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Molecular characterization of a feline calicivirus isolated from tiger and its pathogenesis in cats

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ABSTRACT

Feline calicivirus (FCV) is a virus that causes respiratory disease in cats. In this study, the FCV TIG-1 was isolated from Siberian tiger feces collected in 2014 in Heilongjiang Province, China. Phylogenetic analysis among TIG-1 and other FCVs showed that TIG-1 does not share the same lineage with other FCV isolates from Heilongjiang or other regions in China but is located in the same cluster with the FCV strain Urbana, which was isolated from the United States. The growth kinetics *in vitro* and the pathogenicity in cats between TIG-1 and the domestic cat-origin FCV strain F9 (vaccine strain) and strain 2280 were compared. We found that the growth kinetics of strains TIG-1 and 2280 were faster than that of strain F9 from 12 h to 36 h post-infection, indicating that strains TIG-1 and 2280 produce infectious virions and reach peak yields earlier. Challenge experiments in cats showed that TIG-1 grew faster than the other two strains in the lungs of cats and that TIG-1 is a virulent FCV with 100% morbidity and lethality. In addition, the histopathological results showed that the virulent TIG-1 strain directly led to severe lung tissue damage and indirectly led to intestinal damage. The results presented here show that a tiger-origin FCV exhibits high virulence in cats.

1. Introduction

Feline calicivirus (FCV) is small non-enveloped virus that consists of a non-segmented, polyadenylated, positive-sense ssRNA genome (approximately 7.8 kb in length) enclosed in an icosahedral capsid with a diameter of 27–40 nm (Mikalsen et al., 2014). FCV is a highly contagious pathogen of domestic cats and generally causes oral and upper respiratory tract disease (Radford et al., 2007). The clinical signs during FCV infection include fever, oral ulceration, ocular and nasal discharge, conjunctivitis, and limping (Hurley et al., 2004). Subclinically infected cats or recovered cats from FCV infection can continue shedding virions from ocular and nasal discharges, saliva, and feces (Tian et al., 2015).

FCV belongs to the genus *Vesivirus* in the *Caliciviridae* family, which includes five well-defined genera: *Norovirus*, *Sapovirus*, *Lagovirus*, *Nebovirus*, and *Vesivirus* (Castro et al., 2015). Norovirus (NoV) and Sapovirus can cause acute gastroenteritis in humans (Hansman et al., 2004) and result in family or community-wide outbreaks, especially in the winter season (Iwai et al., 2009), and such outbreaks cause significant morbidity (Taube et al., 2013; Patel et al., 2008). Research on NoV and Sapovirus has been stifled by the absence of a cell culture system for these viruses; therefore, FCV has been used widely as a model system for NoV and Sapovirus to broaden our understanding of these viruses (Taube et al., 2013).

FCV shows higher evolutionary rates than other viruses, and approximately 1.3×10^{-2} to 2.6×10^{-2} substitutions per nucleotide occur per year in the variable regions of the FCV capsid protein (Coyne et al., 2007). Thus, the high genetic plasticity of the virus has led to the emergence of variants (Coyne et al., 2012). In the past, it has been generally accepted that FCV can only infect cats, and reservoirs or alternative hosts for FCV have not been observed (Radford et al., 2007). However, FCV and FCV-like viruses were detected in dogs in recent years (Hashimoto et al., 1999; Martella et al., 2002; Roerink et al., 1999; Di Martino et al., 2009). FCV infections have also been reported in lions and tigers (Harrison et al., 2007; Kadoi et al., 1997). These studies have revealed that the

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host range of FCV is spreading. Because of the limited knowledge of FCV and its host range, the isolation and identification of new isolates, especially those exotic felids origin, are of particular significance.

Here, we describe the isolation of an FCV strain referred to as TIG-1 in 2014 from a Siberian tiger housed in a zoo in Harbin, Heilongjiang Province, China. The molecular characterization of FCV TIG-1 and its pathogenicity in cats were determined.

2. Materials and methods

2.1. Ethics statement

The animal experimental protocols in this study were approved by the Harbin Veterinary Research Institutional Animal Care Committee.

2.2. Clinical samples

A total of 58 Siberian tiger fecal samples, including normal and diarrhea samples, were collected from the Harbin Northern Forest Zoo and Harbin Siberian Tiger Park and stored at −80 °C from 2012 to 2014. The samples were diluted using a nine-fold volume of phosphate-buffered saline (PBS) and then homogenized in a vortex. The samples were then centrifuged at 5,000*g* for 5 min at 4 °C, and the supernatant was immediately filtered through 0.45-μm Pellicon II filters (Millipore, Billerica, MA, USA). The filtrates were stored at −80 °C until use.

2.3. Virus isolation and purification

Crandell-Reese feline kidney (CRFK) cells (ATCC) were grown in Dulbecco's modified Eagle medium (DMEM, Gibco) containing 8% fetal bovine serum (FBS) and 1% penicillin-streptomycin. The cells were incubated at 37 °C in a 5% CO_2 humidified cabinet (Bidawid et al., 2003). The filtered supernatants from the 58 samples were inoculated into the CRFK monolayer and passaged five times, and then the cytopathic effect (CPE) was observed. When the CPE appeared, the cultures were freeze-thawed and then harvested and stored at −80 °C. The virus stocks were purified by a plaque assay. Briefly, the cultures were diluted and inoculated into 100% confluent CRFK cells grown in 6-well plates for 1 h. The inoculum was removed, and then the cells were covered with 3 mL of 1% Bacto Agar (DIFCO) diluted in 2 × MEM containing 1% penicillin-streptomycin. The plaques were visible from 24 to 48 h after infection, and then a single plaque was picked up with a pipette tip into 1 mL DMEM. After centrifuging, the mixture was inoculated into 100% confluent CRFK cells and the purification was performed 3 times according to the method described above.

2.4. Viral identification, genome cloning and sequencing, and phylogenetic analysis

Total RNA was extracted from the culture supernatant using an Axygen Viral DNA/RNA Miniprep Kit (Axygen Biosciences, CA, USA) according to the manufacturer's protocol, and cDNA was synthesized by an oligo(dT) primer using RNA as the template. A PCR assay was performed using Pro-Pol gene-specific primers (Wu et al., 2015). The virus isolates were further identified by an indirect immunofluorescence assay (IFA) using a cat polyclonal antibody against FCV strain 2280 (Wu et al., 2015).

The genome of the FCV strain TIG-1 from 34 to 7667 bp exclude the 5′- and 3′- end sequence was cloned using an LA PCR Kit (TaKaRa, Dalian, China) and specific primers (Table 1) based on the conserved regions of the FCV genome. The 5′- and 3′-end sequence was obtained by using the 5′ and 3′ rapid amplification of cDNA ends (5′ and 3′RACE) kit (Invitrogen). The PCR products were cloned into the pMD18-T vector, and positive clones were sequenced.

Table 1
Primers used in this study.

Primer	Sequence (5′–3′)	Position[a]
FCV-TY-12F	TTGAGACAATGTCTCAAACTCT	12-33
FCV-TY-2420-R	TTAACGGTTACCACATGYTGATT	2432-2454
FCV-TY-2420-F	AACTACCCGCCAATCARCATGTGGT	2421-2445
FCV-TY-5200-R	GCTCAAACTTCGAACACATCACAGT	5289-5313
FCV-TY-5200-F	ACTGTGATGTGTTCGAAGTTTGAGC	5289-5313
FCV-end-R	AAAAACCCTGGGGTTAGRCGC	7668-7683

[a] The position of primers was related to that in FCV strain 2280 (Genbank accession: KC835209.1).

BLAST analyses (http://blast.ncbi.nlm.nih.gov/Blast.cgi) were performed to identify the related reference viruses of the TIG-1 strain. To further explore the genotype and genetic origin of the FCV TIG-1, phylogenetic trees were constructed by the neighbor-joining method using MEGA5.1 software. The bootstrap values were calculated according to 1000 replicates of the alignment.

2.5. Pathogenicity experiments in cats

The experimental cats were screened for feline calicivirus, parvovirus, herpes virus and infectious peritonitis virus by RT-PCR and found to be negative. Three-month-old healthy domestic cats (n = 32) weighing from 1.2 to 1.5 kg were randomly divided into four groups, and every four cats group lived in a single animal house (3m × 3m). To prevent the cross contamination among different rooms, the experimenters were required to wear separate personal protection equipment before entering each room. The FCV strains F9 and 2280 were used to compare the pathogenicity among the TIG-1 isolate and the domestic cat-origin viruses. The cats were anesthetized subcutaneously with Quan Mian Bao (10 mg/kg) (QFM mixture) including lidocain, ketamine, haloperidol, which was developed by Northeast Agricultural University. The anesthesia protocol was according to AAHA Anesthesia Guidelines for Dogs and Cats (Bednarski et al., 2011). Cats were challenged with 0.5 mL (0.2 mL for each nasal passage and 0.05 mL for each eye) of 10^7 $TCID_{50}$ of TIG-1, 2280 and F9 by intranasal and ocular routes, respectively. The control group was mock inoculated with 0.5 mL DMEM. The clinical symptoms and rectal temperatures were monitored daily. The clinical scores, which included respiratory, oral cavity and eye scores, were recorded on a scale of 0–3 (Table 2). Fecal swabs and nasal swabs were collected to determine virus shedding at days 1, 3, 5, 7, 9, 11 and 13 post-infection (p.i.). Three cats from each challenged group were humanely euthanized i.v. on day 5 using 20% sodium pentobarbital (0.3 mL/kg) according to the protocol suggested by World Society for the Protection of Animals about Methods for the euthanasia of dogs and cats (World Society for the Protection of Animals, 2016). A portion of the samples was rapidly fixed with 10% buffered formalin for the histopathologic examination, and the remaining samples were immediately stored at −80 °C for the virus titers analysis. During the experiment, challenged cats were humanely euthanized when they became hunched in pain, barely able to move and breath, and lost their ability to eat and drink, and tissues including lung, kidney, liver, trachea, spleen and intestine were collected to analyse the virus titers.

2.6. Viral titers test

The protocol for virus titration has been described in our previous study (Tian et al., 2015). The viral titers were expressed as