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RESEARCH ARTICLE

Isolation, Genomic Characterization and Pathogenicity of a Feline Calicivirus Strain Ch-Jl4 from Chinese Stray Cats

Kai Wang[1,§], Zhihua Pei[1,§], Hao Dong[2], Songtao Yang[3], Guoying Dong[4] and Guixue Hu[1*]

[1]College of Animal Science and Technology; [2]College of Life Science, Jilin Agricultural University, Changchun, 130118, P. R. China; [3]Institute of Military Veterinary, Academy of Military Medical Sciences, Changchun, 130062, P. R. China; [4]College of Global Change and Earth System Science, Beijing Normal University, Beijing, 100875, P. R. China
*Corresponding author: guixue1964@126.com

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ABSTRACT

Feline calicivirus (FCV) is a small, unenveloped, single-stranded RNA virus that can cause feline upper respiratory tract disease (URTD). In order to understand some information about FCV infection in stray cats (not vaccinated), 145 oral/nasal swabs were randomly collected from different areas in China between 2014 and 2016. A FCV strain (named CH-JL4) was isolated and identified through PCR detection, cell culture, immunofluorescence assay (IFA), electron microscopy observation. Complete gene sequencing and phylogenetic analysis showed that it was 7709 nucleotides (nt) in length, excluding the poly (A) tail, and was genetically similar to other field FCV strains such as the HRB-SS strain (accession no. KM016908). All cats that were experimentally infected developed the typical clinical signs of FCV. However, the lung histology of the dying cats showed pulmonary congestion and hemorrhage, a small amount of fibrin exudation in the alveoli, shedding of alveolar epithelial cells, a small number of scattered neutrophils and oozing of red blood cells. The results indicated that virulent FCV infection in stray cat in China still existed. These results were beneficial for understanding the epidemic and pathogenicity in stray cats.

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INTRODUCTION

Feline calicivirus (FCV) is one of the most common upper respiratory pathogens in cats and is a member of the Vesivirus genus and Caliciviridae family. FCV primarily infects 6- to 84-day-old kittens and induces symptoms of acute, mild to moderate upper respiratory tract disease (URTD), including oral ulcerations, rhinitis, conjunctivitis, fever, anorexia, lethargy, lameness and pneumonia. It was reported that the clinical symptoms of different FCV strain infection are different (Sheeba *et al*., 2017; Rong *et al*., 2014). FCV has a positive-sense, single-stranded RNA genome (approximately 7.7 kb in length) that contains three open reading frames (ORFs). ORF1 encodes the non-structural proteins, ORF2 encodes the capsid protein (VP1), and ORF3 encodes the minor structural protein (VP2); VP1 plays an important role in viral antigenicity, receptor binding, internalization, uncoating and resistance to the environment (Neill *et al*., 1991).

FCV infection alone is unlikely to cause death in cats and felines. However, in recent years, due to the high degree of FCV variability, there have appeared highly lethal variant virulent strains, which seriously threaten health of cats (Schorr-Evans *et al*., 2003; Coyne *et al*., 2006; Reynolds *et al*., 2009; Battilani *et al*., 2013; Willi *et al*., 2016) and even appeared reports on infected dogs (Martella *et al*., 2002). Furthermore, this disease not only causes harm to domestic cats and captive felines, but also severely threatens wild felines since this virus has been isolated from tigers (Tian *et al*., 2016), lions and other animals (Pedersen *et al*., 2000). Therefore, harm of this disease does not limit to cats, its invasion to wild animals that are protected should not be ignored, and should arouse our attention.

MATERIALS AND METHODS

Acquisition and preservation of samples: 145 nasal / swabs were randomly collected from different areas in China between 2014 and 2016, and detected by a novel

[§]These authors contributed equally to this work.

nested PCR assay (Fulvio *et al*., 2005). FCV-positive samples were dissolved in PBS solution and centrifuged. Next, the supernatants were filtered using a 0.22 μm microporous filtering film. The filtered supernatants were used as the inoculum for FCV isolation.

Virus isolation: A 500 μl aliquot of the supernatant was placed into 25 cm^2 flasks containing sub confluent monolayers of F81 cells. The cells were incubated for 1 h, and then DMEM supplemented with antibiotics (without foetal calf serum) was added. Cell cultures were observed every 6 h for the cytopathic effect (CPE). The virus was stored at -80°C when 80% CPE were observed.

The virus was passaged to the fifth generation, diluted with DMEM (containing 2% foetal calf serum) and antibiotics to generate 11 serial 10-fold dilutions. Then, 100 μl of F81 monolayer cells were grown in 96-well plates. For each dilution, four wells were inoculated with 100 μl per well of the viral suspension. Viral titres were calculated using the Karber statistical formula and expressed in log10.

Immunofluorescence assay (IFA): F81 cells were grown in 24-well microtitre plates and infected with FCV, the multiplicity of infection (MOI) was 0.01. After 24 h, the cells were fixed with 4% paraformaldehyde for 16 min, and then washed three times by SPS. Cells were permeabilized by Triton X-100 (0.5%) for 20 min at room temperature and incubated with FCV monoclonal antibody (Ingenasa, Spain) at 4°C for a night, FITC rat anti-mouse secondary antibody (Ingenasa, Spain) for 1 h. Finally, the labelled cells were observed via fluorescence microscope.

Electron microscopy observation: F81 cells were infected with field isolates in a 25 cm^2 flask, and the cultures were observed closely for CPE. Approximately 16 h after the infection, the cell culture medium was decanted and the monolayer was overlayered with cold 2.0% paraformaldehyde and 2.5% glutaraldehyde. The flasks were held for 1 h and then stored at 4°C for a few days. Post-fixation was performed with 1% osmium tetroxide in 0.1 M phosphate buffer (pH 7.4) at 37°C for 1-2 h. Grid staining was performed with 2% aqueous uranyl acetate and lead citrate. The samples were observed under an electron microscope.

The complete genomic analysis: Total RNA was extracted using a Simply P Total RNA Extraction Kit (Bioer Technology Co., Ltd. Dalian, China). Reverse transcription was performed using a Trans Script First-Strand cDNA Synthesis Kit (Beijing TransGen Biotech Co., Ltd. Beijing, China). The PCR were those described by Chen (Chen *et al*., 2015). Finally, the sequence data were entered into a BLAST search of the GenBank, which determined sequence homology. A genome homology analysis was performed with the DNA Star 7.0 software.

Experimental infection test: Eight healthy unvaccinated kittens, approximately 6 to 11 weeks old, were housed in separate air spaces of the animal housing facility. The protocol was approved by the Committee on the Ethics of Animal Experiments of the Jilin Agricultural University. All the cats used in this study were negative for FCV by a novel nested PCR (Fulvio *et al*., 2005).

Eight cats were randomly assigned to a challenge group and a control group. Cats in the challenge group were intranasally inoculated with the FCV isolate at a titer of $10^{7.5}$ $TCID_{50}$/ml (0.25 ml per cat) (Lesbros *et al*., 2013). Cats in the control group received equal volumes of F81 cell culture. In the post-challenge phase, all cats were monitored for clinical signs for 14 days. The lungs were collected for the histopathology.

RESULTS

Virus isolation and IFA: 145 clinical samples were attempted to isolate the virus, but only one FCV strain, designated FCV CH-JL4, was successfully isolated from a sample from Jilin province, China. Typical CPE was observed within 24 hours in F81 cells cultures exposed to the swab supernatant. Compared to the nomal F81 cells Fig. 1A), F81 cells infected with FCV exhibited cytogamy, cell rounding, karyopyknosis and cluster-like forms; they eventually shed completely and remained suspended (Fig. 1B). By applying immune fluorescence assay (IFA), the specificity of the virus was confirmed. Bright green fluorescence was found in F81 cells infecting FCV monoclonal antibody (Fig. 1C), in contrast, there were not fluorescence for normal F81 cells (Fig. 1D).

Electron microscopy observation: After infection with FCV CH-JL4 for 16 h, the ultracryotomy and a transmission electron micrograph of F81 cells were used. The virus particle was spherical with no envelope, which was consistent with the structural characteristics of caliciviruses (Fig. 2A). Numerous viral particles were observed inside the cytoplasm and also aggregated into a crystalline arrangement, next to the myelin sample corpuscle (Fig. 2B).

Analysis of the whole-genome sequence: The percentage of amino acid similarities among the FCV CH-JL4 strain and other FCV strains/isolates ranged from 76.6-80.5%. The FCV CH-JL4 strain was closely related to the HRB-SS sequence (GenBank: KM016908.1), with 80.5% similarity. Thus, the field isolate and the HRB-SS sequence were from the same cluster (Fig. 3).

Pathological features of cats challenged with FCV CH-JL4: In the challenge group, all cats displayed anorexia, weight loss, fever, conjunctivitis discharge Fig. 4A, oral ulcerations Fig. 4B and typical upper respiratory symptoms for 4 days post-challenge. Three of the four cats died on the 7th day after onset. The lung histology showed pulmonary congestion and hemorrhage, a small amount of fibrin exudation in the alveoli, shedding of alveolar epithelial cells, a small number of scattered neutrophils and oozing of red blood cells (Fig. 4C, 4D).

DISCUSSION

FCV is a common upper respiratory pathogen of cats and is characterized by high degree of genetic and antigenic diversity. Cats within 84 days of age are the most susceptible to an FCV infection. Previous studies indicated that different FCV isolates could cause an asymptomatic or mild infection to severe respiratory symptoms and pneumonia. However, the differences in

viral pathogenicity are hard to distinguish using genetic or serologic comparisons (Pedersen *et al*., 2000; Pesavento *et al*., 2004). Most FCV isolates display a different immuno-dominant region of the capsid protein that is closely related to its antigenic diversity (Martino and Marsilio, 2010). The lack of cross-neutralization among FCV strains is related to the variation in the sequences of antigenic region (Radford *et al*., 1999; Brian *et al*., 2011). In the present study, field isolate CH-JL4 was isolated from assay cat. Through virus isolation, IFA, microscopic observation and phylogenetic analysis of the full-length cDNA sequence, the field isolate was finally identified and named CH-JL4. The result from the animal regression test also indicated that FCV CH-JL4 could infect healthy unvaccinated cats and lead to typical clinical signs, which is consistent with a naturally infected appearance. Therefore, our observations support the notion that FCV CH-JL4 confers pathogenicity to host animals with a certain virulence.

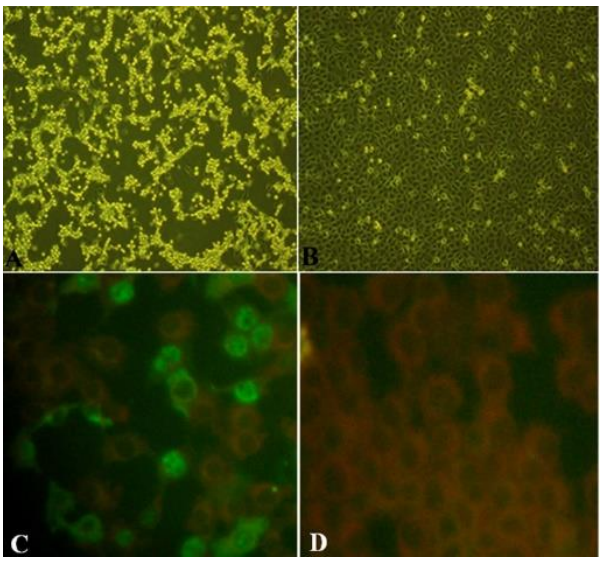

Fig. 1: Virus isolation. (A) Nasal swabs of cats were inoculated in F81 cells and then passaged. The fifth generation showed CPE characterized by cytogamy, cell rounding, karyopyknosis, and clustering in virus-infected cells. (B) Natural F81 cells inoculated with the same dose of physiological saline showed no cytopathic cells. (C) Bright green fluorescence were found in F81 cells infecting FCV. (D) Green fluorescence were not found in normal F81 cells.

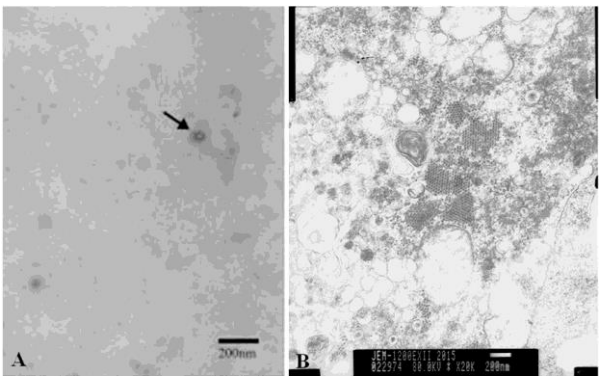

Fig. 2: Transmission electron microscopy technique of negative staining (80000×). (A) The virus particle was spherical with no envelope, which was consistent with the structural characteristics of caliciviruses. (B) Numerous viral particles were observed inside the cytoplasm and also aggregated into a crystalline arrangement, next to the myelin sample corpuscle.

FCV has only one serotype, but the strains isolated in different regions and periods in the same population are diverse (Sato *et al*., 2002; Sun *et al*., 2017; Coyne *et al*., 2012). In a phylogenetic tree of whole genome sequence analysis we found that FCV CH-JL4 had the highest similarity with FCV HRB-SS (GenBank: KM016908.1) (Fig. 3), which may indicate that the two isolates were the variants of the same FCV strain from different regions. However, there is a significant genetic distance between CH-JL4 and the commonly used vaccine strain FCV F9. However, experimental FCV infection of kittens can lead to typical clinical symptoms after re-inoculation. The interstitial manifestations were very obvious via the histopathological examination of dead cats, which indicated that the FCV CH-JL4 had a stronger virulence.

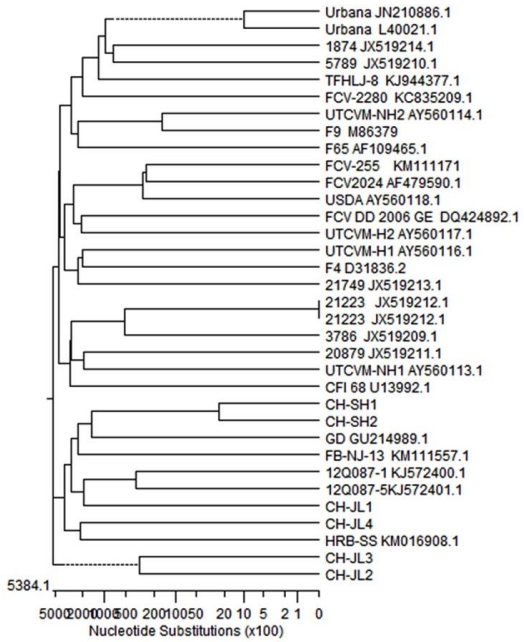

Fig. 3: Phylogenetic trees based on the complete genome of FCV strain CH-JL4. FCV CH-JL4 was closely related to the HRB-SS sequence (GenBank: KM016908.1), with 80.5% similarity, and came from the same cluster.

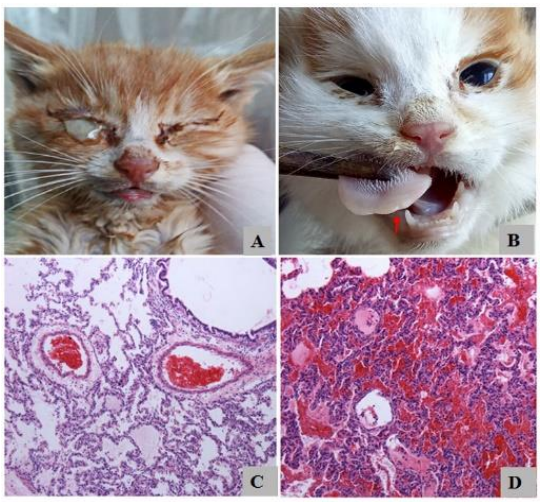

Fig. 4: Clinical symptoms and the lung histology of cats challenged with FCV strain CH-JL4. (A) Representative images of the conjunctivitis. (B) Representative images of the oral ulcer. (C) Comparing group: There were tiny red blood cells in the alveolar cavity. (D) Disease group: There were a large number of red blood cells and alveolar epithelial cells in the alveolar cavity, edematous fluid was found in individual alveolar space.

At present, FCV is widespread in cats around the world (Abd-Eldaim *et al*., 2005; Hou *et al*., 2016), and its harm is also growing from simple nonlethal infection to outbreak of feline calicivirus associated virulent systemic disease (FCV-VSD) (Ossiboff *et al*., 2007), which alerts us the need for early prevention. Similar to the present epizootic, FCV-induced pneumonia requires prompt application of strict quarantine, isolation, personnel sanitation, and disinfection procedures.

Conclusions: Feline calicivirus (FCV) were isolated from stray cats in China, FCV CH-JL4 were able to cause pneumonia symptoms and death in cats.

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Authors contribution: KW and ZHP conceived of the study. HD and GYD participated in verification of data. STY and GYD helped draft the manuscript. GXH participated in its design and coordination. All authors critically reviewed and edited the manuscript.

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