Co-circulation and evolution of genogroups I and II of respiratory and enteric feline calicivirus isolates in cats

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Abstract
Feline calicivirus (FCV) is a highly infectious pathogen that causes upper respiratory tract disease (URTD), but the enteric FCVs raise concerns regarding their role of an enteric pathogen. In this study, between 2019 and 2020, 101 clinical samples from domestic cats with symptoms of URTD, with or without enteritis, were collected for FCV-specific detection. The FCV-positive rate reached to 42.4% (28/66) in cats with respiratory symptoms. The rates were 11.1% (3/27) and 12.5% (1/8) when faeces and serum samples were measured using reverse transcription polymerase chain reaction (RT-PCR), respectively. Ten FCV strains were successfully isolated from respiratory and enteric sources in domestic cats from Guangxi. Phylogenetic analysis based on the genome sequences of 11 isolates (including GX01-13 isolated in 2013) indicated that the newly characterized FCV strains had two recombinant events in comparison with other FCVs and were of respiratory and enteric origins. These strains displayed high genetic diversity, and they were divided into two genogroups (I and II). Of these, the GXNN02-19 isolate was grouped with previously published Chinese isolates that were identified as genogroup II, which contained three specific amino acid residues (377K, 539V and 557S) in the VP1 protein. In addition, the three enteric viruses appeared genetically heterogeneous to each other. All isolates were found to be more sensitive when exposed to low pH conditions, but they were resistant to treatment with trypsin and bile salts. Furthermore, there were no significant differences between the respiratory and enteric FCVs. Our results showed that the genetically distinct FCV strains with genogroups I and II from respiratory and enteric origins were co-circulating in this geographical area. Also, it was revealed that the potential recombinant events between the enteric and respiratory FCVs suggested an important role of enteric FCV during the evolution.

KEYWORDS
cat, co-circulation, feline calicivirus, genogroup, recombinant virus, respiratory and enteric origins
Feline calicivirus (FCV) is classified in the genus Vesivirus, family Caliciviridae, and it can cause a highly contagious disease in domestic cats and other feline species with signs of fever, oral ulceration, anorexia, lethargy, lameness and respiratory clinical manifestations (Radford et al., 2007). A multi-cat environment, high population turnover and presence of healthy carriers can increase the chances of infection, especially from FCV-infected cats that have recovered from acute disease. These conditions can lead to persistent infections with FCV. The latent carriers may shed virus particles for weeks to years via nasal, oral, ocular secretions and faeces (Coyne et al., 2006; Di Martino et al., 2020; Mochizuki, 1992; Pinto et al., 2012). Being genetically diverse, cats infected with FCV strains with wide-ranging virulence can show varying degrees of clinical signs and symptoms. More recently, highly virulent forms of FCV that are associated with systemic disease have emerged in China (Sun et al., 2017; Tian et al., 2016) and Europe (Caringella et al., 2019). These viruses display extensive multi-organ involvement, subcutaneous edema and oral and cutaneous ulcers mainly on the auricle and plantar pads as well as pancreatitis and hepatic necrosis in their cat hosts.

FCV virions are small (30—35 nm in diameter), non-enveloped and icosahedral particles and have a single-stranded positive-sense RNA genome of approximately 7.7 kb. They consist of three open reading frames (ORFs), covalently linked by a virally encoded protein (VPg), and they are polyadenylated at the 5′ and 3′ ends of their genomes, respectively. ORF1 encodes a large polyprotein (~200 kDa) that undergoes proteolytic processing and is cleaved into six small proteins (p5.6, p32, p39, p30, p13 and p76) (Sosnovtsevs et al., 2002; 1999). ORF2 encodes a capsid precursor protein (~60 kDa), which is processed in the trans configuration by a viral protease to release two proteins, the leader capsid (LC) and VP1 (Carter, 1989; Carter et al., 1992; Sosnovtsev et al., 1998; Tohya et al., 1999). The major capsid protein (VP1) is usually divided into six regions (A—F) based on their conserved sequences (Seal et al., 1993). The variable region, E, is responsible for the antigenic variety of the FCV, and it is further divided into two hyper-variable regions (5′HVR E and 3′HVR E) that are separated by a conserved region E (ConE). The latter region can often be used as a tool for epidemiological investigations of FCV outbreaks (Radford et al., 1997). In addition to VP1, there is an essential minor capsid protein (VP2) which is responsible for the production of infectious virions (Sosnovtsev et al., 2005), and it forms a portal-like assembly to help the delivery of the FCV genome into its host cells (Conley et al., 2019).

FCV can usually be regarded as a representative pathogen in relation to respiratory diseases. However, several forms of enteric FCVs from the stools of cats have raised some concerns regarding the role of FCV as an enteric pathogen. FCV was first isolated from an enteric origin in cats by Fastier in 1957 (Fastier, 1957). Marshall et al. identified seven distinct viral types by electron microscopy in 208 faecal samples of cats with or without diarrhoea. The picornavirus-like particles (including FCV particles) were found in about 6% of stool specimens (Marshall et al., 1987). In 1991, Mochizuki et al. obtained five FCV isolated strains from diarrheal or normal faeces, and these displayed a resistance to bile salts. The FCVs were divided into two types, respiratory-type (R-FCV) and enteric-type (E-FCV), based on their susceptibility to bile salts (Mochizuki, 1992). Subsequently, Di Martino et al. obtained the genome of one E-FCV-isolated strain and four capsid proteins. Comparative studies using phylogenetic analysis and physical-chemical tests showed that these viruses were genetically heterogeneous and were more resistant to bile salts, acidic conditions and trypsin when compared to the R-FCV (Di Martino et al., 2020). However, the possible linkage of E-FCV and enteric disease needs to be determined. In this study, more information regarding the genomic sequences of viruses from respiratory and enteric origins was obtained, which will facilitate the exploration of the differences of genetic characterization between R-FCV and E-FCV.

## MATERIALS AND METHODS

### 2.1 Sample collection

From 2019 to 2020, a total of 101 samples were collected from cats with respiratory symptoms and stomatitis, with and without diarrhoea, at veterinary clinics in Guangxi Zhuang Autonomous Region (Guangxi). These were comprised of conjunctival, nasal and oropharyngeal swabs (n = 66), faecal swabs (n = 27) and serum/plasma samples (n = 8). Full characterization of the sampled cats, including their locations, collection dates, clinical symptoms, vaccination status and cat breed, was recorded and collected, except for the strain, GXNN05-20. Most of the samples were collected from pet cats presenting with upper respiratory tract disease (URTLD), oral ulceration, salvation and conjunctivitis. In some of the samples, other pathogens were also detected, including feline herpesvirus (FHV-1), feline panleukopenia virus (FPV) and feline influenza virus (FIV). The detailed characteristics of the 11 FCV-positive samples, including the cat breed, collection dates, locations, ages, sex, vaccination status, clinical signs, origin of the samples and name of the isolate are presented in Table 1. One isolate, GX01-13, was isolated in 2013 from household cats with severe respiratory symptoms (He et al., 2016).

### 2.2 Reverse transcription polymerase chain reaction screening for FCV

Viral RNA was extracted from the nasal swabs or 20% (w/v) faecal samples by using QIAamp Viral RNA Mini Kit (Qiagen Germantown, MD, USA) and following the manufacturer’s instructions. cDNA was generated by using PrimeScript Reverse Transcriptase (Takara Bio, Inc., Dalian, China) and oligo (dT) primer. The samples were screened using reverse transcription polymerase chain reaction (RT-PCR) with CaF (5′-CAACCTGCGCTAACGTGC-3′) and CaR (5′-CCARTGCATTGGNGNAC-3′) primer pair, which targeted a 257-bp length of the capsid gene. The cDNA template was denatured at 94°C for 5 min and amplified for 32 cycles of denaturation at 94°C for 30 s, annealing at 47°C for 30 s and extension at 72°C for 30 s.
| Cat breed          | Date        | Location | Age (months) | Sex | Vaccination status | Clinical signs                                                                 | Samples (swab) | Isolationa | Cell lines | Access number in GenBank |
|--------------------|-------------|----------|--------------|-----|--------------------|--------------------------------------------------------------------------------|----------------|-------------|------------|------------------------|
| Domestic           | 09/10/2013  | Liuzhou  | NA           | NA  | Yes                | URTD, fever (39.3–41.5°C), conjunctivitis                                     | Nasal          | GX01-13     | MDCK      | KT970059               |
| Domestic           | 06/09/2019  | Nanning  | 9            | M   | Yes                | Oral ulceration, anorexia                                                     | Nasal          | GXNN01-19   | CRFK      | OL512761               |
| Domestic           | 02/25/2019  | Nanning  | 3            | M   | No                 | URTD, conjunctivitis, FHV positive                                           | Oral           | GXNN02-19   | CRFK      | MZ712022               |
| Domestic           | 05/21/2019  | Liuzhou  | 2            | F   | Yes                | URTD, conjunctivitis, nasal and oral discharge, FHV positive                | Nasal          | GXLZ01-19   | CRFK      | MZ712025               |
| Domestic           | 07/26/2019  | Liuzhou  | 24           | M   | Yes                | Severe stomatitis                                                            | Nasal          | GXLZ02-19   | CRFK      | MZ712024               |
| Garfield           | 09/01/2019  | Yulin    | 7            | M   | No                 | Oral ulceration, salivation                                                  | Oral           | GXYL01-19   | CRFK      | MZ712016               |
| Domestic           | 07/22/2020  | Nanning  | 3            | F   | No                 | Gastroenteritis, diarrhoea, FPV positive                                      | Faecal         | GXNN03-20   | CRFK      | MZ712021               |
| Hybrid             | 07/22/2020  | Nanning  | 4            | M   | No                 | Gastroenteritis, diarrhoea, FPV positive                                      | Faecal         | GXNN04-20   | CRFK      | MZ712020               |
| British Shorthair  | 08/20/2020  | Nanning  | 5            | M   | No                 | URTD, runny nose, coughing, recurrent fever (39.6–41.6°C), without diarrhoea| Nasal and     | GXNN05-20b  | CRFK      | MZ712019               |
| Chinese garden cat | 08/20/2020  | Nanning  | 24           | M   | Yes                | Severe stomatitis, FHV positive                                              | Oral           | GXNN06-20   | CRFK      | MZ712018               |
| British Shorthair  | 09/03/2020  | Nanning  | 5            | M   | No                 | Conjunctivitis, nasal eyelid edema, anorexia, diarrhoea, FHV positive       | Nasal and     | GXNN07-20   | CRFK      | MZ712017               |

Abbreviations: F, female; FHV, feline herpesvirus; FPV, feline panleukopenia virus; M, male; NA, not available; URTD, upper respiratory tract disease.

aThe GX01-13 strain had been isolated in 2013 with the GenBank accession number of KT970059 [He et al., 2016].

bThe isolates from nasal swab and faecal sample respectively share 100% nt identity with each other.
followed by a final extension at 72°C for 10 min. The PCR products were analyzed using agarose gel electrophoresis and stained with ethidium bromide.

### 2.3 Virus isolation and identification

The RNA-positive clinical samples were homogenized in phosphate-buffered saline (PBS, pH 7.4) with 200 U/ml penicillin, 200 mg/ml streptomycin and 100 µg/ml gentamicin and centrifuged at 2000 rpm for 5 min at 4°C. The supernatants were filtered through 0.45 µM Millipore filters (Bedford, MA) and were added to Crandell–Rees feline kidney (CRFK) or Madin–Darby canine kidney (MDCK) cells. After continuous observation at 6, 12, 24 and 48 h post-infection, passaging was continued until the appearance of obvious cell pathogenic effects (CPEs). The cell supernatants were then used for extraction of total RNA, and the full-length genome was then amplified.

Cells were observed after three washes with PBS under a fluorescence microscope. The virus purification procedure was carried out by using four sets of primers, respectively (Table 2). All of the primers were designed to enable sequencing of the entire segments with ~200 nt overlaps between them. The PCR was performed in a 25 µl reaction mixture containing 3 µl of template cDNA, 12.5 µl of Prime star Max DNA Polymerase (Takara Bio, Inc.), 0.5 µl of each forward and reverse primer, with ddH2O added to reach a total volume of 25 µl. PCR was performed under the following condition which included a pre-denaturation step at 98°C for 2 min, 30 cycles of denaturation at 98°C for 15 s, annealing at 47°C for 15 s and extension 72°C for 25 s, followed by a final extension at 72°C for 10 min. Then, the PCR products were purified with an E.A.N.A. Gel Extraction Kit (USA) and directly sequenced by the method of Sanger (Sangon Bio, Inc., Guangzhou, China). The purified PCR products were sequenced from both the 5′−3′ and 3′−5′ directions to ensure no mutations arose in these segments. Each segment was sequenced at least twice.

### 2.4 Primer design and amplification of the full-length genome

The FCV full-length genome is divided into four overlapping fragments of ~2.5 kb (1–2523 nt), ~1.9 kb (2420–4308 nt), ~1.2 kb (4182–5345 nt) and ~2.5 kb (5288–7710 nt), and these can be amplified by using four sets of primers, respectively (Table 2). All of the primers were designed to enable sequencing of the entire segments with ~200 nt overlaps between them. The PCR was performed in a 25 µl reaction mixture containing 3 µl of template cDNA, 12.5 µl of Prime star Max DNA Polymerase (Takara Bio, Inc.), 0.5 µl of each forward and reverse primer, with ddH2O added to reach a total volume of 25 µl. PCR was performed under the following condition which included a pre-denaturation step at 98°C for 2 min, 30 cycles of denaturation at 98°C for 15 s, annealing at 47°C for 15 s and extension 72°C for 25 s, followed by a final extension at 72°C for 10 min. Then, the PCR products were purified with an E.A.N.A. Gel Extraction Kit (USA) and directly sequenced by the method of Sanger (Sangon Bio, Inc., Guangzhou, China). The purified PCR products were sequenced from both the 5′−3′ and 3′−5′ directions to ensure no mutations arose in these segments. Each segment was sequenced at least twice.

### 2.5 Sequence analysis

The full-length genome sequences and deduced amino acid sequences of the 11 strains were aligned and analyzed with the Seqman and Megalign programmes (DNASTAR, Madison, USA). Multiple alignments were performed between these strains and 34 FCV genomic as well as 44 capsid sequences which were found in the GenBank database. Phylogenetic trees of the full-length genomes and ORF2 sequences were constructed using the maximum likelihood (ML) method with the Tamura-Nei and Kimura 2 parameter models, respectively. This was performed using MEGA X software (http://www.megasoftware.net/) and 1000 bootstrap values. The putative recombinant origin of the 45 FCV genome sequences (including the 11 isolates in this study) was performed using a recombination detection program (RDP5.3) software (Bordicchia et al., 2021). Seven different methods including RDP, GENECONV, MaxChi, Bootscsan, SiScan and 3Seq using default parameters were employed and the p-values obtained were expected to satisfy at least six of the algorithms (p ≤ 10−6). The SimPlot v3.5.1 software was employed to further identify whether there were any possible recombinant events in the 11 FCV strains.

### Table 2

| Primer name | Primers sequences (5′−3′) | Genomic position (nt) | Length of PCR product (bp) |
|--------------|---------------------------|-----------------------|---------------------------|
| FCV-1F       | GCGCGTAAAAGAAATTTGAGAC    | 1–2523                | 2523                      |
| FCV-1R       | AGCACATCATATGCGGCTC       |                       |                           |
| FCV-2F       | GAATCCGGCAGAACATCAC       | 2420–4308             | 1889                      |
| FCV-2R       | CRTCAACCACCCCARATCAT     |                       |                           |
| FCV-3F       | CAAARTGGAARTTGGCTAC       | 4182–5345             | 1164                      |
| FCV-3R       | ATTWAGACCTAGCGCAG         |                       |                           |
| FCV-4F       | CACTGTGATGTGTTCAAGT       | 5288–7710             | 2423                      |
| FCV-4R       | CCGTGGGTTAGCCGCDR         |                       |                           |

*F and R represent upstream and downstream primers, respectively.

*The GX01-13 genomic sequence (KT970059) is used to be a reference sequence for primer designing.*
**RESULTS**

**2.6 Evaluation of susceptibility of viruses to pH, trypsin and bile salts**

The stability of E-FCVs and R-FCVs to acid, trypsin and bile salts were investigated in vitro according to Di Martino's protocol. In the low pH test, hydrochloric acid was added to the virus suspension to reduce its final pH to 3.0. Each mixture was kept at room temperature for 60 min. Susceptibility to trypsin treatment was carried out by mixing 1% trypsin in distilled water to an equal volume of virus suspension to obtain the final concentration of 0.5 (v/v). The mixture was then incubated for 1 h at 37°C in a 5% CO₂ incubator. To test the resistance of FCVs to bile salts, 250 µl of virus suspensions was mixed with an equal volume of bile salt mixture (Sigma-Aldrich) at final concentration of 0.5%. A negative control group (DMEM + virus suspension) was set up at the same time. After treatment, the virus mixture was inoculated into a 96-well plate containing CRFK cells and titrated in quadruplicate. At the same time, the virus mixture was harvested for each sample. The plates were incubated for 72 h at 37°C and in a 5% CO₂ incubator. To test the resistance of FCVs to bile salts, 250 µl of virus suspensions was mixed with an equal volume of bile salt mixture (Sigma-Aldrich) at final concentration of 0.5%. A negative control group (DMEM + virus suspension) was set up at the same time. After treatment, the virus mixture was inoculated into a 96-well plate containing CRFK cells and titrated in quadruplicate.

**3 | RESULTS**

**3.1 Clinical investigation and virus isolation**

From 2019 to 2020, there were 32 viruses sources (31.7%) which tested positive for FCV by RT-PCR, including 42.4% (28/66) in cats with respiratory symptoms, 11.1% (3/27) from faeces samples and 12.5% (1/8) from serum samples. In the present study, 26 of the diarrhoeic samples were screened for FCV, but there were only two FCV-positive faecal samples associated with this condition. In addition, there was another sample that originated from a cat with acute respiratory symptoms but without diarrhoea. There were 21 samples (77.8%) which tested positive for FPV. It was found that most kittens (<1 year old, 32.9%) were susceptible to FCV, when compared with young cats (1-3 year-olds, 37.5%). Also, 11 FCV strains were isolated successfully using MDCK or CRFK cells, and these were named as GX01-13, GXNN01-19, GXNN02-19, GXLZ01-19, GXLZ02-19, GXYL01-19, GXNN03-20, GXNN04-20, GXNN05-20, GXNN06-20 and GXNN07-20 (Table 2). These were identified as FCV by obvious CPE and IFA using anti-FCV serum (Figure 1) and by their direct observation at the electron microscope level showing 35-40 nm diameter particles with characteristic morphological shapes (Figure 2).

**3.2 Genetic and phylogenetic analysis of FCV isolates**

Eleven complete FCV genomes were successfully amplified by using four sets of sequence-specific primers. The total length of FCVs ranged from 7685 to 7710 nt. Each genome encodes ORF1, ORF2, ORF3, 3’UTR and 5’UTR. These were compared with 34 full-length genomic sequences of R-FCV, E-FCV and virulent systemic FCV (VS-FCV) strains are available from the database (Table S1). All of the 11 genomic sequences shared 76.2%-99.8% nt identities to each other. Three ORFs were identified in the 11 genomic sequences and these were analyzed by comparing their identities against the 255 vaccine (Table 3). The ORF1 genes encoded an RNA-dependent RNA polymerase (RdRp) with a length of 5292 nt and the predicted protein of 1763 aa. All the isolates shared high (89.8%-92.1%) aa identities with the 255 strain. The length of the the capsid genes ranged from 2007 to 2010 nt and encoded for a capsid protein of 669 or 670 aa, sharing lower nt/aa identities with 66.3%-73.7%/75.1%-77.9% to the 225 strain. Generally, ORF3 was 321 nt long and encoded a protein of 106 aa, but in this study, the GXNN06-20 strain from a respiratory origin was 324 nt/107 aa long. The overall identities of the deduced aa ranged from 87.9 to 95.3%.

Upon phylogenetic analysis based on the eleven genomic sequences, three E-FCV isolates were distributed among the different branches (Figure 3a). Two E-FCV isolates (GXNN04-20 and GXNN05-20) were grouped with R-FCV (GXNN01-19 and GXLZ01-19) and were related genetically to J4 from the north of China, and shared 82.9%-87.6% nt identities. There were high levels of similarity between GXLZ01-19 and GXYL01-19 with nt identities of 98.8%, and these were clustered with the early isolates (GX01-13) and shared higher nt identities with an average of 84.8%. This suggests that the GX01-13 strain has long been circulating among feline populations. The remaining three isolates (E-FCV-GXNN03-20, GXNN07-20 and GXNN06-20) formed a monophyletic cluster and was genetically related to the Chinese isolates, XH-2015 and WZ1-2016. Notably, the GXNN02-19 isolate was more distantly related to other isolates, only sharing average nt identities of 76.6% with each other.

Comparative analysis of the capsid sequences from 44 FCV strains from the database showed that they were grouped into different branches (Figure 3b). In particular, the E-FCV isolates were distributed in a scattered fashion, sharing average nt/aa identities of 77.4%/86.8%, respectively. Two E-FCV (GXNN05-20 and GXNN03-20) and three R-FCV (GXNN06, GXNN07 and GX01-13) isolates were genetically closer to the VS-FCV strains, including George, Kaos, Jengo, 4b, UTCVM-H1 (Abd-Eldaim et al., 2005; Hurley et al., 2004; Ossiboff et al., 2007) and the E-FCV (160-2015-ITA) isolated from Italy in 2015 (Di Martino et al., 2020). Moreover, the remaining four strains including their energetic origins formed the monophyletic cluster which were genetically related to JL-4, and the GXNN01-19 was clustered with the JL-2 strain obtained from a cat from China with URTD (Zhao et al., 2017). Interestingly, one of 11 isolates (GXNN02-19) was clustered into genogroup II, which included the early Chinese isolates. There were Asn/Ser, Ala/Pro and Gly residues at positions 377, 539 and 557, respectively, in genogroup I, whilst the genogroup II strains kept their Lys, Val and Ser residues in agreement with Sun's report (Sun et al., 2017) (Figure 5).

**3.3 Recombinant analysis**

To investigate whether there were recombinant events in the eleven FCVs with different origins, the full-length genomic sequences were analyzed by using the SimPlot and RDP5.3 software. The results
FIGURE 1  Cytopathic effect (CPE) of the feline calicivirus (FCV) isolate on CRFK and MDCK cells and indirect fluorescence assay (IFA) specific for the FCV isolates. CRFK (a) and MDCK (c) cells were infected with a GX01-13 isolate at 12 h post-infection (200×); (b) and (d) negative controls (the uninfected cells). (e) Green fluorescence was found in MDCK cells infected by the FCV GX01-13 isolate at 24 h post-infection (200×). These were identified by the indirect immunofluorescence assay (200×). (f) There is a cell pathogenic effect (CPE) on MDCK cells in accordance with the results of IFA as observed under white light (200×).

FIGURE 2  Feline calicivirus (FCV) particles are shown in transmission electron microscopy. The bar represents 100 nm showed that the R-FCV GX01-13 genome sequence had the potential for recombinant with scores of 0.736 within the ORF2 region. The recombination breakpoints of the GX01-13 strain were mapped to positions 5817 of the genomic sequence, which was identified in the capsid gene (Figure 4a). The region A (before position 5817) was identified to be closely related to R-FCV GXLZ02-19 (87.3% similarity). Interestingly, the region B (between position 5817 and 7686) shared 86.6% similarity with E-FCV GXNN03-20 (Figure 4b).

3.4 Comparative analysis of amino acids of the capsid proteins

The comparison of amino acid sequences of the capsid protein genes of FCV allowed the capsid to be divided into conserved (A, B, D and F) and hypervariable (C and E) regions (Figure 5). The hyper-variable E region (426–523 aa positions) in the capsid protein was separated by
TABLE 3
Homology analysis of three open reading frames (ORFs) of feline calicivirus (FCV) isolates compared with the 255 FCV strain

| Origin      | Genogroups | Strains               | Length (nt/aa) | ORF1 (%) | Length (nt/aa) | ORF2 (%) | ORF2-E (%) | Length (nt/aa) | ORF3 (%) |
|-------------|------------|-----------------------|----------------|----------|----------------|----------|------------|----------------|----------|
| Respiratory | Respiratory| GX01-13               | 5292/1763      | 79.3     | 91.5           | 76.1     | 86.1       | 80.1           | 94.4     |
| Respiratory | Respiratory| GXLZ01-19             | 5292/1763      | 79.3     | 92.1           | 77.4     | 87.4       | 85.5           | 94.4     |
| Respiratory | Respiratory| GXLZ02-19             | 5292/1763      | 79.7     | 91.2           | 77.2     | 86.2       | 84.2           | 86.9     |
| Respiratory | Respiratory| GXYL01-19             | 5292/1763      | 79.9     | 91.1           | 77.2     | 86.8       | 84.2           | 87.9     |
| Respiratory | Respiratory| GXNN01-19             | 5292/1763      | 79.4     | 92.0           | 77.2     | 86.8       | 84.2           | 87.9     |
| Respiratory | Respiratory| GXNN02-19             | 5292/1763      | 78.1     | 89.8           | 77.5     | 86.5       | 85.1           | 89.7     |
| Respiratory | Respiratory| GXNN03-20             | 5292/1763      | 79.0     | 90.8           | 77.7     | 88.8       | 85.8           | 95.3     |
| Respiratory | Respiratory| GXNN05-20             | 5292/1763      | 79.3     | 91.7           | 78.4     | 87.4       | 85.2           | 92.6     |
| Respiratory | Respiratory| GXNN06-20             | 5292/1763      | 79.6     | 91.0           | 78.0     | 87.0       | 84.8           | 94.4     |
| Respiratory | Respiratory| GXNN07-20             | 5292/1763      | 78.9     | 90.8           | 77.5     | 86.5       | 85.1           | 89.7     |
| Respiratory | Respiratory| GXNN08-20             | 5292/1763      | 79.9     | 91.0           | 77.7     | 88.8       | 85.8           | 95.3     |
| Respiratory | Respiratory| GXNN09-20             | 5292/1763      | 79.0     | 91.4           | 78.4     | 87.4       | 85.2           | 92.6     |
| Respiratory | Respiratory| GXNN10-20             | 5292/1763      | 79.3     | 91.7           | 78.4     | 87.4       | 85.2           | 92.6     |
| Enteric     | Enteric    | GXYL01-19             | 5292/1763      | 79.9     | 91.1           | 77.2     | 86.8       | 84.2           | 87.9     |
| Enteric     | Enteric    | GXNN02-19             | 5292/1763      | 78.1     | 89.8           | 77.5     | 86.5       | 85.1           | 89.7     |
| Enteric     | Enteric    | GXNN03-20             | 5292/1763      | 79.0     | 90.8           | 77.7     | 88.8       | 85.8           | 95.3     |
| Enteric     | Enteric    | GXNN05-20             | 5292/1763      | 79.3     | 91.7           | 78.4     | 87.4       | 85.2           | 92.6     |

Note: The different lengths differed from other FCV isolates are marked in bold.

ConE into two further regions, 5'HVR and 3'HVR. These were responsible for the major antigenic variability and were also involved with the cell receptor for initial attachment (Bhella & Goodfellow, 2011; Bhella et al., 2008; Geissler et al., 2002; Makino et al., 2006; Radford et al., 1999). In our study, there was an insertion of Gly at position 127 of region B, and this was found in GXLZ02-19, GXYL01-19, GXNN01-19, GXNN04-20 and GXNN05-20. This resulted in changes of the length of VP1 and the deletion of three amino acids (at positions 495–497) in the 3'HVR of the region E of all the Chinese isolates reported (Zhao et al., 2017), and these might also be responsible for changes in cell tropism.

The linear B-cell epitopes in regions D and E were highly conserved, but one mutation corresponding to I417L was found. In addition, almost all the amino acid changes were focused in the 5’ HVR of region E which is responsible for the binding of the fJAM-1 receptor (429-440 aa) and linear epitopes (445-457 aa). Although the genetic markers for differentiating virulent systemic disease (VSD) and oral respiratory disease (ORD) have not been completely identified, the physicochemical properties of amino acids located in 5' HRV of region E displayed possible differences in the way VSD and ORD FCVs interacted with the cell receptors (Di Martino et al., 2020). Analysis of these isolates found that there were higher frequencies in the mutations of A448K/R/H and D452E associated with the VSD strains, compared with another positions (Figure S1). Moreover, the GXNN02-19 isolate from genogroup II displayed four amino acid residues (T438V, G440Q, D455T and G465S) which was consistent with the VSD strains (Table 4).

3.5 In vitro sensitivity to acid, trypsin and bile salts

The results of titre changes of the 12 FCV strains, including GXNN05-20 from respiratory and enteric origins, respectively, with respect to the treatment with acid, trypsin and bile salts are shown in Table 5. All the R-FCV and E-FCV strains lost most of their infectivity on CRFK cells at low pH. There were only six FCV isolates (including E-FCV GXNN03-20) which showed lower infectious titres ranging from 2.08 to 4.45 log10. In contrast, most of FCV isolates were stable in the sensitivity test to trypsin. Of these, three R-FCVs showed a limited reduction in TCID50 of 2.48 log10 (GXNN07-20), 2.34 log10 (GXLZ02-19) and 3.11 log10 (GX01-13). After treatment with bile salts, three of the E-FCVs were bile salt-resistant and these showed a loss of infectivity titres ranging from 0.05 to 0.21 log10. The average loss of infectious titres of nine of the R-FCVs was 0.52 log10, suggesting that these viruses were relatively more sensitive to bile salts than the E-FCVs. Interestingly, there was no significant difference for the GXNN05-20 isolates from respiratory and enteric origins in these sensitivity tests.

4 DISCUSSION

FCV usually causes acute oral and upper respiratory tract disease in domestic cats. The variability of the RNA genome led to the emergence of VS-FCV isolates which could cause extensive lesions and high...
FIGURE 3  Phylogenetic trees were constructed on the basis of the full-length genome (a) and the nucleotide sequences of the the capsid gene (b) of 11 Feline caliciviruses (FCVs) as well as other FCV isolates available from the GenBank database. Phylogenetic trees were generated by using the maximum likelihood (ML) method with Tamura-Nei (a) and Kimura two parameter (b) models. The ML bootstraps (1000 replicates) with values ≥70 are shown at the major branches of the trees. The FCVs isolated from respiratory origin were characterized in this study and were marked with black circular symbols. The solid black triangles indicate the FCV isolates from enteric origin and the hollow triangles represent the previously published enteric FCV strains. The eight isolates from respiratory origin are marked with solid black circles. The three isolates from enteric origin were marked with solid black triangles. The hollow triangles represent the published Italian isolates from enteric origin.

mortality rates in the cat populations. Although there were no further experiments to identify the infection of VS-FCV in cats in China, VSD symptoms were observed in some cases (Guo et al., 2018; Wang et al., 2021). Notably, the finding of FCV isolates in the stools of cats with enteritis suggested that FCV may play a role as enteric pathogens or they were localized in the intestinal tissues for shedding. Mochizuki et al. had obtained FCV from the faeces and determined that these viruses could be divided into enteric (E-FCV) and respiratory (R-FCV) types depending on the differences in their resistance to bile acids (Mochizuki, 1992). Here, the full-length genomic sequence information of E-FCVs and R-FCVs was gathered for comparative analysis which will show better insights into the epidemiological links and genetic heterogeneity of these viruses.

More recently, there were more FCV-positive RNA found in the faecal specimens from cats with diarrhoea or enteritis, such as 35.7% in New York, USA, 9.6% in Japan and 25.6% in Italy (Di Martino et al., 2020; Pinto et al., 2012; Takehisa et al., 2015). In the present study, most of the FCV-positive samples (42.4%) were associated with classical respiratory or ulceration symptoms, but FCV was only detected in 11.1% of faecal samples. Interestingly, one faecal sample (GXNN05-20) from a 5-month-old cat that displayed severe URTD, recurrent fever, but without diarrhoea, was found. This suggested that FCVs could not only replicate in the upper respiratory tract but also pass through the alimentary tract for shedding. Although there was a higher prevalence of FPV (77.8%), and only two of the FCV-positive samples were co-infected with FPV. This showed that FCV was only one component of the possible feline viral genome, although it could not be determined whether it triggered a synergistic effect with other enteric pathogens to cause diarrhoea or enteritis.
In addition, there was one serum-derived sample that tested positive for FCV, and this was reported in a cat with VSD (Caringella et al., 2019). During our investigations, it was also found that kittens and cats up to 2 years old were more susceptible to FCV. This may have been due to the lack of maternally derived antibodies and immature immune systems, exposure to higher FCV doses (via shedding in litter trays) or persistent infections in multiple-cat environments. Therefore, more paired respiratory, faecal and serum samples will need to be investigated to allow a better insight into whether there were epidemiological links or genetic differences of FCV isolates from respiratory secretions and faeces.

The present study focused on the comparative analysis of genetic characterization between enteric and respiratory origins. Based on analysis of the full-length FCV genomic sequences, it was found that three E-FCV isolates did not form a well-defined cluster and appeared distantly related to each other (aa identities 77.8%–82.8%).
FIGURE 5  Alignment of the capsid sequences of the 11 FCV isolates (based on the F9 strain). Regions A, B, C, D, E and F are indicated by vertical lines. The 5' HVR, conserved region (ConE) and 3' HVR in the E region are separated by the dotted lines. The amino acids described by Brunet et al. are indicated by the dark arrows (Brunet et al., 2019). The linear B-cell epitopes are indicated using the light grey coloration (Radford et al., 1999). The amino acid residues involved in interactions between the VP1 and fJAM-A are marked with + signs (Bhella & Goodfellow, 2011). Three amino acids marked by the ▲ symbol are common changes between genogroups I and II (Sun et al., 2017). The deleted regions are indicated using the hollow boxes. E represents the viruses of enteric origin, R represents the viruses of respiratory origin and I and II represent the genogroups. The symbol '-' indicates the missing of amino acids indicated that there was no phylogenetic grouping and geographic links based on the identities of their sequences (Table S2). The other eight R-FCV isolates displayed highly genetic diversity, sharing aa identities from 76.1% to 98.8%. When compared using the vaccine strains, it was found that all of these were genetically distant from strains F9, F4 and 2024 and only shared an average of 78.8% nt identity. However, it was shown that the vaccine strain F9 antisera still remained broadly and equally cross-reactive to separated FCV populations (Smith et al., 2020), but cats occasionally developed FCV after vaccination with commercial FCV vaccine (including the 255 FCV strain) which is mostly used to vaccinate cats in China.

In our investigations, there were five FCV isolates from the vaccinated cats, which represent a failure of the vaccine to induce sufficient protective neutralizing antibodies, either due to incorrect vaccine administration or lack of antigenic cross-reactivity. The homology between these isolates and the 255 strain displayed the significantly genetic variability in the major capsid protein with lower identities (on average 77.0%/86.9% nt/aa) (Table 3). The immunodominant region of FCVs in the capsid protein may allow virus evasion of the host immune response by a process of immune-mediated positive selection (Kreutz et al., 1998; Radford et al., 1998). Among these isolates, R-FCV GX01-13 may undergo replacements of the genomic region...
TABLE 4  Comparative amino acids of region E of the feline calicivirus (FCV) capsid proteins with virulent systemic disease (VSD) strains

| Genogroup | Origin     | Strains  | Amino acids associated with VSD |
|-----------|------------|----------|---------------------------------|
| GI        | Respiratory| VSD FCV  | 438 V 440 Q 448 K/R 452 E 455 T 465 S 492 V |
| GI        | Respiratory| ORD FCV  | 438 T 440 G 448 A 452 D 455 D 465 G 492 V |
| GI        | Respiratory| GX01-13  | 438 T 440 S 448 K 452 E 455 D 465 G 492 I |
| GI        | Respiratory| GXLZ01-19 | 438 T 440 G 448 A 452 D 455 D 465 S 492 V |
| GI        | Respiratory| GXLZ02-19 | 438 T 440 G 448 A 452 D 455 D 465 S 492 V |
| GI        | Respiratory| GXNN01-19 | 438 T 440 S 448 S 452 D 455 D 465 G 492 I |
| GI        | Respiratory| GXYL01-19 | 438 T 440 G 448 A 452 D 455 D 465 S 492 V |
| GI        | Respiratory| GXNN06-20 | 438 T 440 S 448 R 452 E 455 D 465 G 492 I |
| GI        | Respiratory| GXNN07-20 | 438 T 440 L 448 R 452 E 455 D 465 G 492 I |
| GI        | Enteric    | GXNN03-20 | 438 T 440 S 448 K 452 E 455 D 465 G 492 I |
| GI        | Enteric    | GXNN04-20 | 438 T 440 G 448 A 452 D 455 D 465 G 492 I |
| GI        | Enteric    | GXNN05-20 | 438 T 440 Q 448 H 452 E 455 I 465 G 492 I |
| GII       | Respiratory| GXNN02-19 | 438 V 440 Q 448 A 452 D 455 D 465 T 492 L |

Note: The amino acid residues with the highest frequency and physiochemical properties associated with VSD and ORD FCV strains (Brunet et al., 2019; Di Martino et al., 2020).
Grey-shaded cells in the table with bold font correspond to amino acid residues with the highest frequency and physiochemical properties associated with VSD strains (Brunet et al., 2019; Di Martino et al., 2020).

TABLE 5  Effects of acid, trypsin and bile salts treatment on infectivity of enteric and respiratory feline calicivirus (FCVs)

| Origin  | FCV strain* | Titre (log10 TCID50) and log10 reduction resulted from the treatment indicated |
|---------|-------------|-----------------------------------------------------------------------------|
|         |             | Control | HCl (pH 3.0) | Trisyn (0.5%) | Bile (0.5%) |
|         |             |          | Treated Δ | Treated Δ | Treated Δ |
| Enteric | GXNN03-20   | 6.77     | 5.00 | 6.77 | 5.98 | 5.67 | 6.56 |
| Enteric | GXNN04-20   | 6.55     | 6.55 | 6.55 | 5.50 | 6.50 | 6.50 |
| Enteric | GXNN05-20   | 6.48     | 6.48 | 6.48 | 5.89 | 6.33 | 6.33 |
| Respiratory | GXNN05-20 | 6.11     | 6.11 | 6.11 | 6.11 | 6.11 | 6.11 |
| Respiratory | GXNN01-19 | 6.33     | 6.33 | 6.33 | 6.33 | 6.33 | 6.33 |
| Respiratory | GXNN02-19 | 6.77     | 6.77 | 6.77 | 6.77 | 6.77 | 6.77 |
| Respiratory | GXNN06-20 | 6.33     | 6.33 | 6.33 | 6.33 | 6.33 | 6.33 |
| Respiratory | GXNN07-20 | 6.57     | 6.57 | 6.57 | 6.57 | 6.57 | 6.57 |
| Respiratory | GXLZ01-19 | 5.67     | 5.67 | 5.67 | 5.67 | 5.67 | 5.67 |
| Respiratory | GXLZ02-19 | 5.67     | 5.67 | 5.67 | 5.67 | 5.67 | 5.67 |
| Respiratory | GXYL01-19 | 5.67     | 5.67 | 5.67 | 5.67 | 5.67 | 5.67 |
| Respiratory | GX01-13   | 5.89     | 5.89 | 5.89 | 5.89 | 5.89 | 5.89 |

Abbreviation: Δ, log10 reduction.
*GXNN05-20 was isolated from nasal and faecal swabs of the same cat.

during virus replication. Recombination of RNA viruses could be regarded as the predominant mechanism and contribute to the emergence and evolution of new variant strains, which was observed in some FCV strains (Bordicchia et al., 2021; Coyne et al., 2006; Lee et al., 2021; Zhou et al., 2021). This is the first time that the recombination of FCV strains occurred within respiratory and enteric origin to our knowledge, suggesting that the high prevalence of co-infection under the ideal environment or in an individual cat, will lead to a high level of virus diversity. Of note, the GX01-13 isolate (including A448K and D452E) was shown to be a virulent strain of FCV after the challenge experiments in cats, which displayed acute respiratory and severe ulceration in the plantar regions (data not shown). Co-infection within E-FCV and R-FCV or emergence of recombination between respiratory and enteric origin increased the complexity of clinical symptoms,
and it will be possible to contribute the tissue tropism or virulence in cats.

Although there was only one serotype of FCV, the high variable region in the the capsid gene made the viruses display genetic diversity. According to the differences of amino acids (370-580) in the VP1 protein (Sato et al., 2002; Sun et al., 2017), most of the Chinese isolates were clustered into genogroup I, but the isolates from Wuhan city and southwestern of China were clustered into genogroup II (Sun et al., 2017; Zhou et al., 2021). The co-existence and interplay of different genogroups or origins will possibly increase the frequency of recombinant events or result in the incomplete protection through vaccination. The further analysis of VP1 of a GXNN02:19 isolate (genogroup II) showed four amino acids (T438V, G440Q, D455T and G465S) associated with the VSD strains which were identified in the hyper-variable E region (Table 4). The mutation of T438V appears to be a key element that could affect the interaction between VP1 and fJAM-1 receptor as well as the intra-chain interaction between G440Q and D455T which would decrease the flexibility of the loop (Brunet et al., 2019). It will be interesting to explore whether these amino acid changes in the E region of capsid genes caused different clinical symptoms or changed the tissue tropism (through respiration and the intestines). This still remains to be determined and require the use of the FCV reverse genetic systems for clarifying the functions of these residues.

The in vitro sensitivity to acid, trypsin and bile salts were compared to all the R-FCVs and E-FCVs, including two GXNN05-20 strains. The analysis found that there were no significant biological differences between two phenotypes groups, but their infectivity in MDCK cells was different (data not shown). Of note, these E-FCV strains were not as resistant to low pH as previously described. The GXNN05-20 strain from nasal and faecal samples of the single cat brought new insights into whether these E-FCVs were shed in the faeces after primary localization in other tissues. Actually, the isolation of GXNN05-20 from different origins displayed high identity at the nucleotide level and similar biological properties were found. Also, the lower positive rate from the cats with enteric disease suggested that although they were occasionally identified in the faeces of cats, it was not clear whether FCV could play a role as an enteric pathogen.

In conclusion, we obtained 11 isolates of FCV from oral/nasal or faecal samples using MDCK or CRFK cells. Phylogenetic analysis showed that there were multiple strains of genogroups I and II circulating in Guangxi. The genetic homology among respiratory and enteric FCVs ranged from 76.3% to 98.8% which displayed high genetic diversity. All of the respiratory and enteric FCVs showed increased sensitivity to low pH, but were resistant to treatment with trypsin and bile salts. Additionally, the acquisition of full-length genome sequences of E-FCVs and R-FCVs displayed the detailed molecular characterization of different FCV pathotypes. This provides a deeper understanding of the genetic relationship between viruses of respiratory and enteric origins. The continued monitoring of emerging FCV strains and preparation of a candidate vaccine could be better controls for dealing with future FCV outbreaks in cats.

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CONFLICT OF INTEREST
The authors declare no conflict of interest.

ETHICS STATEMENT
The authors confirm that the ethical policies of the journal. No ethical approval was required, and ethical statement is not applicable as sample collection from animals has been gathered.

DATA AVAILABILITY STATEMENT
The data that support the findings of this study are available from the corresponding author upon reasonable request.

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Supporting Information

Additional supporting information may be found in the online version of the article at the publisher’s website.