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Short communication

Identification and genome characterization of a novel feline picornavirus proposed in the *Hunnivirus* genus

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ABSTRACT

The genus *Hunnivirus*, which has been identified in sheep, cattle, and rats, was first proposed in the family *Picornaviridae* by the International Committee on Taxonomy of Viruses in 2013. In this study, a hunnivirus was detected in fecal samples collected from a diarrheic cat in Southern China in 2017. Genome sequencing and analysis indicated that the novel hunnivirus has the same genome organization as reported for other hunniviruses, 5′UTR-L-P1(VP4-VP2-VP3-VP1)-P2(2A-2B-2C)-P3(3A-3B-3C^{pro}-3D^{pol})-3′UTR, but is genetically divergent. This hunnivirus is proposed as a novel genotype of the species *Hunnivirus A* and provisionally designated feline hunnivirus. Our study expands the host range of hunnivirus and enriches knowledge on picornaviruses.

1. Introduction

According to the International Committee on Taxonomy of Viruses (ICTV), picornaviruses in the family *Picornaviridae* are currently divided into 40 genera ([www.picornaviridae.com] February 2018). The genus *Hunnivirus*, which contains a single species, *Hunnivirus A*, was included within the family *Picornaviridae* of the order *Picornavirales* in 2013(Adams et al., 2014). To date, three genotypes of hunniviruses have been officially proposed by the ICTV, including one hunnivirus isolated from sheep cell cultures in Northern Ireland in 1965 (https://talk.ictvonline.org/ictv/proposals/2013.008a-dV.A.v2.Hunnivirus.pdf) and bovine and ovine hunniviruses identified in Hungary during 2008 and 2009(Reuter et al., 2012). From 2014 to 2016, hunniviruses have also been detected in rats in America and China, revealing new hunnivirus genotypes(Du et al., 2016; Firth et al., 2014).

This study is the first to report a novel hunnivirus identified in fecal samples collected from a cat with diarrhea in China. The genome of feline hunnivirus (FeHuV) is genetically divergent from that of other hunniviruses and is proposed as a new genotype of the species *Hunnivirus A*.

2. Materials and methods

2.1. Sample collection

To understand whether hunniviruses are present in cats, we screened 47 and 44 archived fecal samples from diarrheic and healthy cats, respectively. These samples were collected from Guangzhou and Shenzhen, Southern China, from 2016 to 2018. The method of sample collection was conducted under the guidance of the South China Agricultural University Experimental Animal Welfare Ethics Committee. Samples ere collected for testing the prevalence of viruses in cats.

2.2. Virus detection and genome sequencing

Viral nucleic acid was extracted from samples using MiniBEST Viral RNA/DNA Extraction Kit (Takara, Japan). The RNA obtained was then reverse-transcribed into cDNA using GoScript Reverse Transcription System with a random primer (Promega, USA). Viral cDNA/DNA was assessed for the presence of known common feline enteric viral pathogens by PCR with PrimeSTAR HS (Takara, Japan). Using a primer

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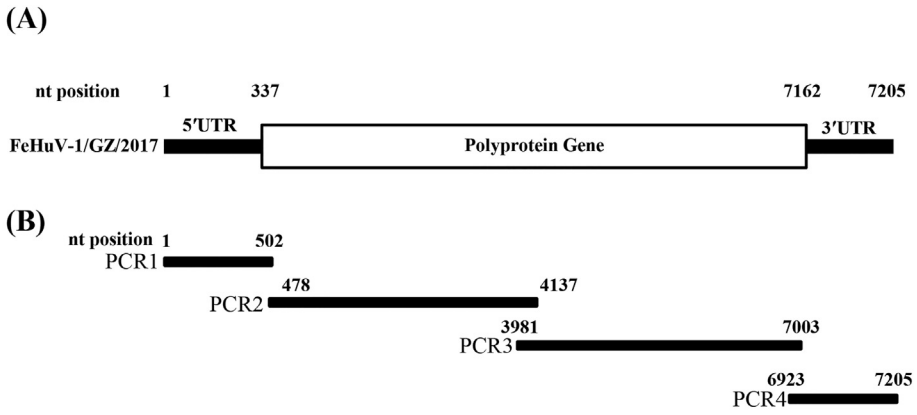

Fig. 1. Sequencing strategy of the feline hunnivirus genome amplified by PCR.

(A) PCR primer-targeting regions are numbered according to the field feline hunnivirus strain FeHuV-1/GZ/2017. The nucleotide (nt) position of each gene is shown above.
(B) Four PCR primer pairs were designed to sequence the feline hunnivirus genome. The primer name and target region are indicated.
(C) Information for the primer sequences. Y: C/T; W: A/T; R: A/G.

(C)

PCR fragment	Primer name	Sequence(5'-3')
PCR1	PCR1F	TTGYSTGGGCTATGAGCCTG
	PCR1R	CCTCAAACCAGCWGTCCTCA
PCR2	PCR2F	CTCTGTTGAGGACAGCTGGTT
	PCR2R	GGWGGTCCTCTCAACACAAC
PCR3	PCR3F	ATGCAAACTGGGAGGTGTGT
	PCR3R	AGCACGRGCRTAGGAGAGGA
PCR4	PCR4F	TACTTTTACCGTGATGGCTTG
	PCR4R	TTAACTAACACTAGAACTAA

walking strategy and long-range PCR method, four sets of overlapping primer pairs targeting the near-complete genome of FeHuV were designed using Oligo 7.0 (Fig. 1), and DNA fragments were amplified using Q5 high-fidelity DNA polymerase (NEB, UK). After sequencing, the near-complete genome of one feline hunnivirus strain was assembled from the raw data using SeqMan 7.1.0.

2.3. Phylogenetic analysis

Before constructing the phylogenetic tree, amino acid substitution models were estimated using the "find best protein model" in MEGA 5.05. Three phylogenetic trees based on the putative amino acid sequences of the P1 (778 aa), 2C (323 aa), and 3C (208 aa) coding regions were inferred using the rtREV + G + F amino acid substitution model, as based on bootstrap values of 1000 replicates (Fig. 2).

2.4. Virus isolation

Fecal samples testing positive for FeHuV RNA were processed for virus isolation. Culture was performed using a previously described method for bovine and ovine hunniviruses, with slight modification (Reuter et al., 2012). Six cell lines (CRFK, MDCK, A549, PK-15, DF-1, Vero) were used; cultures were incubated for 4 days and observed daily for the cytopathic effect (CPE). After five passages, total RNA was extracted from culture lysates and supernatant for detection of FeHuV by RT-PCR with UNIV-kobu-F/UNIV-kobu-R primers.

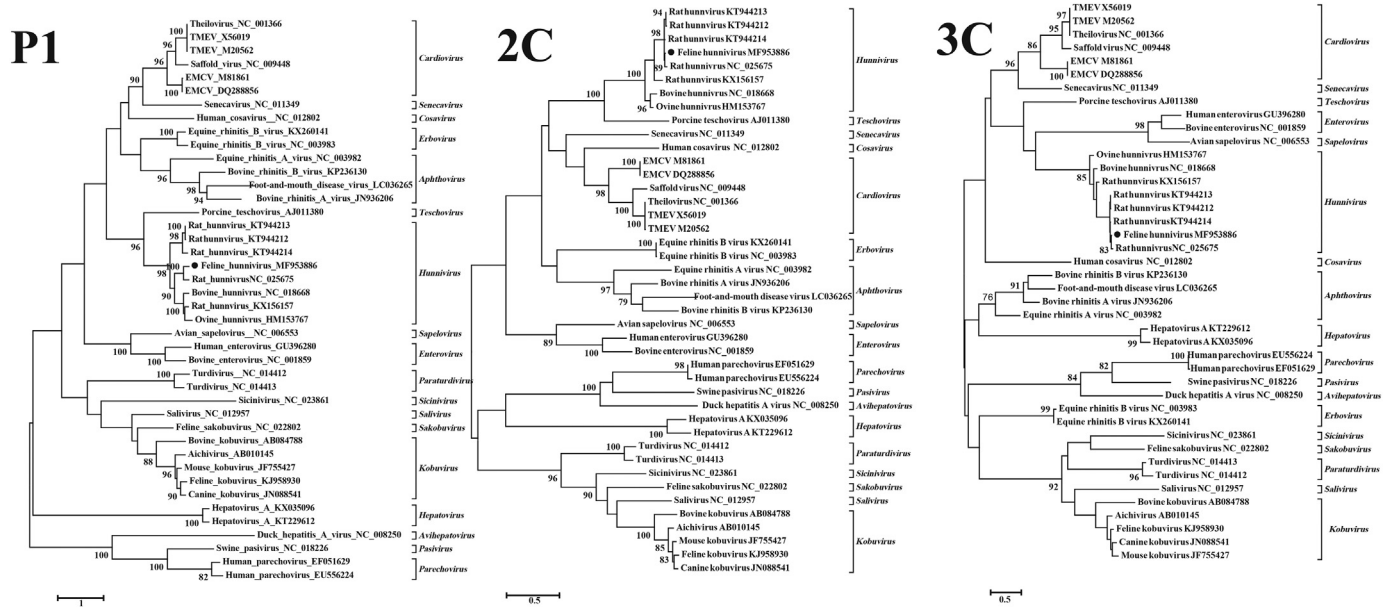

Fig. 2. Phylogram of the feline hunnivirus with other picornaviruses based on the predicted amino acid sequences of P1, 2C, and 3C coding regions. The field feline hunnivirus strain FeHuV-1/GZ/2017 is labeled with a black circle.

3. Results

3.1. Identification of a novel hunnivirus in diarrheic cat

The primers UNIV-kobu-F/UNIV-kobu-R were utilized in this screening study to assess whether hunniviruses are present in cats. These primers amplify a 216-bp fragment of the 3D RNA-dependent RNA-polymerase (RdRp) gene of both kobuviruses and hunniviruses, and previous studies have reported frequent detection of kobuvirus in stools from diarrheic cats using this primer pair(Chung et al., 2013; Di Martino et al., 2015a).

Among the archived diarrheic fecal samples, PCR products of the expected size were generated using UNIV-kobu-F/UNIV-kobu-R with six samples from stray cats and four samples from pet cats (Supplementary Table 1). After sequencing and blast searches, the species *Hunnivirus A* was found to be a match for three PCR-positive samples, showing 86.9%–95.3% nucleotide homology with previously identified hunniviruses. This novel hunnivirus in the present study was provisionally designated feline hunnivirus (FeHuV). The three FeHuV RNA-positive samples included two from stray cats and one from a pet cat in Guangzhou; all other samples producing the expected fragment size by RT-PCR using UNIV-kobu-F/UNIV-kobu-R were positive for feline kobuvirus. In addition, feline parvovirus was detected in one FeHuV RNA-positive sample and feline enteric coronavirus in another FeHuV RNA-positive sample. Based on sequencing results, the feline kobuvirus, feline parvovirus, and feline enteric coronavirus in this study have high nucleotide homology, > 98%, with previously reported strains. No FeHuV or feline kobuvirus was detected in samples from healthy cats.

3.2. Genome characteristic of the novel feline hunnivirus

After PCR and sequencing, the near-complete genome of one field strain, FeHuV-1/GZ/2017, was assembled, including a partial 5′UTR of 337 nucleotides, a single polyprotein gene of 6825 nucleotides, and a partial 3′UTR of 43 nucleotides. Rapid-amplification of cDNA ends (RACE) was not performed to obtain the complete 5′UTR and 3′UTR sequences due to a limited amount of sample. FeHuV has the largest polyprotein gene and the highest genome G + C content (48.2%) among reported hunniviruses (6732–6822 nucleotides; 44.8%–47.7%).

The genome of FeHuV-1/GZ/2017 has the same genome organization as determined for other hunniviruses: 5′UTR-L-P1(VP4-VP2-VP3-VP1)-P2(2A-2B-2C)-P3(3A-3B-3C-3D)-3′UTR. The putative viral proteins are of 83, 81, 240, 223, 234, 21, 243, 324, 112, 27, 208, and 456 amino acids in length. As reported for bovine and ovine hunniviruses, the putative translation initiation site of FeHuV-1/GZ/2017 contains a nearly optimal Kozak consensus sequence (A/GNNAUGG)(Reuter et al., 2012). A polypyrimidine-rich tract (UUUUCCCUUU) is located upstream of the AUG initiation codon, with a 19-nt spacer sequence. The Yn-Xm-AUG formula is Y_{10}-X_{17}-AUG (n: length of the pyrimidine tract; m: length of the spacer sequence), which is identical to that of bovine hunnivirus (Y_{10}-X_{17}-AUG) and ovine hunnivirus (Y_{11}-X_{17}-AUG)(Reuter et al., 2012).

Protease-cleavage sites between the hunnivirus viral proteins were predicted, including E/G, A/D, Q/G, P/S, and E/F, as shown in Table 1. The protease-cleavage sites between VP4 and VP2 (A/D), 2A and 2B (G/P), 3B and 3C (Q/G), and 3C and 3D (Q/G) are conserved among all hunniviruses. The protease-cleavage site between L and VP4 is E/G for all hunniviruses, except for bovine hunnivirus (Q/G). The protease-cleavage site between VP3 and VP1 is Q/G for all hunniviruses, except for ovine hunnivirus (E/G). Conserved motifs in hunnivruses were also analyzed (Supplementary Fig. 1).

A novel picornavirus genus is proposed if its P1, P2, and P3 coding regions have amino acid identities < 40%, 40%, and 50% with other picornaviruses, respectively. According to this principle, the novel feline picornavirus found in our study was classified into the newly established genus *Hunnivirus*. Homology analysis for the FeHuV polyprotein gene showed 70.8%–83.5%, 68.8%, and 68.9% nucleotide homology with rat hunnivirus, bovine hunnivirus, and ovine hunnivirus, respectively. This finding indicates that FeHuV is substantially genetically divergent from other hunniviruses. It is thus proposed as a novel *Hunnivirus A* genotype.

3.3. Phylogenetic analysis of feline hunnivirus

Phylogenetic analysis of hunnivirus and representative picornaviruses using the amino acid sequences of the P1, 2C, and 3C proteins indicated that FeHuV clusters with other hunniviruses (Fig. 2). Furthermore, among the analyzed picornavirus genera, the P1, 2C, and 3C proteins of the *Hunnivirus* genus have the closest relationship with those of the *Teschovirus* genus.

3.4. Virus isolation

CPE was not observed after five passages. In addition, FeHuV RNA was not detected in the culture lysate or supernatant.

4. Discussion

Based on the 3D conserved viral RdRp regions of the Aichi virus, bovine kobuvirus, and porcine kobuvirus, the primer pair UNIV-kobu-F/UNIV-kobu-R was initially designed by Gábor Reuter et al. for screening kobuviruses(Reuter et al., 2009). Using this primer pair, new numbers classified in the genus *Kobuvirus* have been determined in various animals, including sheep, goat, dog, deer, wolf, fox, and cat (Chung et al., 2013; Di Martino et al., 2013; Di Martino et al., 2015b; Di Martino et al., 2014; Melegari et al., 2018; Oem et al., 2014; Reuter et al., 2010). However, these primers are more generic for picornavirus

Table 1
Protease-cleavage sites of the hunnivirus.

Cleavage betwwen	Feline hunnivris FeHuV-1/GZ/2017	Rat hunnivirus NrHuV/NYC-E21	Rat hunnivirus 83GR-70-RAT130	Rat hunnivirus 05VZ-75-RAT099	Rat hunnivirus 83GR-70-RAT106	Rat hunnivirus rodent/Rn/PicoV/SX2015_2	Bovine hunnivirus BHUV1/2008/HUN	Ovine hunnivirus OHUV1/2009/HUN
L/VP4	FEFE/GPGQ	FEFE/GPGQ	FEFE/GPGQ	FEFE/GPGQ	FEFE/GPGQ	FEFE/GPGQ	FEFQ/GPGQ	FEFE/GPGQ
VP4/VP2	PLLA/DGLT	PLLA/DGLT	PLLA/DSTT	PLLA/DSTT	PLLA/DSTT	PLLA/DGLT	PLLA/DGLT	PLLA/DGLT
VP2/VP3	ARFQ/GLPK	GQFQ/GLPK	ADFQ/GLPK	ADFQ/GLPK	ADFQ/GLPK	AQFE/GLPK	SEFE/GLPK	AEFE/GLPK
VP3/VP1	LNYQ/GEDT	LTYQ/GEET	LSLQ/GEDA	LALQ/GEDA	LSLQ/GEDA	LALQ/GEDT	LALQ/GEDT	LLLE/GEDS
VP1/2A	PPTP/SPGM	PPAP/SPGM	PPLP/SRGA	PPMN/SRGA	PPLP/SRGA	PPAP/SRGA	PPIQ/SSGA	PPAQ/SSGA
2A/2B	LNPG/PFFM	LNPG/PFFM	SNPG/PFFM	ENPG/PFFM	SNPG/PFFM	LNPG/PFLF	LNPG/PPIL	LNPG/PSVL
2B/2C	FKFQ/GPVG	FKFQ/GPVG	FKFQ/GPVG	FKFQ/GPVG	FKFQ/GPVG	FKFE/GPLS	PKME/GPIM	PKLE/GPIL
2C/3A	FKFE/FNFQ	FKFE/FNFQ	FKFE/FNFQ	FKFE/FNFQ	FKFE/FNFQ	FKFE/GPKK	FNFQ/GPKE	FNFQ/GPRR
3A/3B	FSYQ/GAYG	FSYQ/GAYG	FSYQ/GAYG	FSYQ/GAYG	FSYQ/GAYG	FSME/GAYS	FNME/GAYS	FSLE/GAYS
3B/3C	ASFQ/GPMN	ASFQ/GPMN	ASFQ/GPMN	ASFQ/GPMN	ASFQ/GPMN	ASFQ/GPMN	ASLQ/GPMN	ASLQ/GPMN
3C/3D	GEFQ/GKIH	GEFQ/GKIH	GEFQ/GKIH	GEFQ/GKIH	GEFQ/GKIH	GEFQ/GRIH	AEFQ/GRIH	AEFQ/GRIH

than for kobuvirus(Reuter et al., 2012). One novel quail picornavirus and two novel hunniviruses, bovine hunnivirus and ovine hunnivirus, were identified in 2012 using UNIV-kobu-F/UNIV-kobu-R(Pankovics et al., 2012; Reuter et al., 2012). Our study further confirmed the universal applicability of UNIV-kobu-F/UNIV-kobu-R in determining novel picornaviruses. The family *Picornaviridae* contains clinically important human and animal pathogens and is associated with a series of diseases in the central nervous system, respiratory tract, heart, liver, pancreas, skin and eye(Zell, 2017). However, the transmission routes and pathogenicity of hunniviruses have not been investigated. Both bovine and ovine hunniviruses have been detected in apparently healthy animals. In contrast, FeHuV in this study was detected in diarrheic animals. Further analyses are needed to isolate FeHuV and to clarify its transmission route and relationship with diarrhea in an animal model as well as to assess its zoonotic potential.

In summary, a novel hunnivirus was identified in cats, expanding the host range of hunniviruses. Genomic sequencing and analysis indicated that this hunnivirus is a new genotype. Continued and more large-scale surveillance of this hunnivirus in cat populations is necessary to assess the pathogenicity and risk of zoonotic transmission of this novel pathogen.

Supplementary data to this article can be found online at https://doi.org/10.1016/j.meegid.2019.03.011.

Author contributions

GL and SJL conceived and designed the experiments; GL, MH, XJC, YKS, JH and RJH performed the experiments; GL, MH, XJC, YKS, JH and RJH analyzed the data; JH and RJH contributed reagents/materials/analysis tools; GL drafted the manuscript; SJL revised the manuscript; SJL supervised and approved the message for publication.

Conflict of interest

All authors declare that they have no competing interests.

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