found that the four virus strains isolated in this study belonged to the same evolutionary branch as the previously isolated WUXV strain (SXWX1813-2). On the basis of the sequences of the *S* (including the NS and N coding sequences), *M* and *L* gene, these viruses belonged to the same evolutionary population as the TORV isolated from Turkey sandflies in 2012–2013 (Fig. 2).

Identification of Sandfly Species

To identify the species of the collected sandflies, we used PCR to amplify the COI gene. We found that all four pools of sandflies used to isolate the viruses belonged to the species *Phlebotomus chinensis* (Table 2).

Detection of Neutralizing Antibody of WUXV

In this study, 46 serum samples of healthy people were collected. The neutralizing antibody level of WUXV in serum samples was detected by plaque neutralization test (when the ratio of WUXV and neutralizing antibody in serum was 1:10, the sample was positive). The results showed that 8.70% (4/46) of the serum samples were positive in local healthy people. The neutralization titers of WX14, WX44 positive specimens were 1:10, WX28 and WX34 positive specimens were 1:20. The serum neutralizing antibodies of four chickens were all positive with titers ranging from 1:10 to 1:160. The results of neutralizing antibody test were shown in Table 5.

Table 4 Homology analysis of the newly isolated virus (SXYQ1916) and other Phleboviruses.

Virus strains	M segment		S segment			
	GP		NS		N	
	nt (%)	aa (%)	nt (%)	aa (%)	nt (%)	aa (%)
SXYQ1916	4089	1362	783	260	741	246
SXWX1813-2	4089(96.7)	1362(97.6)	783(97.1)	260(97.7)	741(98.0)	246(99.6)
TORV(213/Turkey/2012)	4080(72.0)	1359(75.1)	783(75.4)	260(85.1)	741(82.2)	246(96.4)
TORV(292/Turkey/2012)	4081(72.0)	1359(75.1)	783(75.2)	260(85.1)	741(81.9)	246(96.0)
CFUV(Pa Ar 814/Greece/1981)	4080(71.3)	1359(75.7)	783(74.5)	260(84.3)	741(81.9)	246(96.4)
SFSV(Ethiopia-2011/Ethiopia / 2011)	4026(62.6)	1341(57.1)	/	/	741(74.4)	246(84.2)
SFSV(Sabin/Italy/1943)	/	/	783(53.3)	260(63.6)	741(75.3)	246(83.8)
DASHV(131/Iran/2011)	4029(65.2)	1342(58.6)	786(58.1)	261(61.7)	741(76.5)	246(85.0)
RVFV(ZH-548/Egypt/1977)	3594(36.0)	1197(39.9)	798(15.5)	265(24.1)	738(55.4)	245(52.8)

“/” indicates that the sequence was not available in the GenBank database.

Fig. 2 Phylogenetic analyses of the genetic sequences of the four virus strains isolated from sandflies collected in Yangquan in 2019. **A** Phylogenetic analysis of isolates SXYQ1916, SXYQ1927-2, SXYQ1930, SXYQ1931 (black dots) based on the *M* gene sequence. **B** Phylogenetic analysis of isolates SXYQ1916, SXYQ1927-2, SXYQ1930, SXYQ1931 based on the *S* gene sequence (black dots). **C** Phylogenetic analysis of isolates SXYQ1927-2 (black dots) based on the *L* gene sequence. The phylogenetic analysis was conducted using MEGA 6.0 software and the neighbor-joining method (bootstrap value of 1000). *Note* For phylogenetic comparison, we used the gene sequences of Phleboviruses from the ICTV 2020 guidelines; “/” indicates that the sequence was not available in the GenBank database.

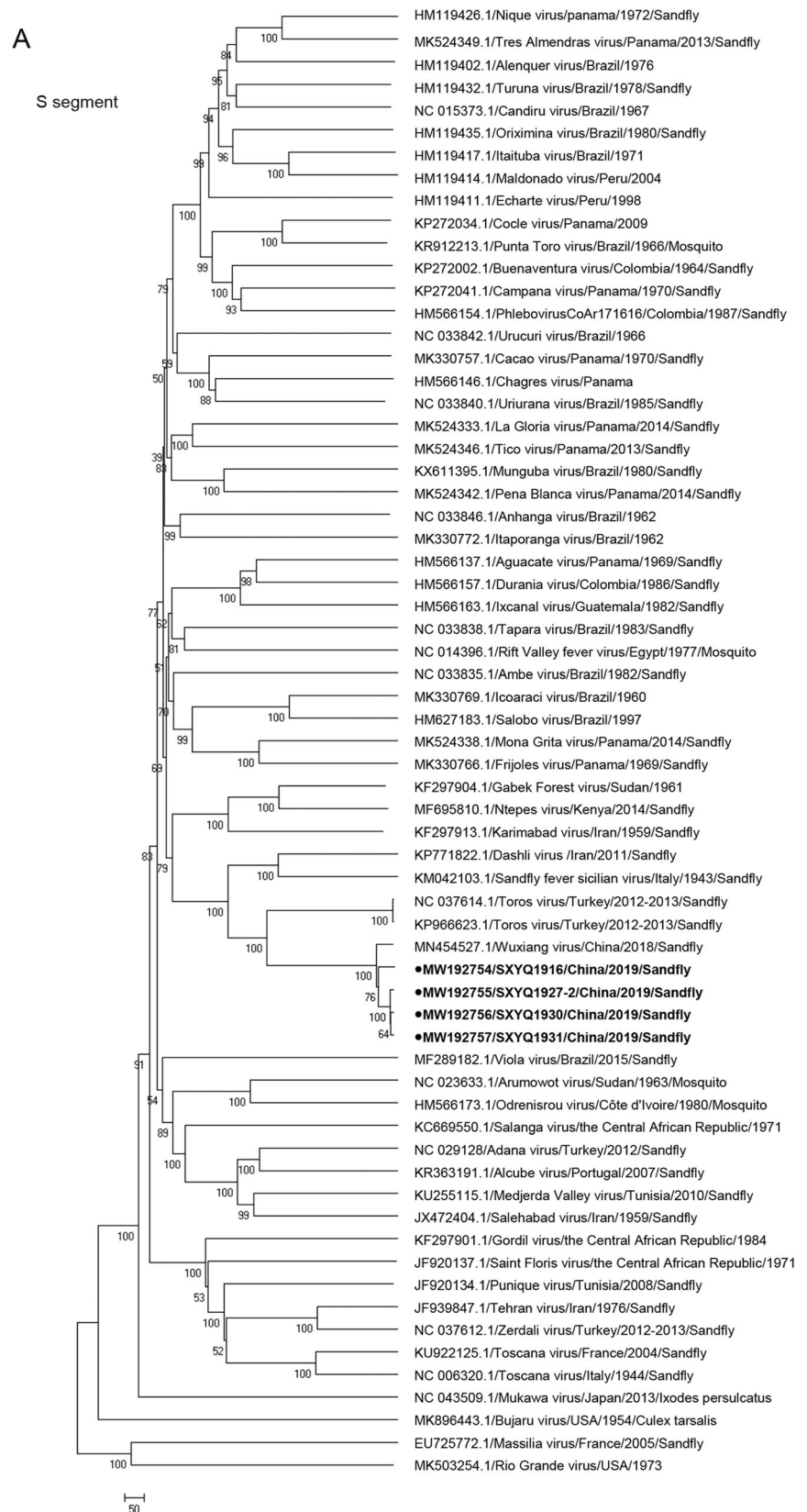


Fig. 2 continued

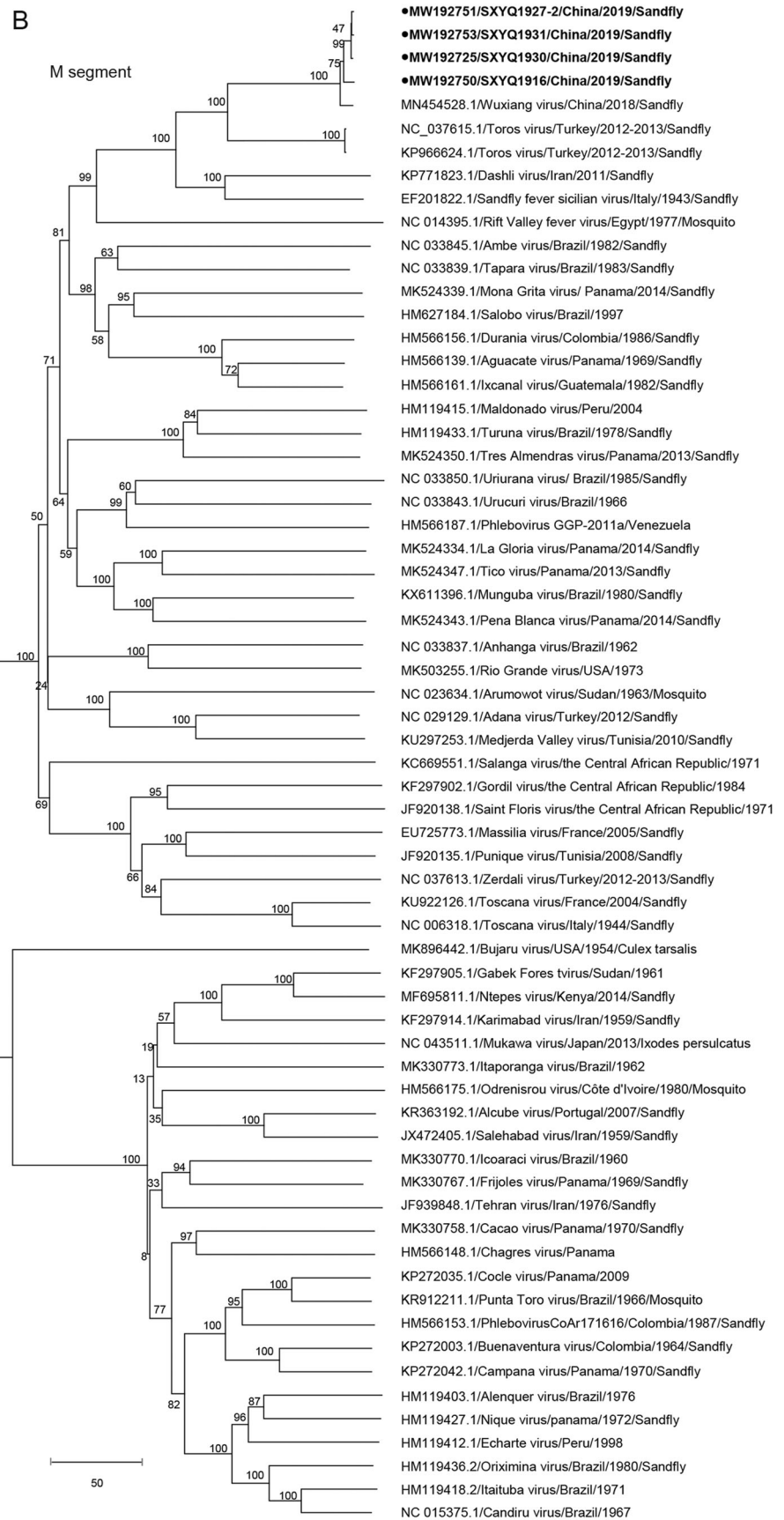


Table 5 Detection of neutralizing antibody against WUXV.

Number	Gender	Age	Livestock raised			Neutrization test (Serum dilution)	WUXV gene amplification
			Chicken	Sheep	Dog		
WX14	Female	66	10	–	1	+ (1:10)	–
WX28	Male	68				+ (1:20)	–
WX34	Male	61	–	–	1	+ (1:20)	–
WX44	Male	59	–	–	1	+ (1:10)	–

Table 5 shows the background information of healthy people and chickens with neutralizing antibody positive in serum specimens. The blood donors of WX14 and WX28 came from the same family, which raised 10 chickens and 1 dog. Other positive individual families raised only one dog but not sheep, chickens and other livestock and poultry. “–” means that the experimental result is negative.

Discussion

We have previously isolated the sandfly-borne virus WUXV from wild sandflies collected from Wuxiang County, Shanxi Province, China (Wang *et al.* 2020). In this study, we re-isolated WUXV from wild sandflies collected from Yangquan County, Shanxi Province, China. Molecular phylogenetic analyses revealed that these virus isolates represented different geographical isolates of WUXV viruses, suggesting that the WUXV can also be found in wild sandflies outside Wuxiang County.

Yangquan County (east longitude 112°5′–114°4′, north latitude 37°40′–38°31′) is located in Shanxi Province in central China, on the west side of the central Taihang Mountains with an average elevation of 700–1700 m. Yangquan has a warm, arid continental climate characterized by limited rainfall, and an ecological environment favoring the growth and reproduction of blood-sucking insects, such as sandflies and mosquitoes. In this study, we collected sandfly specimens from two regions of Yangquan County. One sampling point was located 9 km southeast of the center of Yangquan town, and the other was 13 km from the town; the distance between the two specimen collection points was approximately 4 km. The farmland around the two villages was mainly planted with corn and sorghum. Specimens were collected from sheep and chicken pens at each of the two villages. From the first village, we also collected samples from a cattle pen. All specimens were collected on June 11, 2019. Specimen collectors were placed at two collection points at the same time, and each of the sheep and chicken pens had one collector. Collectors were left overnight, and a total of 1535 sandflies were collected from both villages. Most of the sandflies were collected from the sheep pens ($n = 935$); only 290 samples were from the chicken pens. Three (SXYQ1916, SXYQ1930, SXYQ1927-2) of the four virus isolates in this study were derived from the sheep pen, and one isolate (SXYQ1931) came from the chicken pen. These

results demonstrate that sandflies were abundant in Yangquan County in June 2019.

Four different virus strains were isolated from wild sandflies collected from Yangquan County. The infection rate of these virus isolates differed significantly. The infection rate of the WUXV pool from the sheep pen was 21.43%, and the MIR was 3.21; the infection rate of the viral pool from the chicken pen was 20.00%, and the MIR was 3.45. In previous studies conducted in coastal countries in the Mediterranean region, the MIR of the TOSV isolated from Turkey sandflies was 2.2 (Calzolari *et al.* 2018), the MIR of Ntepes virus isolated from Kenyan sandflies was 0.26 (Tchouassi *et al.* 2019), and the MIR of Zerdali virus isolated from Turkey sandflies was 0.35 (Alkan *et al.* 2016). TORV was isolated from sandflies collected from Turkey in 2012, and its MIR was 0.26 (Alkan *et al.* 2016). Although phylogenetic analyses revealed that the WUXV isolated in Yangquan County, China, and the TORV isolated from Turkey were closely related, the MIR of the WUXV was significantly higher than that of the TORV (2.61 vs. 0.26). The pooled infection rate and MIR of WUXV from local sandflies were significantly higher than those of viruses isolated from Mediterranean region sandflies. The biological significance and pathogenicity of the virus carried by sandflies requires further investigation. In this study, the positive rates of Wuxiang virus neutralizing antibodies detected in the serum samples of local healthy people and domestic chickens were 8.4% (4/46) and 100% (4/4), respectively. Although the number of serum specimens collected in this study is limited, and only serum specimen from one animal (chicken) was collected, the above results suggest Wuxiang virus can infect humans and domestic chickens. Therefore, in the future, it is necessary to investigate the infection status of Wuxiang virus in local humans and animals to clarify the harm of the virus to public health.

The revised guidelines from ICTV meeting in 2020 recommended the addition of 53 new Phleboviruses (Kuhn *et al.* 2020). The previous ICTV classification guidelines

included only 10 *Phlebovirus* species (Abudurexiti *et al.* 2019), including the SFTSV isolated in China (Yu *et al.* 2011) and the Hartland virus isolated in the USA (Shen *et al.* 2018). These two viruses are not classified as Phleboviruses according to the new ICTV guidelines, although 53 newly identified Phleboviruses were added. In this study, we conducted a molecular evolution analysis of the WUXV isolated in China in 2018 (Wang *et al.* 2020), and the newly identified Phleboviruses included in the revised ICTV classification guidelines. Among the new *Phlebovirus* species, 37 were isolated in the Americas (16 in North America and 21 in South America), 10 were isolated in Africa (1 in West Africa, 1 in East Africa, 3 in Central Africa, and 5 in North Africa), 8 were first isolated in Asia (1 in East Asia and 7 in West Asia), and 5 were from Europe (5 from Western Europe). A significant number of these Phleboviruses ($n = 37$ strains) uses sandflies as their transmission vector; 5 viruses are transmitted through mosquitoes, and one via ticks; the transmission vector is unknown for the remaining 17 viruses (Fig. 2). Here, we analyzed the *M* and *S* gene sequences of the four WUXV strains isolated from Yangquan sandflies (Fig. 2), and found that these isolates belonged to the same evolutionary branch as the TORV (Alkan *et al.* 2016); this suggested that WUXV may have derived from the TORV isolated from Turkey sandflies in 2012 (Alkan *et al.* 2016).

In this study, we re-isolated the WUXV from wild sandflies collected from Yangquan County, a different region from where the WUXV was originally isolated. These findings suggest that the WUXV has a relatively wide geographical distribution range. In addition, the neutralizing antibody of Wuxiang virus was detected in both human and chicken serum specimens (Table 5), suggesting that the local people and domestic chickens were infected with Wuxiang virus. Therefore, it is of great significance to strengthen the seroepidemiological investigation of Wuxiang virus in local humans and animals.

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Author Contributions QW and GL drafted and revised the manuscript. QW, SF, JC, XX, JW, BW, XT, YL, YH, FL, KN and SX participated in collection of sandfly specimens and acquisition of data. QW, SF, JC and GL participated in the analysis and interpretation of data. HW, XL and GL contributed to conception and design of the manuscript. All authors read and approved the final manuscript.

Compliance with Ethical Standards

Conflict of interest The authors declare no conflict of interests.

Animal and Human Rights Statement All animal experiments were conducted in strict compliance with the regulations set by the Animal Ethics Committee of China CDC.

References

- Abudurexiti A, Adkins S, Alioto D, Alkhovsky SV, Avšič-Županc T, Ballinger MJ, Bente DA, Beer M, Bergeron É, Blair CD *et al.* (2019) Taxonomy of the order Bunyavirales: update 2019. *Arch Virol* 164:1949–1965
- Alkan C, Erisoz Kasap O, Alten B, de Lamballerie X, Charrel RN (2016) Sandfly-borne phlebovirus isolations from Turkey: new insight into the sandfly fever sicilian and sandfly fever naples species. *PLoS Neg Trop Dis* 10:e0004519
- Calzolari M, Chiapponi C, Bellini R, Bonilauri P, Lelli D, Moreno A, Barbieri I, Pongolini S, Lavazza A, Dottori M (2018) Isolation of three novel reassortant phleboviruses, Ponticelli I, II, III, and of Toscana virus from field-collected sand flies in Italy. *Parasit Vectors* 11:84
- Cao L, Fu S, Gao X, Li M, Cui S, Li X, Cao Y, Lei W, Lu Z, He Y, Wang H, Yan J, Gao GF, Liang G (2016) Low protective efficacy of the current Japanese encephalitis vaccine against the emerging genotype 5 Japanese encephalitis virus. *PLoS Neg Trop Dis* 10: e0004686
- Depaquit J, Grandadam M, Fouque F, Andry PE, Peyrefitte C (2010) Arthropod-borne viruses transmitted by Phlebotomine sandflies in Europe: a review. *Euro Surveill* 15:19507
- Feng Y, Fu S, Zhang H, Li M, Zhou T, Wang J, Zhang Y, Wang H, Tang Q, Liang G (2012) Distribution of mosquitoes and mosquito-borne viruses along the China-Myanmar border in Yunnan Province. *Jpn J Infect Dis* 65:215–221
- Feng Y, Li Y, Fu S, Li X, Song J, Zhang H, Yang W, Zhang Y, Pan H, Liang G (2017) Isolation of Kaeng Khoi virus (KKV) from Eucampsipoda sundaica bat flies in China. *Virus Res* 238:94–100
- Feng Y, Ren X, Xu Z, Fu S, Li X, Zhang H, Yang W, Zhang Y, Liang G (2019) Genetic diversity of the Yokose virus, XYBX1332, isolated from bats (*Myotis daubentonii*) in China. *Virol J* 16:8
- Folmer O, Black M, Hoeh W, Lutz R, Vrijenhoek R (1994) DNA primers for amplification of mitochondrial cytochrome c oxidase subunit I from diverse metazoan invertebrates. *Mol Mar Biol Biotech* 3:294–299
- Fu S, Song S, Liu H, Li Y, Li X, Gao X, Xu Z, Liu G, Wang D, Tian Z, Zhou J, He Y, Lei W, Wang H, Wang B, Lu X, Liang G (2017) ZIKA virus isolated from mosquitoes: a field and laboratory investigation in China, 2016. *Sci China Life Sci* 60:1364–1371
- Kuhn JH, Adkins S, Alioto D, Alkhovsky SV, Amarasinghe GK, Anthony SJ, Avšič-Županc T, Ayllón MA, Bahl J, Balkema-Buschmann A, *et al.* (2020) 2020 Taxonomic update for phylum Negarnaviricota (Riboviria: Orthornavirae), including the large orders Bunyavirales and Mononegavirales. *Archives Virol* 165:3023–3072
- Liang G, Li X, Gao X, Fu S, Wang H, Li M, Lu Z, Zhu W, Lu X, Wang L, Cao Y, He Y, Lei W (2018) Arboviruses and their related infections in China: a comprehensive field and laboratory investigation over the last 3 decades. *Rev Med Virol* 28

- Maes P, Adkins S, Alkhovsky SV, Avšič-Županc T, Ballinger MJ, Bente DA, Beer M, Bergeron É, Blair CD, Briese T *et al* (2019) Taxonomy of the order Bunyavirales: second update 2018. *Arch Virol* 164:927–941
- Ren X, Fu S, Dai P, Wang H, Li Y, Li X, Lei W, Gao X, He Y, Lv Z, Cheng J, Wang G, Liang G (2017) Pigsties near dwellings as a potential risk factor for the prevalence of Japanese encephalitis virus in adult in Shanxi. *China Infect Dis Poverty* 6:100
- Shen S, Duan X, Wang B, Zhu L, Zhang Y, Zhang J, Wang J, Luo T, Kou C, Liu D, Lv C, Zhang L, Chang C, Su Z, Tang S, Qiao J, Moming A, Wang C, Abudurexiti A, Wang H, Hu Z, Zhang Y, Sun S, Deng F (2018) A novel tick-borne phlebovirus, closely related to severe fever with thrombocytopenia syndrome virus and Heartland virus, is a potential pathogen. *Emerg Microbes Infect* 7:95
- Song S, Li Y, Fu S, Liu H, Li X, Gao X, Xu Z, Liu G, Wang D, Tian Z, Zhou J, He Y, Lei W, Wang H, Wang B, Lu X, Liang G (2017) Could Zika virus emerge in Mainland China? Virus isolation from nature in *Culex quinquefasciatus*, 2016. *Emerg Microbes Infect* 6:e93
- Tchouassi DP, Marklewitz M, Chepkorir E, Zirkel F, Agha SB, Tigoi CC, Koskei E, Drosten C, Borgemeister C, Torto B, Junglen S, Sang R (2019) Sand fly-associated phlebovirus with evidence of neutralizing antibodies in humans, Kenya. *Emerg Infect Dis* 25:681–690
- Walker PJ, Siddell SG, Lefkowitz EJ, Mushegian AR, Adriaenssens EM, Dempsey DM, Dutilh BE, Harrach B, Harrison RL, Hendrickson RC, Junglen S, Knowles NJ, Kropinski AM, Krupovic M, Kuhn JH, Nibert M, Orton RJ, Rubino L, Sabanadzovic S, Simmonds P, Smith DB, Varsani A, Zerbini FM, Davison AJ (2020) Changes to virus taxonomy and the Statutes ratified by the International Committee on Taxonomy of Viruses (2020). *Adv Virol* 165:2737–2748
- Wang J, Fu S, Xu Z, Cheng J, Shi M, Fan N, Song J, Tian X, Cheng J, Ni S, He Y, Lei W, Li F, Peng H, Wang B, Wang H, Lu X, Ma Y, Liang G (2020) Emerging sand fly-borne phlebovirus in China. *Emerg Infect Dis* 26:2435–2438
- Yu X-J, Liang M-F, Zhang S-Y, Liu Y, Li J-D, Sun Y-L, Zhang L, Zhang Q-F, Popov VL, Li C *et al* (2011) Fever with thrombocytopenia associated with a novel bunyavirus in China. *N Engl J Med* 364:1523–1532