Isolation and characterization of a novel arenavirus harbored by Rodents and Shrews in Zhejiang province, China

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ABSTRACT

To determine the biodiversity of arenaviruses in China, we captured and screened rodents and shrews in Wenzhou city, Zhejiang province, a locality where hemorrhagic fever diseases are endemic in humans. Accordingly, arenaviruses were detected in 42 of 351 rodents from eight species, and in 12 of 272 Asian house shrews (*Suncus murinus*), by RT-PCR targeting the L segment. From these, a single arenavirus was successfully isolated in cell culture. The virion particles exhibited a typical arenavirus morphology under transmission electron microscopy. Comparison of the S and L segment sequences revealed high levels of nucleotide ($>32.2\%$ and $>39.6\%$) and amino acid ($>28.8\%$ and $>43.8\%$) sequence differences from known arenaviruses, suggesting that it represents a novel arenavirus, which we designated Wenzhou virus (WENV). Phylogenetic analysis revealed that all WENV strains harbored by both rodents and Asian house shrews formed a distinct lineage most closely related to Old World arenaviruses.

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Introduction

Arenaviruses (genus *Arenavirus*, family *Arenaviridae*) are enveloped, single-stranded RNA viruses of ambisense polarity (Neuman et al., 2005). The virus genome comprises two segments. The small (S) segment encodes the nucleocapsid protein (NP) and envelope glycoproteins (GP), while the large (L) segment encodes the viral RNA-dependent RNA polymerase (RdRP) and a zinc-binding protein (ZP). Additionally, in both segments, the genes are separated by a non-coding region which has the potential to form hairpin configurations and which plays a role in transcription termination (Gonzalez et al., 2007). Arenaviruses are important human pathogens, and associated with central nervous system disease and hemorrhagic fever (Charrel and de Lamballerie, 2010).

Rodents are the primary natural reservoir hosts of the known arenaviruses. The only known exceptions are a single virus isolated from fruit bats (*Artibeus spp.*) and three recently discovered viruses from snakes (Downs et al., 1963; Stenglein et al., 2012; Bodewes et al., 2013; Hetzel et al., 2013). Each rodent-borne arenavirus species appears to be primarily associated with one (or a few closely related) rodent species, compatible with the long-term co-divergence of these viruses with their hosts (Gonzalez et al., 2007). To date, 25 established arenaviruses have been identified worldwide, largely from the New World, as well as several newly discovered viruses whose taxonomic status has not yet been confirmed by the International Committee on Taxonomy of Viruses (Salvato et al., 2011; Coulibaly-N'Golo et al., 2011; Kronmann et al., 2013; Stenglein et al., 2012; Bodewes et al., 2013; Hetzel et al.,

2013). These viruses form two phylogenetic groups according to their geographic origins – the New World and the Old World arenaviruses – although Lymphocytic choriomeningitis virus (LCMV) is more globally distributed.

Several viral pathogens are known to cause hemorrhagic fevers in China (Gao et al., 2010; Zhang et al., 2010; Zhang et al., 2012; Chen et al., 2014). Indeed, China is one of the most important endemic areas for Hemorrhagic Fever with Renal Syndrome (HFRS), which is caused by hantaviruses and transmitted by rodents (Zhang et al., 2010). Although more than 103 species of *Muridae* rodents are present in China (Zhang et al., 1997), the only arenavirus that has been described in China to date is LCMV (Morita et al., 1996). The purpose of this study was to determine whether other arenaviruses are present in China and which mammalian hosts act as viral reservoirs.

Results

RT-PCR detection of arenaviruses in rodents and shrews

A total of 623 small mammals comprising rodents and insectivores were captured in Wenzhou city, Zhejiang province, southeast China (Fig. 1). Overall, we sampled 351 rodents from eight species and 272 insectivores of a single species (*Suncus murinus*, the Asian house shrew) as identified by analysis of the mt-*cyt b* gene (Table 1). A nested RT-PCR protocol targeting the RdRP gene within the viral L segment was performed to determine the presence of arenaviruses in stool samples. This revealed an extremely high positive rate for arenavirus RNA. Specifically, in rodents, the arenavirus infection rates in *Rattus norvegicus*, *R. flavipectus*, *R. losea* and *R. rattus* were 17.07%, 15.38%, 11.76% and 75.00%, respectively, with an overall positive rate of 11.97% (42 of 351 animals). In the case of insectivores, 12 of the 272 animals (4.41%) tested positive for arenavirus RNA. The infected animals were found in all four districts and counties of Wenzhou city

Fig. 1. A map of Wenzhou city, Zhejiang province, China, showing the location of trap sites from which small animals were captured. Red circles: locations of the sampled rodents and shrews. Blue triangle: location where Wenzhou virus was isolated from rodents. Purple star: location where the rodents were sampled to obtain the virus genome.

(Table 1, Fig. 1). In addition to fecal materials, viral RNA was identified in liver, lung, heart, kidney and spleen tissue samples, suggesting that the infected rodents and shrews are experiencing chronic viremic infections.

Isolation of an arenavirus from the liver tissue of the rat Rattus norvegicus

To better characterize the arenaviruses circulating in rodents and shrews, liver tissues from two *R. norvegicus* rats, one *R. flavipectus* rat and one *R. rattus* rat were homogenized and inoculated onto DH82 cell monolayers. After 10 days of viral cultivation, partial L segment sequences were recovered from the harvested culture supernatant inoculated with the liver homogenate of a *R. norvegicus* rat (strain Wencheng-Rn-366), indicating that the virus was successfully isolated. However, amplification from the remaining culture supernatant was unsuccessful. To observe the virus particles, the cells and the supernatant were both subjected to transmission electron microscopy (TEM), which revealed spherical to pleomorphic particles observed in both the cell and supernatant samples (Figs. 2 and S1). The diameter of these virons in each case was approximately 100 nm. In addition, the 'sandy' appearance of the virion particles could be easily observed, further supporting the successful isolation of this arenavirus.

To investigate virus propagation in cultured cells, the isolate was inoculated into fresh DH82 cells, and the extent of virus propagation was approximately estimated by quantitative real-time RT-PCR targeting the RdRP gene. Intracellular viral RNA began to increase steadily at one day post infection (p.i.) (Fig. 3), and levels of viral RNA increased exponentially between day three to five day p.i. However, no increase of viral RNA was observed after seven day p. i.

Genetic analysis of arenavirus sequences recovered from rodents and shrews

The complete viral S and L segments were first successfully recovered from an infected *R. norvegicus* rat (designated strain 'WENV/Wencheng-Rn-242') by high-throughput sequencing and confirmed by RT-PCR. As a typical arenavirus, this virus has two segments of length 7,146 (L) and 3,337 (S) nucleotides (nt), respectively. Highly conserved 3′ and 5′ non-coding regions were identified in both segments (Fig. S2). The S segment has two open reading frames (ORF) encoding a NP of 567 amino acids (aa) and a GP precursor of 493 aa, with a 62 nt non-coding region between the two ORFs. The L segment also has two ORFs encoding a RdRP of 2,228 aa and a ZP of 91 aa, with a 109 nt non-coding sequence between the two ORFs. In addition, complete (or near complete) genome sequences were also recovered from the RNA samples of 1 *R. rattus* rat (Wencheng-Rr-233), 1 *S. murinus* shrew (Wencheng-Sm-247), as well as from the isolate (Wencheng-Rn-366) by RT-PCR. Details of the viral segments and their encoded proteins are presented in (Table 2), while sequence characteristics of the non-coding regions, the 3'-5' exonuclease domain within the C-terminus of NP, and the late domain motifs within the C-terminus of ZP are shown in Fig. S2.

Genetic analysis of the four genome sequences recovered from rodents and shrews in this study indicated that they exhibited less than 7.6% nt difference in the complete S and L segments. This suggests that they are variants of the same virus species, albeit with a relatively high genetic diversity among them. Although the Wenzhou viruses were more closely related to Old World arenaviruses (see below), the differences between them were substantial; > 32.2% and > 39.6% at the nt level, and > 28.8% and > 43.8% at the aa level for the S and L segments, respectively (Table 3). This suggests that the Wenzhou viruses comprise a new
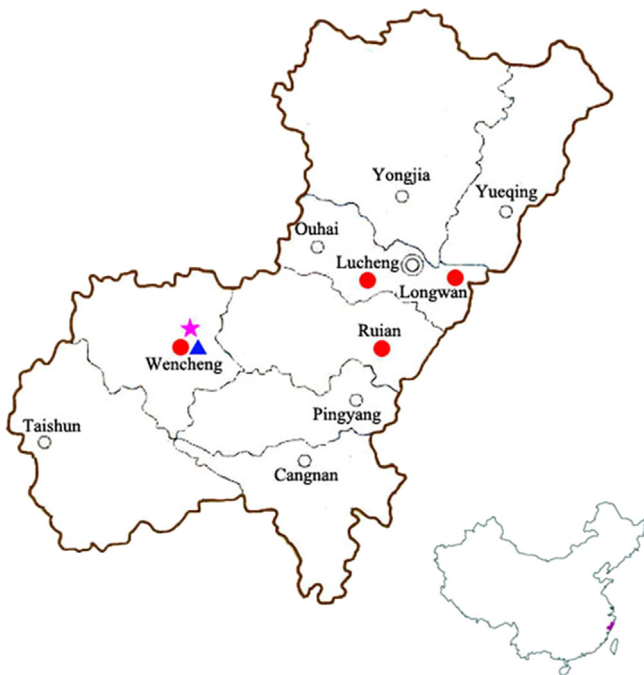
Table 1
Prevalence of Wenzhou virus in rodents by species and district in Wenzhou, China.

District	Species								
	R. norvegicus	*R. rattus*	*R. flavipectus*	*R. losea*	*R. edwardsi*	*N. niviventer*	*M. musculus*	*A. agrarius*	*S. murinus*
Wencheng	23/88*	3/4	7/38	2/4	0/2	1/53	0/11	0/41	11/190
Lucheng	2/17	0/0	0/3	0/0	0/0	0/0	0/0	0/0	0/0
Ruian	2/53	0/0	0/9	0/10	0/0	0/0	0/2	0/4	0/38
Longwan	1/6	0/0	1/2	0/3	0/0	0/0	0/1	0/0	1/44
Total	*28/164*	*3/4*	*8/52*	*2/17*	*0/2*	*1/53*	*0/14*	*0/45*	*12/272*

Abbreviations: R, *Rattus*; N, *Niviventer*; M, *Mus*; A, *Apodemus*; S, *Suncus*.

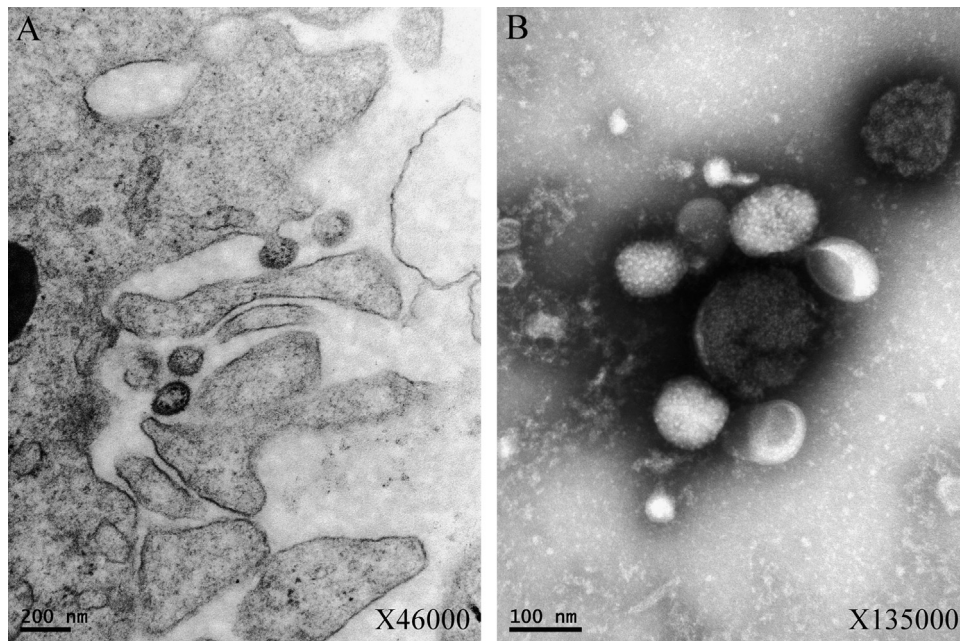* The number of rodents and shrews number PCR positive/captured.

Fig. 2. Transmission EM images of Wenzhou virus in (A) infected DH82 cells and (B) cell culture supernatant.

viral species within the genus *Arenavirus*. As a consequence, we named this new virus as Wenzhou virus (WENV).

Phylogenetic analysis of viral sequences

We estimated phylogenetic trees of the entire coding sequences of the NP, GP, RdRP and ZP genes using a maximum likelihood (ML) method. In all four phylogenies, the viral sequences recovered from small mammals captured in Wenzhou clustered with the Old World arenaviruses group, although as a distinct lineage (Fig. 4). Also of note was that WENV strains were most closely related to Ippy virus isolated from *Arvicanthis niloticus* rats in Central African (Swanepoel et al., 1985) in the NP tree, albeit with weak bootstrap support (Fig. 4A). In contrast, the WENV strains clustered strongly with Lassa viruses isolated from *Mastomys natalensis* mice in Sierra Leone (Auperin and McCormick, 1989) (Fig. 4C) in the GP tree, and formed a distinct lineage in both the RdRP and the ZP trees.

Discussion

We detected and isolated a novel arenavirus – WENV – harbored by rodents and shrews in Wenzhou, Zhejiang province, southeastern China. As indicated by TEM, the virion particle shows a typical
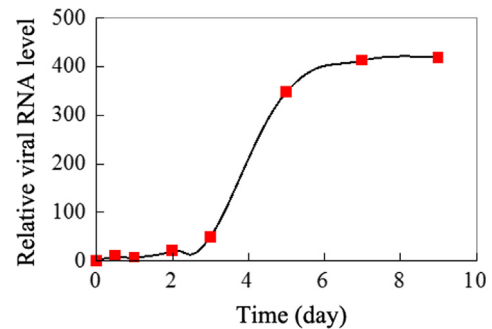

Fig. 3. The propagation of Wenzhou virus in DH82 cells as estimated by the amount of viral RNA in cells. At the time points indicated (0.5, 1, 2, 3, 5, 7, 9 days post infection) the intracellular viral RNA levels were measured using quantitative real-time RT-PCR. The relative number of viral RNA molecules were then estimated using a comparative Ct method.

arenavirus morphology. Phylogenic analysis of viral genome sequences indicated that WENV is distinct from known arenaviruses, suggesting that it represents a new virus species within the genus *Arenavirus*.

Rodents are considered to be the primary natural hosts of arenaviruses. Notably, most arenavirus species are believed to be primarily associated with one (or a few closely related) rodent

Table 2
Genome organization of Wenzhou virus strains identified in this study and of reference strains.

Segment	Virus	Full length	Conservative termini	Length of intergenic region	L-domain in ZP	Exonuclease domain in NP	Cytoplasmic region of GP	Reference
L	Wencheng-Rn-366	7113	+	62	+	+	+	
	Wencheng-Rn-242	7146	+	62	+	+	+	
	Lassa virus	7279	+	61	+	+	+	Auperin and McCormick (1989)
	Ippy virus	7316	+	58	+	+	+	Emonet et al. (2006)
	Chapare virus	7107	+	95	+	+	+	Delgado et al. (2008)
S	Wencheng-Rn-366	3350	+	120	+	+	+	
	Wencheng-Rn-242	3337	+	109	+	+	+	
	Lassa virus	3402	+	100	+	+	+	Auperin and McCormick (1989)
	Ippy virus	3366	+	164	+	+	+	Emonet et al. (2006)
	Chapare virus	3357	+	72	+	+	+	Delgado et al. (2008)

Note: + represents "presence".

Table 3
Nucleotide and amino acid identities (%) of Wenzhou virus (strain Wencheng-Rn-242) with other arenaviruses.

Virus	S segment (nucleotide/amino acid)			L segment (nucleotide/amino acid)		
	S	NP	GP	L	RdRP	ZP
Lassa virus	67.8/71.2	68.2/69.7	67.6/72.9	60.4/54.1	60.2/50.8	49.3/57.1
Ippy virus	67.1/70.1	69.5/72.0	65.2/67.9	59.7/56.2	60.9/51.8	52.5/57.1
Lujo virus	47.3/50.4	63.4/58.5	28.4/41.6	46.6/44.2	49.9/42.6	39.9/45.1
LCMV	62.3/59.0	63.4/62.6	60.9/54.7	56.1/47.0	58.7/43.8	48.0/45.6
Mopeia virus	66.3/70.3	69.8/71.1	64.6/69.7	59.3/53.6	54.7/50.8	50.7/50.5
Mobala virus	65.0/71.1	68.1/70.2	65.4/72.5	60.0/54.0	60.9/51.2	48.6/51.6
Morogoro virus	66.7/71.2	68.4/70.8	66.2/72.2	58.9/53.0	60.0/49.8	38.4/49.5
Luna virus	67.0/71.0	68.5/70.2	66.7/71.5	59.2/53.7	53.5/50.6	43.5/57.1
Chapare virus	57.8/45.1	56.9/50.9	29.5/40.3	43.9/34.1	47.0/32.2	37.0/37.4
Junín virus	44.6/44.4	59.4/49.6	27.8/40.2	42.5/35.9	45.4/33.2	17.0/34.1

Abbreviations: LCMV, Lymphocytic choriomeningitis virus.

species, which likely reflects long-term virus-host co-divergence (Gonzalez et al., 2007). For instance, Lassa virus is carried by *Mastomys* mice (*M. huberti* and *M. erythroleucus*) of the *Muridae* family, while Machupo virus is only associated with *Calomys callosus* mice of the Cricetidae family (Gonzalez et al., 2007). However, recent studies have revealed that cross-species transmission might also play a role in arenavirus evolution (Coulibaly-N'Golo et al., 2011). Notably, we observed that WENV exhibits a very wide host range. In addition to four species of *Rattus* rats (*R. norvegicus*, *R. rattus, R. flavipectus*, and *R. losea*) and *Niviventer* rats, Asian house shrews (*S. murinus*) are also hosts for WENV, although the infection rate (4.41%) is not as high as in rodents. Hence, these data indicate that arenaviruses are not as host specific as previously suggested, and additional small animals may be the natural reservoir of pathogenic arenaviruses.

Our study was also notable for the very high infection rate of Wenzhou virus in rodents, including *R. norvegicus* (28/164, 17.07%), *R. flavipectus* (8/52, 15.38%), *R. losea* (2/17, 11.76%) and *R. rattus* (3/4, 75.00%). Importantly, all these rodents are widely distributed in villages and cities throughout China, where they live in close association with humans. Hence, it is possible that humans are sometimes exposed to infected rodents or infective rodent excreta and secreta. Although the human pathogenicity of Wenzhou virus is unclear, both vigilance and more research are clearly necessary as many arenavirus species are known to cause severe human diseases. Hemorrhagic fever diseases, which are caused by Hantavirus, Huaiyangshan virus, Rickesttsiales bacteria, are highly

prevalent in China and a significant public health burden (Zhang et al., 2010; Zhang et al., 2012; Chen et al., 2014). Importantly, these diseases are often misdiagnosed due to a similarity in symptoms, and the etiologic agents are often unknown (Chen et al., 2014), although it is clear that some are likely to be rodent-borne. Because of the wide distribution of *Rattus* rats in China, determining whether the commonly carried WENV is pathogenic to humans is clearly of importance.

Materials and methods

Sample collection and treatment

From February to August 2013, rodents and insectivores were trapped by cages using fried foods as bait. In total, 351 rodents and 272 insectivores were captured in four regions of Wenzhou city: the Longwan and Lucheng districts, and the Ruian and Wencheng counties (Table 1). All animals were captured alive and then anesthetized to minimize suffering. The tissue and stool samples were collected and stored at −80 °C. Total RNA was extracted from stool samples and tissue samples of heart, liver, spleen, lung and kidney using TRIzol reagent (Invitrogen, Carlsbad, CA). Using the DNeasy Blood & Tissue kit (QIAGEN), total DNA was extracted from tissue samples for species identification. Animals were identified by trained field biologists and confirmed by sequencing mt-*cyt b* gene.
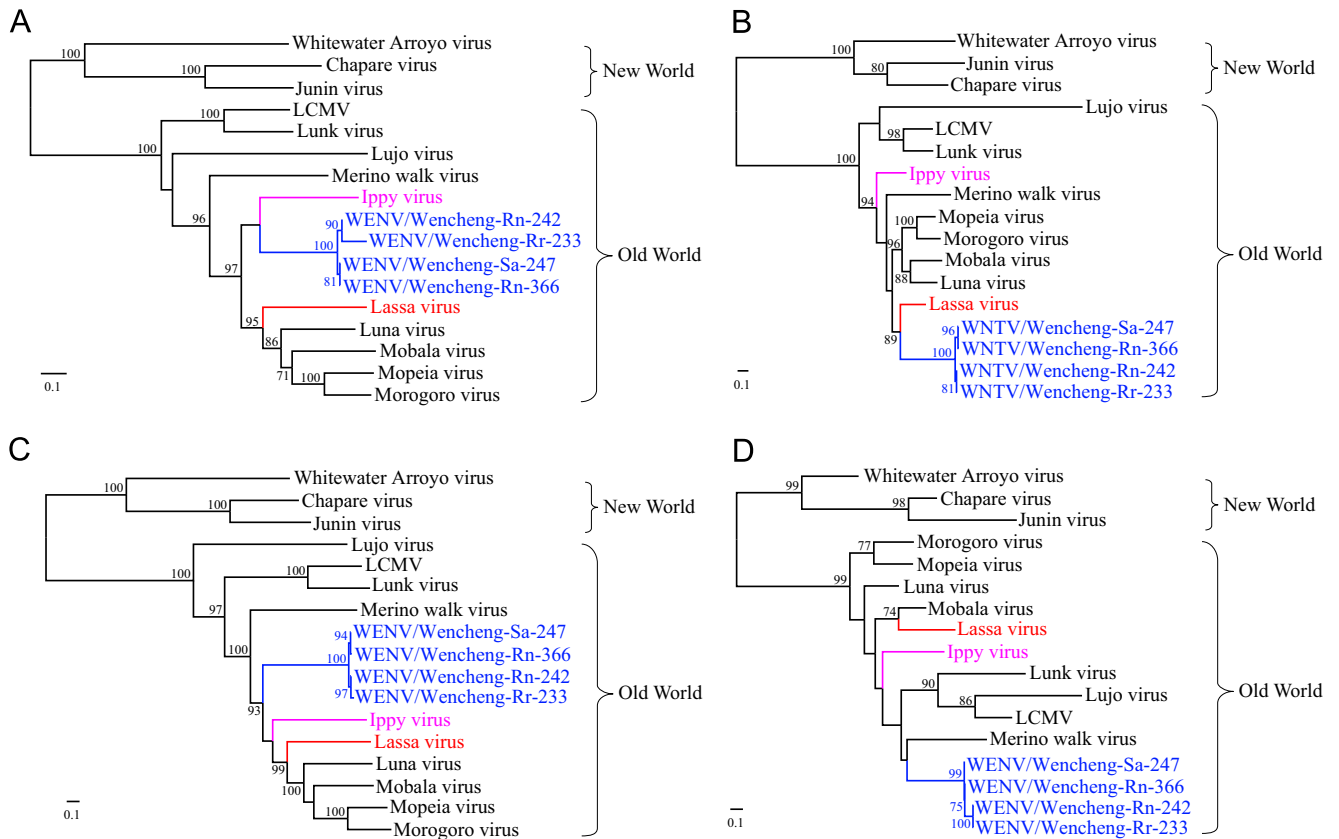
Fig. 4. Phylogenetic analysis of Wenzhou virus based on coding region nucleotide sequences. Genome regions are as follows: A) NP, B) GP, C) RdRP and D) ZP genes. Bootstrap values (> 70%) are shown at appropriate nodes. Scales bars indicate the number of nucleotide substitutions per site. The tree is rooted between the Old World and New World arenaviruses. All sequences recovered in this study have been deposited in GenBank as follows: Wencheng-Rn-242 S segment: KJ909794, L segment: KJ909794; Wencheng-Rr-233 S segment: KM051423, L segment: KM051421; Wencheng-Sm-247 S segment: KM051422, L segment: KM051420; Wencheng-Rn-366 S segment: KM386660, L segment: KM386661.

PCR detection of arenaviruses in rat and shrew samples

Stool samples were screened for the presence of arenavirus by using nested RT-PCR. Two pairs of primers, based on the conserved regions of the L segment sequences from known arenaviruses, were used to amplify the viral L segment (the first pair: 5-AYNGGNACNCCRTTNGC-3 and 5-TCHTAYAARGARCARGTD-GGDGG-3, the second pair: 5-GGNACYTCHTCHCCCCANAC-3 and 5-AGYAARTGGGGNCCNAYKATG-3). The primer (5-AYNGGNACN-CCRTTNGC-3) was used for reverse transcription of the partial L segment from total RNA by using a TaKaRa PrimeScript™ One Step RT-PCR kit ver. 2 according to the manufacturer's protocol. The RT-PCR screening results were confirmed by sequencing of the amplified products and by BLAST search. Tissue samples from infected animals including heart, liver, lung and kidney were also screened.

Acquisition of the complete genome sequence

To obtain the full viral genome, RNA samples from infected *Rattus norvegicus* rats were subjected to library preparation following the standard Illumina protocol. After removal of rRNA, the remaining RNA was fragmented using the Agilent 2100 Bioanalyzer and then reverse-transcribed, adaptored, purified, and examined by the ABI StepOnePlus Real-Time PCR System. The sequencing steps were performed by the BGI Tech Corporation (Shenzhen, China). The resulting sequencing reads were assembled by the Trinity program into 13 contigs (> 100 bp) and aligned to existing database (i.e. GenBank) sequences. BLASTx was performed to retrieve the complete arenavirus genome sequences from the assembled contigs.

In total, complete (or near complete) viral genome sequences were recovered from the RNA positive samples of one *Rattus rattus* rat (sequence Wencheng-Rr-233) and one *Suncus murinus* shrew (sequence Wencheng-Sm-247) using primers based on the complete S and L sequences of WENV/Wencheng-Rn-242 (see Results) and known arenaviruses. The complete viral genome sequence was also recovered from the isolate (WENV/Wencheng-Rn-366).

Phylogenetic analysis

The complete sequences of Old World arenaviruses were downloaded from GenBank. These sequences were: Lassa virus (LASV, S/NC_004296.1, L/NC_004297.1), Ippy virus (IPPYV, S/NC_007905.1, L/NC_007906.1), Lujo virus (LUJV, S/NC_007906.1, L/NC_012777.1), Merino walk virus (MWV, S/NC_023764.1, L/NC_023763.1), Mopeia virus (MOPV, S/NC_006575.1, L/NC_006574.1), Morogoro virus (MORV, S/NC_013057.1, L/NC_013058.1), Luna virus (LUNV, S/NC_016152.1, L/NC_016153.1), Mobala virus (MOBV, S/NC_007903.1, L/NC_007904.1), Lymphocytic choriomeningitis virus (LCMV, S/AY847350.1, L/AY847351.1), Lunk virus (LUNK, S/NC_018710.1, L/NC_018711.1), Mopeia Lassa virus reassortant 29 (MOP/LASV, S/NC_006573.1, L/NC_006572.1). Three New World arenaviruses, Chapare virus (CHPV, S/NC_010562.1, L/NC_010563.1), Junin virus (JUNV, S/NC_005081.1, L/NC_005080.1) and Whitewater Arroyo virus (WWAV, S/NC_010700.1, L/NC_010703.1) were used as outgroup.

Phylogenetic trees were inferred for coding sequences of the NP, GP, RdRP and ZP genes using the maximum likelihood (ML) method implemented in the PhyML v3.0 program (Guindon et al., 2009) and assuming the best-fit GTR+I+Γ_4 model of nucleotide substitution determined by jModelTest (Posada, 2008). To assess

support for individual nodes, 1000 bootstrap replicates were obtained under the same procedure.

Isolation of Wenzhou virus

We used the canine macrophage cell DH82 for virus isolation (Qin et al., 2014). Cells were cultured at 28 °C in MEM medium with 100 U/mL penicillin, 100 μg/mL streptomycin, 5% FBS and 2 mM L-glutamine. The liver homogenate of four infected rodents (two *R. norvegicus* rats, one *R. flavipectus* rat and one *R. rattus* rat) was filtered and inoculated onto the cells after 10-fold dilution. The cells were then washed and incubated in the culture medium. On the 10th day, the cell supernatant was harvested and injected into new DH82 cells. Culture supernatant was sampled on the 10th day and tested for the presence of Wenzhou virus by nested RT-PCR.

Electron microscopy

The Wenzhou virus-infected DH82 cells and supernatant were harvested on the 10th day. The cells were washed twice in PBS and centrifugated at 1000 g × 5 min. The cell pellets were fixed with 2.5% (wt/vol) glutaraldehyde overnight at 4 °C and postfixed with 1% OsO_4 in cacodylate buffer for 1 h at room temperature. The pellets were then dehydrated in ethanol and rinsed with propylene oxide for 30 min at room temperature. After embedding in resin, the samples were subjected to a transmission electron microscope (JEM 1230; JEOL). The supernatant was also treated and observed by negative EM as described (Qin et al., 2014).

Virus propagation

The propagation of WENV in DH82 cells was estimated approximately using quantitative real-time RT-PCR targeting the RdRP gene (primers available on request). The cells infected by WENV were harvested at 12 h, and one, two, three, five, seven, and nine days post-infection. The quantitative real-time PCR was performed using a TaKaRa One Step PrimeScript[TM] RT-PCR kit. Canine glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as the internal control. A comparative Ct method was used to estimate the relative amount of viral RNA in cells. RNA levels were normalized to GAPDH mRNA in the same sample, and then presented as copy numbers relative to mRNA from the sample that contains the smallest ΔΔCt value.

Acknowledgments

Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at http://dx.doi.org/10.1016/j.virol.2014.11.026.

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