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BRIEF REPORT

Reston virus in domestic pigs in China

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Abstract Historically, Reston virus (RESTV) has been found to be associated with outbreaks of disease only in nonhuman primates. Its spread to domestic pigs was reported for the first time in 2008. In this study, we report the discovery, molecular detection, and phylogenetic analysis of Reston virus (RESTV) in domestic pigs in China. A total of 137 spleen specimens from pigs that died after showing typical clinical signs of porcine reproductive and respiratory syndrome (PRRS), and for which infection with porcine reproductive and respiratory syndrome virus (PRRSV) was confirmed by RT-PCR, were collected from three farms in Shanghai from February to September 2011. Of these samples, 2.92 % (4/137) were found to be positive for RESTV. All of the positive piglets were under the age of 8 weeks and were co-infected with PRRSV. Sequences were found that shared 96.1 %-98.9 % sequence similarity with those of two RESTV variants that had been discovered previously in domestic pigs and cynomolgus macaques from the Philippines. We therefore conclude that RESTV was present in domestic pigs in Shanghai, China.

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Reston virus (RESTV) is the sole member of the species *Reston ebolavirus*, which is included in the genus *Ebolavirus,* family *Filoviridae*, order *Mononegavirales*. It was discovered in 1989 in cynomolgus macaques that had been imported into the United States from the Philippines [1]. Subsequently, three outbreaks of disease in nonhuman primates in the United States and Italy were also traced back to imported cynomolgus macaques from the same facility in the Philippines, which had been reported to have RESTV-infected animals [2–4]. In 2008, RESTV was isolated for the first time from domestic pigs (*Sus scrofa*) during an outbreak of porcine reproductive respiratory syndrome (PRRS) [5].

In the present study, three farms, located 35 km from each other in Shanghai, with a total of 137 pigs, were studied from February to September 2011. A total of 137 spleen specimens were collected from pigs that had died after showing typical clinical signs of PRRS, such as high fever or blue ears, and infection by PRRSV was confirmed using RT-PCR as described previously [6]. These spleen specimens (20 to 40 mg) were homogenized separately in 500 μl PBS and centrifuged, and 200 μl of supernatant of the centrifuged homogenized spleen was used for RNA extraction. Total RNA was extracted using TRIzol Reagent (Invitrogen, USA) according to the manufacturer's instructions. The primers RESTV-ER3-F and RESTV-ER3-R were designed [5], and RESTV was detected by RT-PCR. The amplified products were analyzed by electrophoresis in 1.2 % agarose gels containing ethidium bromide (0.5 μg/ml), and a 385-bp amplicon was purified using a QIAquick Gel Extraction Kit (QIAGEN, Germany) following the manufacturer's instructions. The purified PCR products were ligated into the PMD18-T vector (TaKaRa, Japan) at 16 °C overnight using T4 DNA ligase (TaKaRa, Japan). Competent *E. coli* DH5a cells (TaKaRa,

Table 1 Differences in the nucleotide sequences of the four RESTV variants in the present study and those of published RESTV reference variants, Ebola virus, Bundibugyo virus, Taï Forest virus and Sudan virus reference variants. The evolutionary distances were computed by the ClustalW method using DNAMAN 6.0 software

Percent Identity

Divergence

	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24		
1		95.1	95.6	95.1	98.7	79.8	80.3	80.1	99.0	80.1	80.1	80.3	78.0	99.0	97.9	76.7	76.2	76.2	78.0	78.0	98.2	98.7	76.7	78.0	1	JNB872215
2	5.1		97.2	96.1	95.6	76.2	76.7	76.4	95.1	76.4	76.4	76.7	75.9	95.1	94.5	74.6	74.1	73.6	74.6	74.6	94.3	96.1	74.6	75.9	2	JNB872216
3	4.5	2.9		97.9	95.6	76.7	77.2	76.9	95.1	76.9	76.9	77.2	75.4	95.1	94.5	74.1	73.6	73.6	74.4	74.4	94.3	96.1	74.1	75.4	3	JNB872217
4	5.1	4.0	2.1		95.1	75.9	76.4	76.2	94.5	76.2	76.2	76.4	75.9	94.6	94.0	74.6	74.1	73.8	74.4	74.4	93.8	95.6	74.6	75.9	4	JNB872218
5	1.3	4.5	4.5	5.1		79.5	79.5	79.3	99.0	79.3	79.3	79.5	77.7	99.2	98.4	76.4	75.9	75.9	77.5	77.5	97.9	99.5	76.4	77.7	5	AB050936
6	23.7	28.8	28.1	29.3	24.0		99.5	99.7	80.0	99.7	99.7	99.0	76.4	80.3	80.0	74.9	74.4	78.0	80.6	80.6	80.6	79.0	74.9	76.9	6	AF086833
7	22.9	28.1	27.4	28.5	24.0	0.5		99.7	80.3	99.7	99.7	99.0	76.9	80.3	80.3	75.4	74.9	78.0	80.6	80.6	81.1	79.5	75.4	77.5	7	AF272001
8	23.3	28.5	27.8	28.9	24.4	0.3	0.3		80.0	100.0	100.0	99.2	76.7	80.1	80.0	75.1	74.6	78.2	80.8	80.8	80.8	79.3	75.1	77.2	8	AF499101
9	1.0	5.1	5.1	5.7	1.0	23.4	23.0	23.4		80.0	80.0	80.3	77.7	99.7	98.5	76.4	75.8	76.4	78.2	78.2	98.7	98.7	76.4	78.2	9	AF522874
10	23.3	28.5	27.8	28.9	24.4	0.3	0.3	0.0	23.4		100.0	99.2	76.7	80.1	80.0	75.1	74.6	78.2	80.8	80.8	80.8	79.3	75.1	77.2	10	EU224440
11	23.3	28.5	27.8	28.9	24.4	0.3	0.3	0.0	23.4	0.0		99.2	76.7	80.1	80.0	75.1	74.6	78.2	80.8	80.8	80.8	79.3	75.1	77.2	11	AY142960
12	22.9	28.1	27.4	28.5	24.0	1.0	1.0	0.8	23.0	0.8	0.8		76.9	80.3	79.7	75.4	74.9	77.7	81.1	81.1	81.1	79.5	75.4	77.5	12	AY354458
13	26.2	29.3	30.1	29.2	26.6	29.0	28.2	28.6	26.8	28.6	28.6	28.2		77.7	78.4	97.2	96.6	78.2	77.5	77.5	77.7	78.2	97.2	99.5	13	AY729654
14	1.0	5.1	5.1	5.7	0.8	22.9	22.9	23.3	0.3	23.3	23.3	22.9	26.6		98.7	76.4	75.9	76.4	78.2	78.2	98.7	98.7	76.4	78.2	14	AY769362
15	2.1	5.7	5.7	6.2	1.6	23.4	23.0	23.4	1.6	23.4	23.4	23.7	25.6	1.3		77.1	76.6	76.4	78.2	78.2	97.7	98.2	77.1	79.0	15	FJ621583
16	28.2	31.4	32.2	31.3	28.6	31.5	30.7	31.2	28.8	31.2	31.2	30.7	2.9	28.6	27.5		99.5	79.5	77.7	77.7	76.9	76.9	100.0	96.6	16	EU338380
17	28.9	32.2	33.0	32.1	29.3	32.3	31.5	31.9	29.5	31.9	31.9	31.5	3.5	29.4	28.2	0.5		79.0	77.2	77.2	76.4	76.4	99.5	96.1	17	EVU23458
18	28.8	32.8	32.9	32.5	29.2	26.4	26.4	26.0	28.5	26.0	26.0	26.8	26.0	28.4	28.5	24.0	24.8		81.4	81.4	76.9	75.9	79.5	78.8	18	FJ217161
19	26.2	31.2	31.7	31.7	26.9	22.7	22.7	22.4	25.9	22.4	22.4	22.0	27.1	25.8	25.9	26.7	27.4	21.8		100.0	78.5	77.5	77.7	78.0	19	FJ217162
20	26.2	31.2	31.7	31.7	26.9	22.7	22.7	22.4	25.9	22.4	22.4	22.0	27.1	25.8	25.9	26.7	27.4	21.8	0.0		78.5	77.5	77.7	78.0	20	NC014372
21	1.8	6.0	5.9	6.5	2.1	22.6	21.9	22.2	1.3	22.2	22.2	21.9	26.7	1.3	2.4	27.9	28.6	27.6	25.4	25.4		97.9	76.9	78.2	21	FJ621584
22	1.3	4.0	4.0	4.5	0.5	24.8	24.0	24.4	1.3	24.4	24.4	24.0	25.9	1.3	1.9	27.8	28.6	29.2	27.0	27.0	2.1		76.9	78.2	22	FJ621585
23	28.2	31.4	32.2	31.3	28.6	31.5	30.7	31.2	28.8	31.2	31.2	30.7	2.9	28.6	27.5	0.0	0.5	24.0	26.7	26.7	27.9	27.8		96.6	23	FJ968794
24	26.2	29.3	30.1	29.2	26.6	28.1	27.4	27.8	26.0	27.8	27.8	27.4	0.5	25.9	24.8	3.5	4.0	25.2	26.3	26.3	25.9	25.9	3.5		24	JN638998
	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24		

Japan) were transformed with the recombinant plasmid. Four clones were sequenced for each sample, and similarity searches using these sequences were carried out using BLAST (http://www.ncbi.nlm.nih.gov/BLAST/). After multiple alignment with CLUSTAL W (version 1.4), four 385-bp nucleotide sequences obtained in the present study were used for phylogenetic analysis. The reliability of the different phylogenetic groupings was evaluated by using the bootstrap test (1,000 bootstrap replications) available in MEGA4.0 [7].

RT-PCR results indicated that four specimens were positive for RESTV (2.92 %, 4/137), suggesting for the first time the presence of RESTV infection in domestic pigs in Shanghai. All four of the positive spleen specimens were from piglets that were under the age of 8 weeks and were co-infected with PRRSV. The nucleotide sequences were 95.1 % to 97.2 % similar to each other (Table 1). The four L gene sequences amplified in the present study, together with their primer sequences, have been submitted to GenBank under accession numbers JNB872215–JNB872218. A phylogenetic tree was constructed based on 385-bp L gene sequences from the RESTV variants from the current study and 15 reference viruses (Figure 1). The present four RESTV variants were closely related, with 96.1 %-98.9 % sequence similarity, to two RESTV variants (FJ621585 and AB050936) that were discovered previously in domestic pigs and cynomolgus macaques from the Philippines [5, 8], but they formed a separate branch in the phylogenetic tree.

It is unlikely that the RT-PCR sequences that match the earlier RESTV sequences were derived from another as yet undescribed filovirus. Ebolavirus L genes are characteristically well conserved within members of each ebolaviral species (typically >96 %) as compared to between members of ebolaviral species (typically <80 %). Furthermore, while some ebolavirus- and marburgvirus-like sequences have been found integrated into some mammalian genomes [9], this has thus far not been demonstrated for pigs, and no sequences with this level of similarity to those of RESTV have been found. It is therefore likely that the detection of these sequences was due to the presence of RESTV in the animals.

An asymptomatic experimental infection of pigs with RESTV has been shown recently by Marsh et al. [10], while experimental infection with Ebola virus (EBOV) in pigs by Kobinger et al. [11] produced severe respiratory

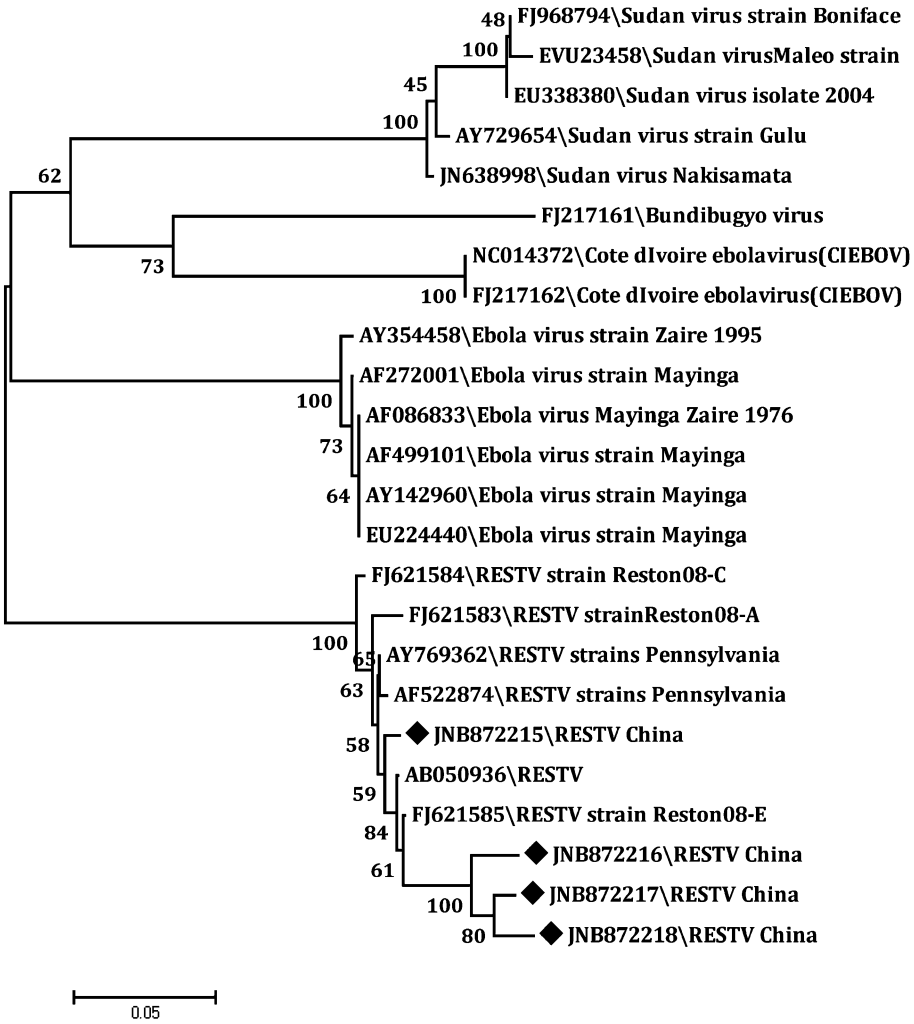

Fig. 1 Phylogenetic analysis of partial 385-bp nucleotide sequences of the L gene of our RESTV variants together with those of published RESTV reference variants, Ebola virus, Bundibugyo virus, Taï Forest virus and Sudan virus reference variants, using the neighbor-joining method with Mega 4.0 software. Bootstrap values >50 % are indicated for the corresponding nodes based on 1000 bootstrapping replicates. GenBank accession numbers for the reference strains are shown at each branch point. The four newly identified RESTV strains from the present study (GenBank accession numbers: JNB872215- JNB872215) are indicated with ''◆'

disease and virus shedding. While the impact of RESTV by itself on public and animal health remains to be determined, it is possible that asymptomatic infections in pigs could contribute to disease severity in the context of other infections such as PRRSV. In this study, the detection of RESTV in pigs was associated with the presence of PRRSV, and the pigs that were infected with RESTV were under 8 weeks old, which is consistent with the results from the Philippines [5], where it was demonstrated that pigs infected with RESTV were immunocompromised or were coinfected with a relative of PRRSV, simian hemorrhagic fever virus (SHFV).

Of the four RESTV sequences, the JNB8782215 sequence was most closely related to the previously reported RESTV sequences from isolates from pigs and cynomolgus macaques originally from the Philippines, while the other three sequences were more distantly related to the previously reported Reston virus sequences, and also to each other. This indicates that the Reston virus genes sequenced in this study had mutated. Because all of the pigs in this study were bred and raised in Shanghai, and bats of the species *Rousettus amplexicaudatus* in the Philippines were recently shown to have antibodies against RESTV [12], it cannot be ruled out that bats are a possible vehicle for spread of RESTV, but further study is needed.

In conclusion, the outbreaks of RESTV infection in nonhuman primates and domestic pigs suggest the zoonotic potential of this virus [13, 14]. We report the molecular detection and phylogenetic analysis of RESTV in domestic pigs in China. Detection and further analysis of RESTV variants from different geographic areas will be helpful for understanding the worldwide distribution and heterogeneity of RESTV variants in domestic pigs and their potential in zoonotic infection. This study indicates the presence of RESTV in domestic pigs in Shanghai, China. It provides additional information on its genetic diversity, possible coinfections, and infection of immunocompromised pigs. It is expected that future epidemiology and pathogenesis studies of RESTV will shed light on its potential reservoirs, mode(s) of transmission, mechanisms of pathogenesis,

prevalence in nature, and consequences for the agricultural industry and trade.

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References

1. Jahrling PB, Geisbert T, Dalgard DW, Johnson ED, Ksiazek TG, Hall WC, Peters CJ (1990) Preliminary report: isolation of Ebola virus from monkeys imported to USA. Lancet 335:502–505
2. Miranda ME, Ksiazek TG, Retuya TJ, Khan AS, Sanchez A, Fulhorst CF, Rollin E, Calaor AB, Manalo DL, Roces MC, Dayrit MM, Peters CJ (1996) Epidemiology of Ebola (subtype Reston) virus in the Philippines. J Infect Dis 179:S115–S119
3. Hayes CG, Burans JP, Ksiazek TG, Del Rosario RA, Miranda ME, Manaloto CR, Barrientos AB, Robles CG, Dayrit MM, Peters CJ (1992) Outbreak of fatal illness among captive macaques in the Philippines caused by an Ebola-related filovirus. Am J Trop Med Hyg 46:664–671
4. Rollin PE, Williams RJ, Bressler DS, Pearson S, Cottingham M, Pucak G, Sanchez A, Trappier SG, Peters RL, Greer PW, Zaki S, Demarcus T, Hendricks K, Kelley M, Simpson D, Geisbert TW, Jahrling PB, Peters CJ, Ksiazek TG (1999) Ebola (subtype Reston) virus among quarantined nonhuman primates recently imported from the Philippines to the United States. J Infect Dis 179:S108–S114
5. Barrette RW, Metwally SA, Rowland JM, Xu L, Zaki SR, Nichol ST, Rollin PE, Towner JS, Shieh WJ, Batten B, Sealy TK, Carrillo C, Moran KE, Bracht AJ, Mayr GA, Sirios-Cruz M, Catbagan DP, Lautner EA, Ksiazek TG, White WR, McIntosh MT (2009) Discovery of swine as a host for the Reston virus. Science 325:204–206
6. Chittick WA, Stensland WR, Prickett JR, Strait EL, Harmon K, Yoon KJ, Wang C, Zimmerman JJ (2011) Comparison of RNA extraction and real-time reverse transcription polymerase chain reaction methods for the detection of Porcine reproductive and respiratory syndrome virus in porcine oral fluid specimens. J Vet Diagn Invest 23:248–253
7. Kumar S, Tamura K, Nei M (2004) MEGA3: integrated software for molecular evolutionary genetics analysis and sequence alignment. Brief Bioinforma 5(2):150–163
8. Ikegami T, Calaor AB, Miranda ME, Niikura M, Saijo M, Kurane I, Yoshikawa Y, Morikawa S (2001) Genome structure of Ebola virus subtype Reston: differences among Ebola subtypes. Arch Virol 146:2021–2027
9. Belyi VA, Levine AJ, Skalka AM (2010) Unexpected inheritance: multiple integrations of ancient bornavirus and ebolavirus/marburgvirus sequences in vertebrate genomes. PLoS Pathog 29(6):e1001030
10. Marsh GA, Haining J, Robinson R, Foord A, Yamada M, Barr JA, Payne J, White J, Yu M, Bingham J, Rollin PE, Nichol ST, Wang LF, Middleton D (2011) Ebola Reston virus infection of pigs: clinical significance and transmission potential. J Infect Dis 204:S804–S809
11. Kobinger GP, Leung A, Neufeld J, Richardson JS, Falzarano D, Smith G, Tierney K, Patel A, Weingartl HM (2011) Replication, pathogenicity, shedding, and transmission of Zaire ebolavirus in pigs. J Infect Dis 204:200–208
12. Taniguchi S, Watanabe S, Masangkay JS, Omatsu T, Ikegami T, Alviola P, Ueda N, Iha K, Fujii H, Ishii Y, Mizutani T, Fukushi S, Saijo M, Kurane I, Kyuwa S, Akashi H, Yoshikawa Y, Morikawa S (2011) Reston Ebolavirus antibodies in bats, the Philippines. Emerg Infect Dis 17:1559–1560
13. Miller RK, Baumgardner JY, Armstrong CW (1990) Update: filovirus infections among persons with occupational exposure to nonhuman primates. MMWR 39:266–273
14. Mary Elizabeth G, Miranda Noel Lee, Miranda J (2011) Reston virus in Humans and Animals in the Philippines: A Review. J. Infect. Dis 204:S757–S760