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Novel parvovirus sublineage in the family of *Parvoviridae*

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Abstract *Parvoviridae*, which are classified into two subfamilies *Parvovirinae* and *Densovirinae*, can infect both vertebrate and insects and are related to a wide range of diseases in insects, animals, and humans. In this report, several new parvoviruses were identified in swine sera collected in southeastern China. The sequence analyses showed that the parvoviruses detected in southeastern China formed a distinct sublineage within the subfamily *Parvovirinae*. Based on these results, we propose a novel parvovirus sublineage, Cnvirus, to describe these parvoviruses.

Keywords *Parvoviridae* · Novel sublineage · China

The parvoviruses are the smallest among the animal DNA viruses. The virion has a diameter of 18 to 26 nm and is composed entirely of protein and DNA. *Parvoviridae*, which are classified into two subfamilies *Parvovirinae* and *Densovirinae*, can infect both vertebrate and insects and are related to a wide range of diseases in insects, animals, and humans. The genome is a linear, single-stranded polydeoxynucleotide chain with typically about 5 kilobases (kb) in size and it usually contains two large open reading frames (ORF1 and ORF2) [1]. According to the report of International Committee on Taxonomy of Viruses, the subfamily *Parvovinidae* contains Parvovirus, Erythrovirus, Dependovirus, Amdovirus, and Bocavirus (http://www.ncbi.nlm.nih.gov/ICTVdb/Ictv/index.htm). Recently, Lau et al. proposed another genus, Hokovirus, including porcine hokovirus (PHoV) and bovine hokovirus (BHoV) [2]. To facilitate an improved taxonomic understanding of this subfamily, it is necessary to gain further information about the poorly characterized members. In this report, we identified several new parvoviruses from swine sera collected in southeastern China. Based on the nucleic acid sequence, these new identified parvoviruses formed a distinct cluster within the subfamily *Parvovirinae*.

In early 2006–2007, severe field outbreaks of "High Fever" disease [3, 4] were observed in commercial swine with Porcine Respiratory and Reproductive Syndrome and/or Post-weaning Multisystemic Wasting Syndrome in Zhejiang province, southeast China. Serum samples were harvested from swines with suspected infection from three field outbreak. Random detection of unknown virus genomic DNA or RNA from serum samples was performed using the method as previously described with some modification [5]: Restriction enzyme TaqI (Sangon) was used to replace Csp6.I. And hence NTaq I Adaptor (which is composed of hybridized oligonucleotides NTaq24 and NTaq11, whose sequences will be available upon request) was used to replace NCsp Adaptor and NTaq24, instead of NBam24, was used as primer to amplify target DNA. The Amplified PCR products were then analyzed by agarose gel electrophoresis. The distinct DNA bands were gel-purified using Gel Extraction Mini Kit (WATSON Biotechnologies). The target DNA fragments were cloned into pMD18-T vector (Takara) and sequenced using universal M13 primers (Invitrogen). To obtain the full-length sequences of the target virus genomes, PCR was used to amplify the intervening DNA sequences and a modified RACE method [6] was performed to amplify its 5′ and 3′

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ends. Finally, the sequences were assembled and edited using Seqman program in DNAStar software. The three strains with nearly full-length sequences were named as YW8, JH13, and YH14. All the sequences were deposited in the GenBank with Accession Nos. GU938299, GU938300, and GU938301, respectively.

Sequence analyses and alignments were performed with the aid of ClustalX 2.0, MEGA 4.0 and DNAstar 7.1 softwares. Seventy-eight (including our new sequences) of *Parvovirinae* with nearly full-length sequences were included in the analysis and one BmDNV sequence of *Densovirinae* was used as outgroup. These sequences were all obtained from GenBank. The sequences (total of 79 sequences) were aligned with ClustalX 2.0 and the phylogenetic tree for parvoviruses was then generated using the neighbor-joining method with 1000 bootstrap replicates with the MEGA 4.0 software program. The phylogenetic relationship of the cloned virus with other members of subfamily *Parvovirinae* was obtained by comparing nucleotide sequences of their entire genomes. The resulting phylogenetic tree is depicted in Fig. 1. The overall branching patterns of the tree constructed for the nucleotide sequence of parvoviruses was split into several distinct branches and each branch represents an individual genus of the subfamily *Parvovirinae*, as follows: *Hokovirus*, *Erythrovirus*, *Bocavirus*, *Dependovirus*, *Amdovirus*, *Parvovirus*, and our cloned

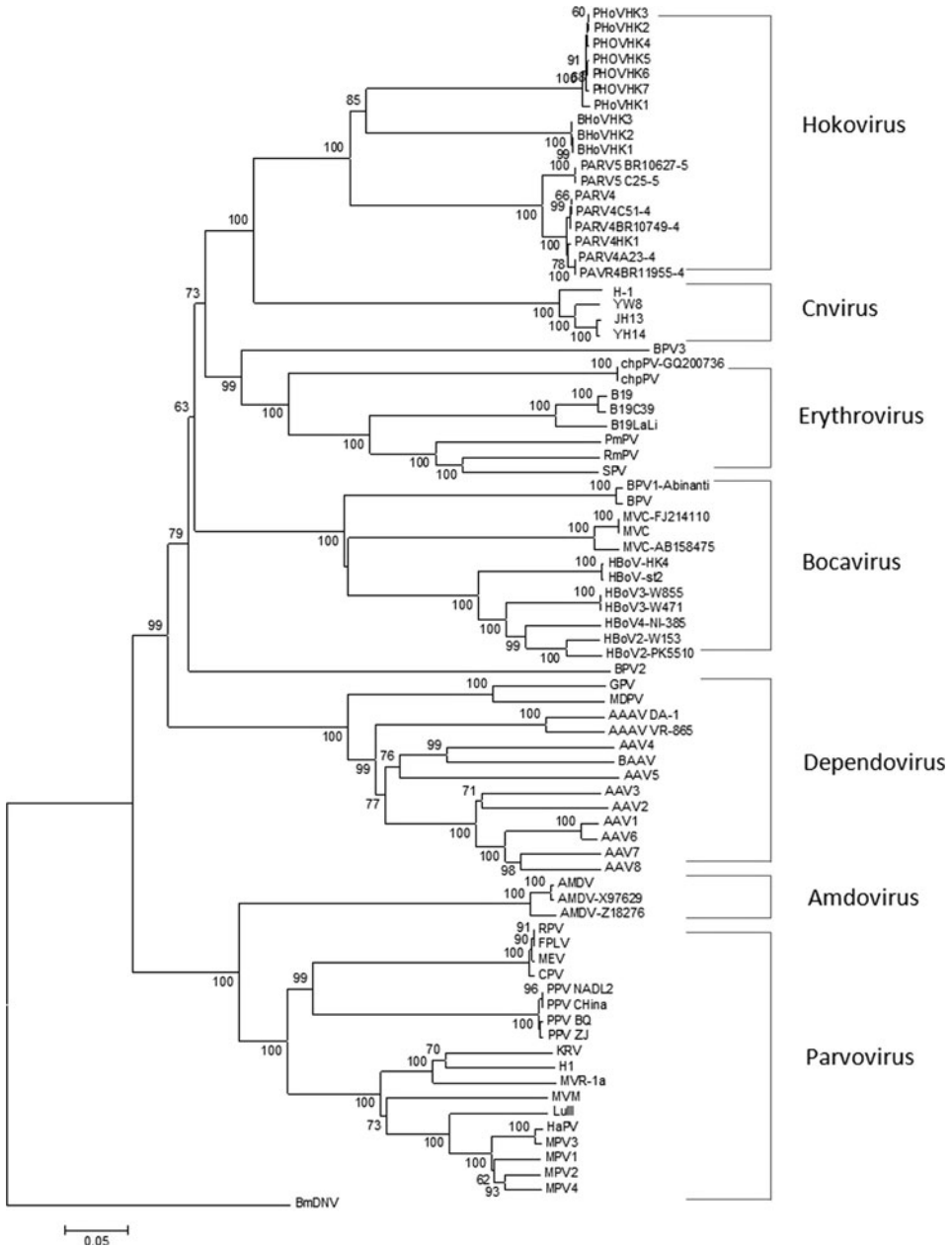

Fig. 1 The phylogentic tree depicting evolutionary relationship between the members of the subfamily *Parvovirinae*. Phylogenetic analysis was based on the nucleotide sequence, using the neighbor-joining method with 1000 bootstrap replicates. Bootstrap values based on the consensus tree are plotted at the main internal branches to show support values. BmDNV was included in the analysis and used as outgroup. All sequences of other 76 parvoviruses were obtained from GenBank

strains (Fig. 1). The phylogenetic trees were also obtained from the deduced amino acid sequences that were encoded by ORF1 and ORF2. Both the representative phylogenetic trees were similar to Fig. 1 (data not shown). Analysis of full genome sequence identity and evolutionary distance additionally supported the observed tree topology. Seventy-eight sequences were aligned with the build-in ClustalW method and the sequence identity between different strains was calculated using the MegAlign program of DNAstar. The evolutionary distances were calculated with the MEGA 4.0 software program using ClustalX aligned sequences. Both the sequence identity and evolutionary distance within and between genera were then summarized in Tables 1 and 2. For the full genome sequence, over 40% identity was observed between different strains within a genus, while less than 30% sequence identity between species of different genera (Table 1). The evolutionary distance value between genera was 0.62–1.97, whereas it was 0.00–0.55 between species in the same genus (Table 2). Analysis of sequence identity and evolutionary distance obtained from the deduced amino acid sequences that encoded by ORF1 and ORF2 yielded comparable results (data not shown). All data above supported the fact that our cloned strains together with the reported strain H-1 [7] represented a different novel parvovirus which are named as Cnvirus.

To investigate the molecular epidemiology of Cnvirus in southeastern China, a total of 296 serum samples were obtained from pig farms in several cities in eastern China, including YiWu, YuHang, JinHua, DeQing, LongYou, JiangShan, HangZhou, JianDe, JinYun, WenLing, YuHuan, WenZhou, and ZhuJi. The results of the PCR detection in all the serum samples are summarized below: among the 296 samples, PCR was positive in 9 (16.3%) of the 55 serum samples in YiWu, 3 (15.0%) of the 20 in YuHang, 2 (4.0%) of the 50 in JinHua, 4 (20.0%) of the 20 in DeQing, 6 (20.0%) of the 30 in LongYou, 1 (3.3%) of the 30 in JiangShan, 1 (2.4%) of the 42 in HangZhou, and no positive in other cities' samples. We cloned and sequenced several PCR products. The identities between determined sequences were 95.1–99.6%. To date, there is only one similar virus from Myanmar [7]. It is not known whether such viruses are currently circulating in other countries as well.

Though the three nearly full-length genomes were obtained from sera collected from three different outbreaks, we do not know whether there was any relation between this virus and the so-called "High Fever" disease. In a survey of a farm (3 times in a 12 months period) with minor problem in early growing finishing pigs, the peak of the Cnvirus DNA detected in sera was about 2–3 weeks earlier than the appearance of symptom. However, there was no relation between the levels of prevalence and the severity of the outbreak (data not showed). The "High Fever" disease, with Porcine Respiratory and Reproductive Syndrome and/or Post-weaning Multisystemic Wasting Syndrome, broke out first in the summer of 2006 in southern China. The

Table 1 Sequences identity(%) within and between genera of *Parvovirinae*

	Hokovirus	Cnvirus	Erythrovirus	Bocavirus	Dependovirus	Amdovirus	Parvovirus
Hokovirus	60.6–99.9						
Cnvirus	4.6–10.3	95.1–98.6					
Erythrovirus	2.4–8.1	2.3–4.3	56.6–98.7				
Bocavirus	0.7–5.0	1.4–4.0	2.7–6.6	40.1–99.9			
Dependovirus	3.6–8.5	3.7–9.4	3.1–7.4	1.9–10.1	43.2–95.9		
Amdovirus	1.5–3.9	1.7–2.9	2.3–4.3	0.9–5.4	0.5–3.6	96.3–99.5	
Parvovirus	1.0–5.0	0.7–3.3	1.9–5.5	1.0–6.8	0.7–5.7	9.3–27.2	56.6–99.8

Table 2 Sequence distances within and between genera of *Parvovirinae*

	Hokovirus	Cnvirus	Erythrovirus	Bocavirus	Dependovirus	Amdovirus	Parvovirus
Hokovirus	0.00–0.42						
Cnvirus	0.75–0.82	0.00–0.06					
Erythrovirus	1.00–1.25	1.20–1.25	0.00–0.45				
Bocavirus	1.09–1.30	1.30–1.39	1.28–1.57	0.00–0.55			
Dependovirus	1.27–1.54	1.44–1.67	1.53–1.82	1.50–1.78	0.00–0.55		
Amdovirus	1.53–1.63	1.62–1.70	1.73–1.86	1.63–1.85	1.59–1.60	0.00–0.03	
Parvovirus	1.32–1.59	1.64–1.84	1.57–1.76	1.63–1.86	1.53–1.72	0.62–0.73	0.00–0.51

outbreak was severe in the summer and early fall of 2006. In many farms, the outbreak could be in all pigs including suckling piglets, weaned piglets, growing finishing pigs, sows and boars and the mortality was also high (even up to over 60%). Though there were still a few farms outbreak severely later, the morbidity and mortality in many farms were lowed and usually restricted to piglets (mainly after weaning) or even to growing finishing pigs.

In conclusion, we identified several new parvoviruses in southeastern China. Extensive genome sequence analysis showed that the new detected parvoviruses formed a distinct cluster within the subfamily *Parvovirinae*. To investigate the growth characteristic of our cloned strains in vitro, several kinds of cell, including PK15 cell, 293 cell plus a help plasmid (a generous gift from Prof. David Pintel, University of Missouri) and 293 cell plus adenovirus (a generous gift from Prof. Hangping Yao, Medical School of Zhejiang University) etc. were used to propagate the viruses. It was reported that the parvovirus adeno-associated virus type 2 (AAV2) is a single-stranded DNA virus which utilizes a helper virus (typically adenovirus or herpesvirus) in the establishment of productive infection [8–10]. However, there was no apparent sign of growth yet observed to date.

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