The Journal of Veterinary Medical Science

FULL PAPER

Public Health

PCR-based screening and phylogenetic analysis of rat pegivirus (RPgV) carried by rodents in China

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J. Vet. Med. Sci.
82(10): 1464–1471, 2020
doi: 10.1292/jvms.19-0530

Received: 26 September 2019
Accepted: 23 June 2020
Advanced Epub:
24 July 2020

ABSTRACT. Rodent-borne *pegiviruses* were initially identified in serum samples from desert wood-rats in 2013, and subsequently in serum samples from commensal rats in 2014. However, the prevalence and phylogenetic characteristics of rodent *pegiviruses* in China are poorly understood. In this study, we screened serum samples collected from wild rats in southern China between 2015 and 2016 for the presence of rat *pegivirus* (RPgV) by PCR. Among the 314 serum samples from murine rodents (*Rattus norvegicus*, *Rattus tanezumi*, and *Rattus losea*) and house shrews (*Suncus murinus*), 21.66% (68/314) tested positive for RPgV. Out of these, 23.81% (62/219) of samples from *R. norvegicus* tested positive, which was significantly higher than that for the other species: 7.69% (1/13), 5.88% (2/34), and 6.25% (3/48) for *R. tanezumi*, *R. losea*, *and S. murinus*, respectively (χ^2=18.91, P<0.001). Phylogenetic analysis revealed clustering of viral sequences in the main rodent clade. Analysis of the 3 near-full-length genome sequences of RPgV obtained in this study showed that these viruses exhibited mean nucleic acid and amino acid identities of 94.1% and 98.5% with Chinese RPgV strains, and 90.3 and 97.1% with an RPgV strain from the USA, respectively. This study provides novel insights into the geographic distribution of rodent *pegiviruses* in China, and identifies potential animal hosts for future studies of these *pegiviruses*.

KEY WORDS: epidemiological investigation, *pegivirus*, phylogenetic analysis, rat, shrew

Since 1995, a number of novel members of the family *Flaviviridae* have been discovered, and tentatively assigned to a new genus named *Pegivirus* (formerly GB virus) [10]. The members of this genus are positive-sense, single-stranded RNA viruses of approximately 10 kb in length, which contain only one open reading frame (ORF). Their genomic structure resembles that of *hepacivirus* [13]. Intriguingly, unlike *hepacivirus*, *pegivirus* is primarily lymphotropic, results in asymptomatic infection, and is benign in humans and other animals [5, 6, 9, 14, 17]. A comprehensive understanding of the natural reservoirs for this emerging virus would provide valuable insights into the transmission and geographic distribution of pegivirus. Although *pegivirus* was first identified in humans, the identification of tamarins infected with the GB hepatitis agent suggested a wider host range for this virus. Homologous *pegiviruses* were subsequently reported in various mammalian species, including non-human primates (GBV-A or simian *pegivirus*, SPgV), horses (equine *pegivirus*, EPgV), pigs (porcine *pegivirus*, PPgV), bats (GBV-D or bat *pegivirus*, BPgV), and wild rodents (rodent *pegivirus*, RoPgV) [1, 2, 8, 10–12].

The order *Rodentia* comprises over 2,200 living species, which represents nearly 40% of the total number of mammals [18]. Numerous rodent species act as wild and synanthropic reservoirs of myriad zoonotic agents. In southern China, *Rattus norvegicus* and *Rattus tanezumi* represent 2 of the predominant commensal rodent species. The impact of the frequent interactions between rodents and humans has been actively studied. The identification of new rodent-borne and insectivore-borne viruses has advanced our understanding of both host range and epizootiology. In this study, we screened serum samples from rodents and house shrews in southern China for infection with rodent *pegivirus*. In addition, partial sequences of the *NS3*/*NS5B* gene and 3 near-full-length genome (NFLG) sequences were determined for detailed genetic characterization of this class of viruses.

MATERIALS AND METHODS

Sample collection

Between August 2015 and March 2016, a total of 314 rodents and shrews were captured using cage traps (280 × 120 × 100

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Fig. 1. A representative map with information of the collection sites. Triangles indicate the sampling locations.

mm) from Baiyun (GZ.BY, n=189) and Yuexiu (GZ.YX, n=56) districts in Guangzhou city in Guangdong province and from Xiamen city in Fujian province (FJ.XM, n=69). The collection sites are depicted in Fig. 1. The animals were all captured in or close to human dwellings, and the respective ages were determined based on body weight, as previously described [3]. The animals captured included 43 juveniles (<80 and <23 g for rats and shrews, respectively), 97 sub-adults (80–180, 80–200, and 23–28 g for female rats, male rats, and shrews, respectively), and 174 adults (>180, >200, and >28 g for female rats, male rats, and shrews, respectively). The male-to-female ratio was 1.32. The animals were anesthetized with diethyl ether prior to drawing cardiac blood. All the serum samples were separated by centrifugation, frozen, and stored immediately at −80°C; the stored samples were thawed at 4°C prior to processing.

Identification of rodent species

Rodent species were confirmed by preliminary morphological identification and sequencing of the cytochrome *b* (*cyt-b*) gene fragment of the mitochondrial genome, which is a widely recognized technique for classifying biological samples. The primers cytb-F (forward, 5′- CGAAGCTTGATATGAAAAACCATCGTTG-3′) and cytb-R (reverse, 5′- AAACTGCAGCCCCTCAGA ATGATATTTGTCCTCA-3′) were used to amplify a fragment containing a partial nucleic acid sequence of the *cyt-b* gene. The following amplification conditions were used: 95°C for 11 min; followed by 35 cycles of 95°C for 45 sec, 50°C for 45 sec, and 72°C for 90 sec; followed by 72°C for 30 min [7]. The sequences obtained were compared to known DNA sequences from reliable databases and voucher specimens, and analyzed to make high-confidence taxonomic assignments.

RNA extraction and targeted molecular analysis

RNA was extracted from 200 *µl* of each blood sample using a High Pure Viral RNA kit (Roche, Mannheim, Germany) according to the manufacturer's instructions, and eluted in a final volume of 50 *µl*. cDNA was generated from the RNA using random hexamers and a Transcriptor First Strand cDNA Synthesis kit (Roche). Then, *pegivirus* partial helicase gene sequences were amplified using nested PCR. In addition, RNA-dependent RNA polymerase genes were amplified and compared to reference sequences to confirm the positive *pegivirus* test result. Specific degenerate primer sets were designed. The rat *Pegivirus* (RPgV)*NS3* gene was amplified using primer pairs as described previously [3]. PCR of the RPgV *NS5B* gene employed the outside primer pair of RPgV-NS5b-1F (5′-ACCCGACCAACTTTCAAGCCMCC-3′) and RPgV-NS5b-1R (5′-CAGCCGAAGTGAGGTCTGCGTGC-3′), and the inside primer pair of RPgV-NS5b-2F (5′- CCGACCAACTTTCAAGCCMCCYC-3′) and RPgV-NS5b-2R (5′-AAGTGAGGTCTGCGTGCCTAGTA-3′). Two microliters of cDNA were added to a 25 *µl* reaction mixture using the Premix Taq™ reagents (Takara, Dalian, People's Republic of China), according to the manufacturer's protocol. The first and second rounds of RPgV-*NS3* gene amplification were carried out under the following conditions: 94°C for 5 min (1 cycle); 94°C for 40 sec, 57°C

for 40 sec, and 72°C for 1 min (35 cycles); followed by incubation at 72°C for 10 min; and storage at 4°C. The first and second rounds of RPgV-*NS5B* gene amplification were carried out under similar conditions with minor modifications: 94°C for 5 min (1 cycle); 94°C for 40 sec, 55°C for 40 sec, and 72°C for 1 min (35 cycles); and 72°C for 10 min (1 cycle). For amplification of the RPgV NFLG sequence, 15 pairs of primers were designed according to the reference sequence (GenBank accession number KJ950934) and used with PrimeSTAR HS (Premix) reagents (Takara, Dalian, People's Republic of China). The PCR products were separated in a 1% agarose gel, and directly sequenced by the company Sangon Co. (Sangon, Guangzhou, People's Republic of China). Any anomalies or bases that were ambiguous were clarified by sequencing in both directions.

Sequence and phylogenetic analysis

Sequence editing was performed using BioEdit and DNAMan. The nucleotides were compared to sequences reported in the GenBank non-redundant nucleotide database (NT) using BLASTn and aligned using Clustal W. Nucleic acid similarity were performed online using BLAST (https://blast.ncbi.nlm.nih.gov/). All data were exported to the MEGA6 software. A maximum-likelihood phylogenetic tree was constructed using the Tamura-Nei model with 1,000 bootstrap replicates. The similarity between *pegivirus* sequences was plotted using the SimPlot 3.5.1 software.

Ethics statement

Animals were caught by snap traps with permission of respective authorities. The treatment was carried out in conformity with the guidelines for the Laboratory Animal Use and Care from Southern Medical University and the Rules for the Implementation of Laboratory Animal Medicine from the Ministry of Health, China. The study protocol has been approved by the Animal Ethics and Welfare Committee of the School of Public Health, Southern Medical University.

Accession numbers

The 3 NFLG sequences have been submitted to the GenBank database with the accession numbers MG273686-MG273688.

RESULTS

Prevalence of pegivirus in rodents and shrews

Specimens of rodent and shrew serum were collected, including 219 samples from *R. norvegicus*, 13 from *R. tanezumi*, 34 from *Ruttus losea*, and 48 from *Suncus murinus* (Table 1). To investigate the frequency of *pegivirus* infection, each of the 314 samples was individually processed and screened by nested PCR, and 21.66% (68/314) of the serum samples from both Guangzhou and Xiamen cities yielded positive results for *pegiviruses*. The highest prevalence of *pegivirus* infection, 51.59% (162/314), was found in Norway rats (*R. norvegicus*). This was significantly higher than that for the other species: 7.69% (1/13), 5.88% (2/34), and 6.25% (3/48) for *R. tanezumi*, *R. losea*, and the insectivore *S. murinus* (χ^2=18.91, P<0.001). The percentages of juvenile (11.63%, 5/43) and sub-adult (12.37%, 12/97) rats positive for infection were similar; however, for adult rats the percentage was higher (29.31%, 51/174).

Phylogenetic analysis of partial helicase and RNA-dependent RNA polymerase gene sequences

Partial (238 bp) *NS3* gene sequences and partial (602 bp) *NS5B* gene sequences were successfully amplified from the serum samples. BLAST was used to align the sequences and determine their similarity. The data obtained are indicative of a close relationship between the rodent *pegiviruses* identified in the present study and the 2 previously reported (accession numbers KJ950934 and KC815311). Next, 10 out of the 46 typical sample sequences were chosen for further phylogenetic analyses. All the sequence variants in *NS3* genome region were congruent with branching orders in the *NS5B* region, indicating the lack of recombination in these sequences. In general, the *pegiviruses* formed 3 distinct phylogenetic lineages. The first lineage comprised sequence variants from humans, primates, and bats; while the second lineage was composed of novel porcine *pegivirus* clustered together with variants from bats and primates. The second human *pegivirus* (HPgV-2) and sequence variants from rodents and bats

Table 1. Distribution of rodent *pegivirus* in rats and shrews according to geographic locations (%, n)

Species	Collection sites			Total (Positive, %)
	GZ.BY (2015.3-2016.4)	GZ.YX (2015.10)	FJ.XM (2015.10)	
Rattus norvegicus	29.08 (41/141)	30.36 (17/56)	18.18 (4/22)	28.31 (62/219)
Rattus tanezumi	33.30 (1/3)		0 (0/10)	7.69 (1/13)
Rattus losea			5.88 (2/34)	5.88 (2/34)
Suncus murinus	6.67 (3/45)		0 (0/3)	6.25 (3/48)
Subtotal	23.81 (45/189)	30.36 (17/56)	8.70 (6/69)	21.66 (68/314)

GZ.BY, Baiyun district, Guangzhou city in Guangdong province; GZ.YX, Yuexiu district, Guangzhou city in Guangdong province; FJ.XM, Xiamen city in Fujian province. There were 2 sample collection regions in Xiamen city: Tong'an district and Huli district.

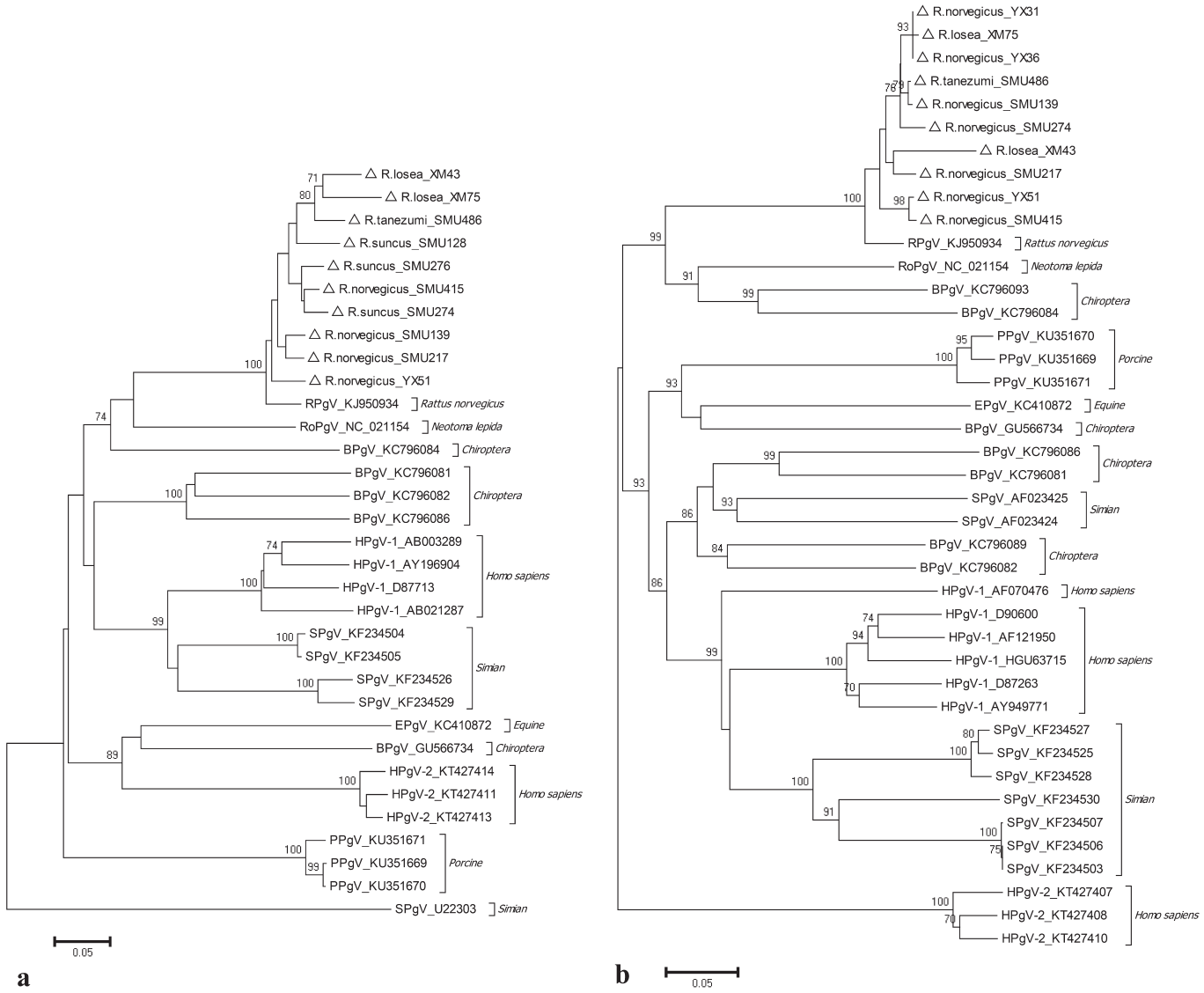

Fig. 2. Phylogenetic tree constructed by the neighbor-joining method based on partial nucleotide sequences of the (a) *NS3* (238 bp) gene and (b) *NS5B* (602 bp) gene of rat *pegivirus* (RPgV), rodent *pegivirus* (RoPgV), bat *pegivirus* (BPgV), human *pegivirus* (HPgV), human *pegivirus* type 2 (HPgV-2), porcine *pegivirus* (PPgV), simian *pegivirus* (SPgV), and equine *pegivirus* (EPgV). The RPgV sequences obtained in this study are marked by triangles. Bootstrap values (1,000 replications) >70% are indicated at nodes.

were classified into the third lineage. Multiple sequence alignment showed that all the sequences had high levels of nucleotide identity (bootstrap >70) to each other over the *NS3* and *NS5B* regions. These sequences formed a separate clade within the genus *Pegivirus* (Fig. 2a and 2b) together with 2 other distinct rodent *pegiviruses* identified from blood samples of wood rats and Norway rats.

Characterization of the pegivirus genome in rodents

For a detailed phylogenetic analysis of the rodent *pegivirus*, we included 3 NFLG sequences (missing only the terminal regions) from *R. norvegicus*, namely YX51 (9,945 nt), SMU217 (9,883 nt), and SMU415 (9,963 nt), representing different collection sites in the Yuexiu and Baiyun districts. The lengths of these genomes ranged from 9,883 to 9,963 nt and contained only one ORF, which encoded a putative multi-functional polyprotein predicted by comparison with polyproteins of rat (GenBank accession number KJ950934) and rodent (GenBank accession number KC815311) *pegiviruses*. A phylogenetic tree created based on the whole reading frame demonstrated that rat *pegivirus* belonged to the genus *Pegivirus*, which is consistent with our analysis using partial *NS3* and *NS5B* sequences (Fig. 3). Overall, the 3 rat *pegivirus* sequences generated in our study branched very deeply next to the sequences of *R. norvegicus* and *Neotoma lepida*, and were appreciably different from the sequences of *pegivirus* from humans, primates, and other original hosts in this group. All the *pegivirus* variants found in *R. norvegicus* were clustered together. The sequences identified in the present study showed a mean nucleic acid identity of 94.1 and 90.3% with the Chinese RPgV strains

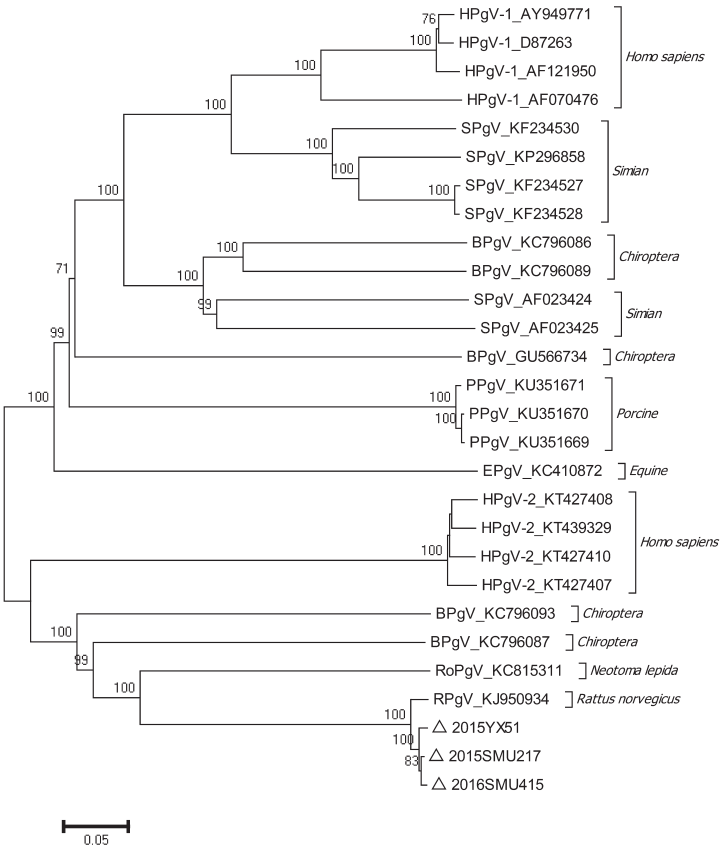

Fig. 3. Phylogenetic tree constructed by the neighbor-joining method based on amino acid sequences of rat *pegivirus* (RPgV), rodent *pegivirus* (RoPgV), bat *pegivirus* (BPgV), human *pegivirus* (HPgV), human *pegivirus* type 2 (HPgV-2), porcine *pegivirus* (PPgV), simian *pegivirus* (SPgV), and equine *pegivirus* (EPgV). The RPgV sequences obtained in this study are marked by triangles. Bootstrap values (1,000 replications) >70% are indicated at nodes.

and the RPgV strain from New York, USA (GenBank accession number KJ950934), respectively. The amino acid identity was similarly high: 98.5% with the Chinese RPgV strains, and 97.1% with both the Chinese strains and the RPgV strains from USA. The differences between the strains identified here and the USA. RPgV sequences were mainly in terms of synonymous mutations at the nucleotide level (Table 2). When compared with *pegiviruses* from other hosts, the nucleic acid (amino acid) identities of rat-derived *pegiviruses* isolated in this study were 91.1% (97.3%), 59.3% (56%), 43.8% (32.4%), 45% (34.8%), 42.7% (33.2%), 42.9% (32.9%), 42.3% (31.9%), and 42.2% (32.6%) compared to the complete coding regions of RPgV, RoPgV, BPgV, HPgV-2, human *pegivirus* type 1 (HPgV-1), PPgV, SPgV, and EPgV, respectively (Table 3). We further analyzed possible recombinations and the differences between RPgV and *pegivirus* sequences from other hosts using the Simplot software: no recombination event was identified (Fig. 4).

DISCUSSION

The detection and characterization of animal virus homologs yields valuable insights into host range, epidemiology, and pathogenesis. This is, to the best of our knowledge, the first report of the prevalence of *pegivirus*-infected rodents in southern China. Rodent *pegivirus* was first found in 2 mouse species, white-throated wood rats (*Neotoma albigula*) and deer mice (*Peromyscus maniculatus*), with the infection rate estimated to be less than 1.5% [5]. Rats are considered to be pests that exist in large population in urban environments and impact human health. One research group assessed the presence and prevalence (15%) of *pegivirus* in *R. norvegicus* in New York city [3]. In addition, Nguyen *et al.* carried out a screening in Vietnam for the presence of rodent *pegivirus* in 638 serum and 470 liver samples from 8 and 6 rodent species, respectively. In their study, no liver samples tested positive for *pegivirus*, although 2.3% (2/87) of their *R. tanezumi* serum samples were *pegivirus* positive [16]. Likewise, we used PCR to identify rodent *pegivirus* sequences in serum samples from *R. norvegicus* and *R. tanezumi,* which are predominant in southern China. A total of 314 serum samples were collected from 4 regions of 2 cities in China: the Baiyun and Yuexiu districts of Guangzhou city, and the Tong'an and Huli districts of Xiamen city. The prevalence of rodent *pegivirus* RNA was 21.66%, which was higher than the prevalence reported in USA [3]. The detection of *pegivirus* infection in *R. tanezumi* is interesting, since only one sample tested positive in this study. The Asian house rat (*R. tanezumi*) is closely related to the brown rat (*R. norvegicus*) and is sympatric with

Table 2. Identity of the near-full-length genome sequences of rat *pegivirus*

Aligned region	Polyprotein	*Y*	*E1*	*E2*	*X*	*NS2*	*NS3*	*NS4A*	*NS4B*	*NS5A*	*NS5B*
Comparison with the Chinese strains											
Nucleotide identity (%)	94.1	92.6	94.3	93.9	92.1	91.4	96.6	96.2	94.9	95.4	95.9
Amino acid identity (%)	98.5	94.6	99.3	98.4	99.1	96.6	99.9	100	100	99.5	99.6
Comparison with both the Chinese strains and the American strain (RPgV)											
Nucleotide identity (%)	90.3	85.2	92.0	90.8	88.5	88.6	93.3	91.1	91.5	92.5	92.8
Amino acid identity (%)	97.1	83.0	97.1	98.0	97.5	94.1	99.6	99.1	99.6	98.1	99.1

Table 3. Nucleotide and translated amino acid sequence identity of the near-full-length sequence of rat *pegivirus* and other *pegiviruses*

	Nucleotide identity (%)									Genbank accession No.
Virus	Present study	RPgV	RoPgV	BPgV	HPgV-2	HPgV-1	PPgV	SPgV	EPgV	
Amino		91.1	59.3	43.8	45.0	42.7	42.9	42.3	42.2	Present study
Acids	97.3		59.2	43.8	44.5	42.9	43.2	42.2	42.2	RPgV KJ950934
	56.0	55.4		43.1	45.1	43.1	43.5	42.8	41.6	RoPgV KC815311
	32.4	32.3	32.3		41.7	49.2	47.9	49.2	46.5	BPgV KC796083
	34.8	34.5	34.9	27.8		39.5	41.4	40.1	40.5	HPgV-2 KX528230
	33.2	32.9	32.5	41.6	28.9		47.0	63.6	45.8	HPgV-1 AB003292
	32.9	32.7	32.8	39.2	29.1	40.8		46.9	46.0	PPgV MH345724
	31.9	31.8	31.9	41.7	28.6	65.4	40.0		44.5	SPgV AF070476
	32.6	32.3	32.2	37.1	27.7	38.3	36.7	37.9		EPgV KC410872

RPgV, rat *pegivirus*; RoPgV, rodent *pegivirus*; BPgV, bat *pegivirus*; HPgV-1, human *pegivirus* type 1; HPgV-2, human *pegivirus* type 2; PPgV, porcine *pegivirus*; SPgV, simian *pegivirus*; EPgV, equine *pegivirus*.

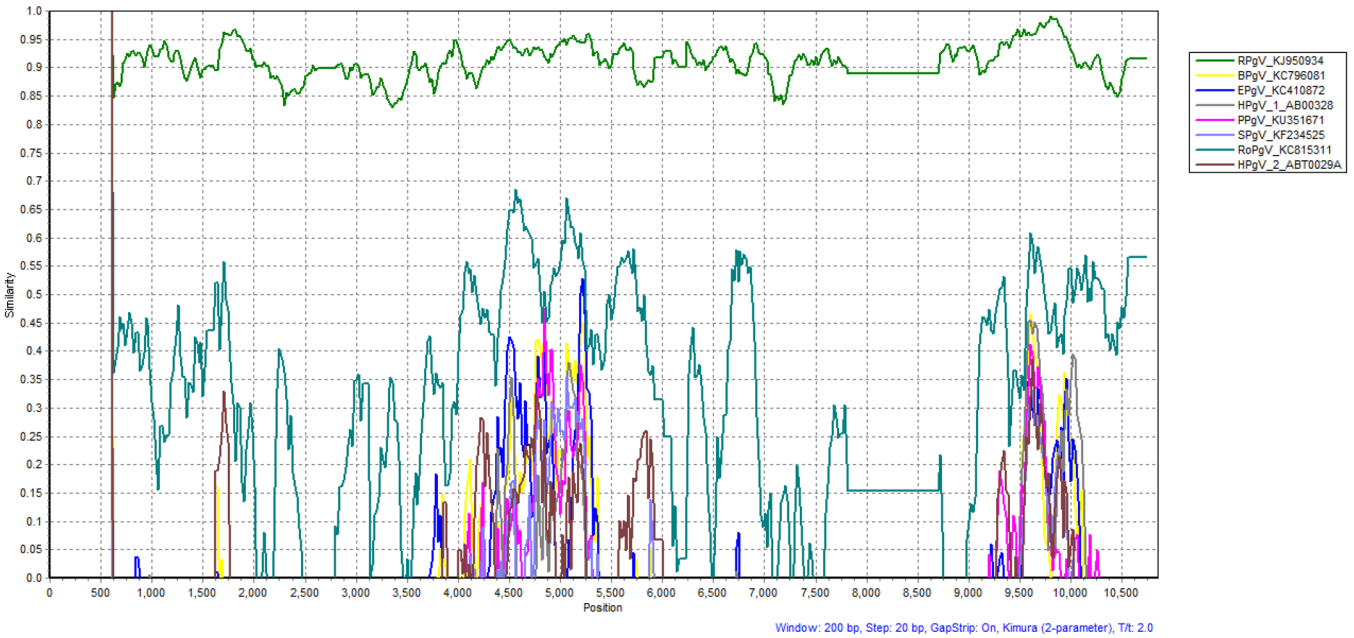

Fig. 4. Characterization of rat *pegivirus*_SMU217 strain. The similarity between RPgV_SMU217 and the reference *pegivirus* sequences was plotted using Simplot (version 3.5.1) software. The sequences used in the phylogenetic analysis were rat *pegivirus* (RPgV, KJ950934), rodent *pegivirus* (RoPgV, KC815311), bat *pegivirus* (BPgV, KC796081), equine *pegivirus* (EPgV, KC410872), human *pegivirus* type 1 (HPgV-1, AB00328), human *pegivirus* type 2 (HPgV-2, ABT0029A), porcine *pegivirus* (PPgV, KU351671), and simian *pegivirus* (SPgV, KF234525).

them in southern China. Both are primarily commensal murine rodents, which inhabit human dwellings and livestock sheds. Genetic analysis of the *pegivirus* sequence from *R. tanezumi* showed high identity (91–95%) with the Norway rat *pegivirus* isolates. These observations might provide some initial indication of the route of rodent *pegivirus* transmission. In addition, the RPgV positivity rates varied from 5.88% in *R. losea* to 6.25% in house shrews, which mainly inhabit fields. Many different zoonotic viruses are

carried by insectivore hosts, for instance, *hantavirus*, *cytomegalovirus,* and *herpesvirus*. However, the only report of an insectivore-borne *pegivirus* was a partial *Tupaia glis NS3 pegivirus* sequence, with 64–67% similarity to bat *pegivirus* (GenBank accession numbers JQ922487-JQ922489). In our study, 3 out of 48 serum samples from the insectivore *S. murinus* tested positive for *pegivirus*. As house shrews and murine rodents have overlapping habitats that are in or close to human dwellings, *pegivirus* may be transmitted between them via fecal-oral contact, exposure of bite wounds to body fluids, close contact, or other transmission routes. Further investigations with an expanded shrew sample set are needed to confirm our findings.

The rat *pegivirus* has been detected in the bladder, urine, fecal matter, heart, kidney, spleen, liver, and even in brain samples of rats [3], suggesting that RPgV is able to infect rats effectively. However, the route of transmission of rodent *pegivirus* between individuals has not been investigated in detail. Much more attention must be paid to tissue tropism in this context.

Studies of virus reservoirs are a critical component of any integrated public-health response to emerging zoonotic diseases [4]. The phylogenetic analysis with partial *NS3* and *NS5B* nucleotide sequences from *pegiviruses* revealed that these viruses were part of the main rodent-derived *pegiviruses* clade, whilst the *pegiviruses* found in *R. norvegicus* and *R. tanezumi* have expanded the taxonomy of rodent *pegiviruses*. The *pegiviruses* tended to be species-specific, and the phylogenetic analysis revealed co-evolution between most of the identified *pegivirus* and their hosts, except for bat *pegivirus* (Fig. 2a and 2b). The rat *pegivirus* occupied the same branch of the phylogenetic tree, indicating that they are adapted to their hosts. However, additional sampling data is required to improve the reliability of this evolutionary history. Also, further studies on the relationship between viral protein and hosts are needed to clarify this issue in a detailed manner. Additionally, the 3 NFLG polyprotein sequences generated in the present study have contributed to our understanding of the phylogeny and biology of *pegivirus*. Recombination and the difference between RPgV and *pegivirus* sequences from other hosts were further analyzed in our study using the Simplot software, and no recombination event was identified (Fig. 4), which was consistent with a previous study showing lack of interspecific recombination in the *pegiviruses* [15]. Rodent *pegivirus* sequences from distant geographic origins, exemplified by samples collected from New York city in the USA and southern China, were aligned and compared. There was over 90% similarity at the nucleic acid level among the sequences from these regions, which suggests that the dominant strain isolated from *R. norvegicus*, which has invaded China and the USA, has acquired an indigenous virus due to horizontal transmission.

The genus *Pegivirus* is a novel genus of the family *Flaviviridae*. Analysis of conserved gene motifs involved in enzymatic function may elucidate the evolutionary relationship of *pegiviruses* to each other and to other members of the family *Flaviviridae*. Comparative phylogenetic analysis of conserved regions in the partial helicase and RNA-dependent RNA polymerase regions were congruent with other rodent *pegiviruses*, which clustered consistently with *pegiviruses* of bats, primates, and humans (HPgV-2), suggesting these viruses are ancient members within the same genus. Kapoor *et al.* has reported that the most conserved regions within viruses of the genus *Pegivirus* were the *NS3* and *NS5B* genes, with highly divergent sequence in the envelope glycoprotein [5]. In our study, RPgV sequences revealed both similarities and differences from previously identified *pegivirus* isolates. The prediction of cleavage sites of nonstructural proteins gave comparable sizes to homologs from other *pegiviruses*. However, 6 *N*-linked glycosylation sites were present in the pegivirus envelope, compared to 9 sites in the deduced envelope protein of *N. lepida pegivirus*. Due to constraints in time and facilities, we were unable to determine the whole genome sequences including the 5′- and 3′-flanking regions. The next step could, therefore, be to use the rapid amplification of cDNA ends method for this purpose. Further, the biophysical characterization of rodent *pegivirus* particles has not been fully elucidated, and their origin and composition remain a mystery. Further studies may help unravel the mechanisms of virus infection and persistence.

ACKNOWLEDGMENT. This work was supported by the Science and Technology Planning Project of Guangdong Province, China.

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