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Letter

First isolation and genomic characterization of a novel inter-genotype recombinant feline calicivirus from domestic cats: Implications for viral evolution

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Dear editor,

As of 2023, the domestic cat population in China reached 65 million, surpassing dogs to become the most numerous companion animal in the country. Feline calicivirus (FCV) infection, one of the three most prevalent infectious diseases in cats, poses a severe threat to feline health. FCV, classified under the *Caliciviridae* family (genus *Vesivirus*), exhibits broad age-specificity host range in cats and is associated with severe respiratory disease manifestations, with certain strains posing a risk of mortality (Prikhodko et al., 2014). The viral genome encodes three open reading frames (ORFs): ORF1, which encodes non-structural protein multimers; ORF2, which encodes the primary capsid protein VP1; and ORF3, which encodes the minor capsid protein VP2 (Abente et al., 2013). The structural protein VP1, characterized by high variability, serves as a crucial protective antigen against FCV. Genetic variation in VP1 allows for the classification of FCV into two genogroups, I and II, with significant differences observed at three amino acid sites: N/D377K, A539V, and G557S. The GI group is the most prevalent, while the GII group is primarily found in Japan, China, and South Korea, indicating a distinct regional distribution (Kim et al., 2021). Recent evolutionary developments in FCV variant strains suggest that vaccination based on classical strains may not provide complete protection for cats, leading to frequent vaccination failures (Zhou et al., 2021).

Like other highly virulent viruses in the *Caliciviridae* family, FCV exhibits considerable genetic variability. Under immune selection pressure, the viral genome undergoes rapid evolution through frequent gene recombination and mutation, with VP1 showing the highest mutation rate (Ozaki et al., 2019). Recombination events have been discovered in FCV strains from diverse geographic regions, enhancing the virus's genetic diversity (Mao et al., 2022). Notably, FCV has demonstrated cross-species transmission and recombination, as evidenced by the first isolation of FCV from young canines and a recombination event involving an early northern strain of the GI group from China (Sun et al., 2023). These findings indicate that accumulated mutations and recombination in natural environments can accelerate the evolution of the virus. However, there are no reports about FCV recombination between GI and GII to date. In this study, two FCV strains were isolated and characterized. Whole-genome sequencing revealed the first documented cross-genotype recombinant FCV, providing critical insights into viral evolution and genetic diversity.

Between 2021 and 2023, fifty-eight nasopharyngeal swabs were collected from veterinary clinics across diverse geographic locations in China. Two FCV RNA-positive samples from Shanxi and Anhui provinces were successfully isolated and purified using Crandell-Rees Feline Kidney (CRFK) cells. An IFA test using a monoclonal antibody against VP1 was also employed to detect the virus after three rounds of purification (Fig. 1A). Based on the above findings, two FCV strains, designated as AH17 and XA20, were successfully isolated and purified. To improve the understanding of the genetic diversity of strains from different locations, the whole genomes of both strains were successfully amplified. The genome of AH17 (GenBank accession number: OR791865; ScienceDB: https://doi.org/10.57760/sciencedb.26350) and XA20 (GenBank accession number: OQ814183; ScienceDB: https://doi.org/10.57760/sciencedb.26345) were 7686 and 7705 nucleotides in length, respectively, with respective virus titers of $1 \times 10^{8.95}$ and $1 \times 10^{8.76}$ $TCID_{50}$/mL. To identify the genomic characteristics of the two FCV strains, we conducted sequence homology analysis using the whole genome sequences, as well as ORF1, ORF2, and ORF3 gene sequences of ten FCV strains selected from GenBank. The results showed that the

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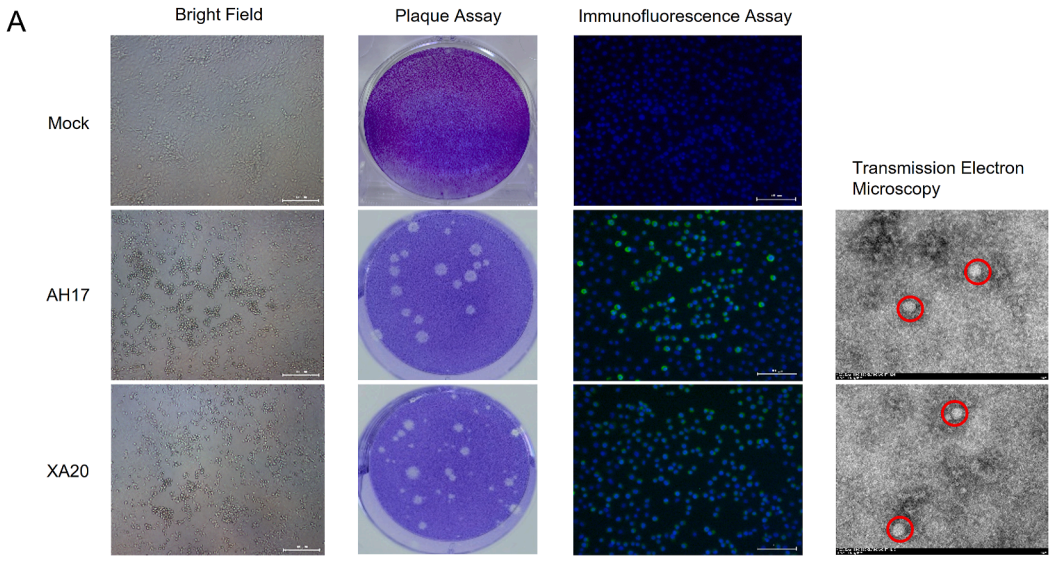

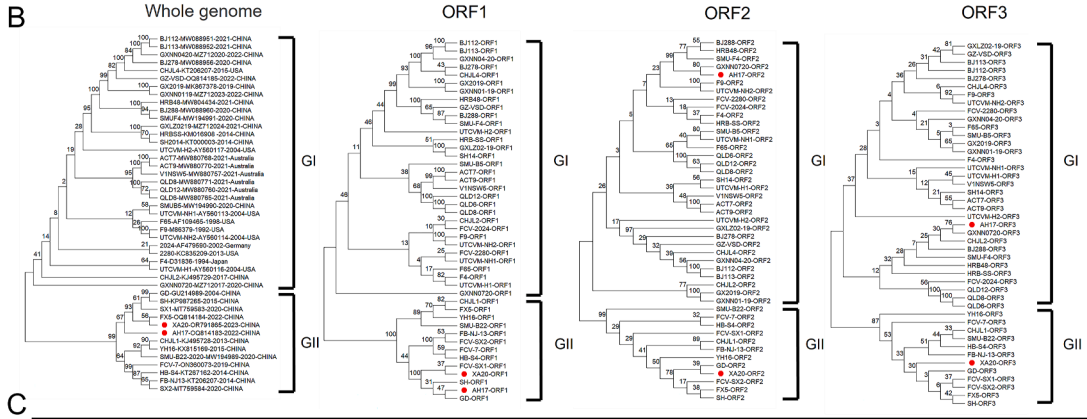

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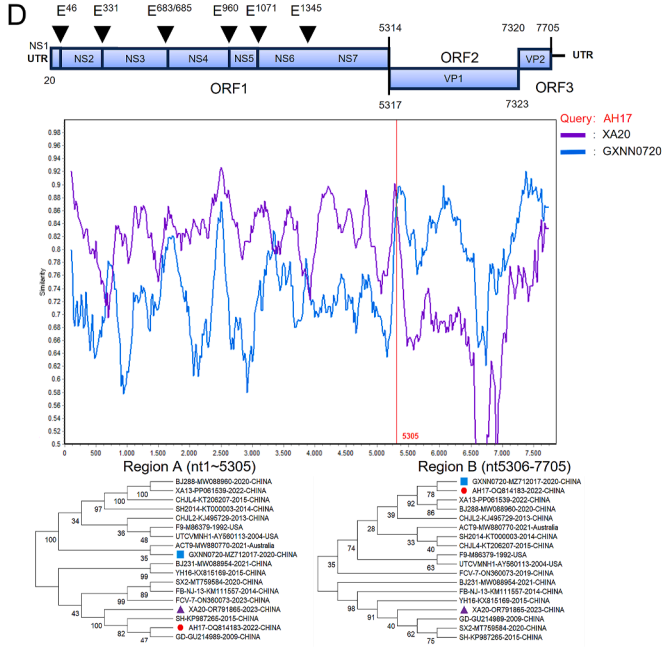
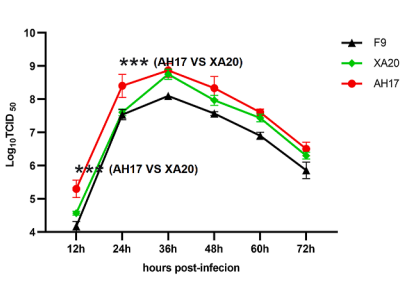
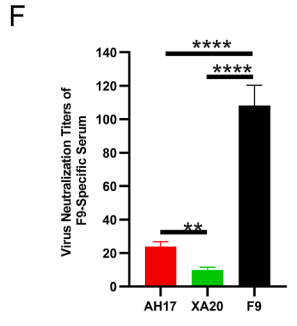
Recombinant	Major (Similarity)	Minor (Similarity)	*P*-Value of the Detection Methods						
			RDP	Geneconv	BoostScan	MaxChi	Chimaera	SiScan	3Seq
AH17	XA20 (85%)	GXNN07-20 (84.4%)	7.831×10^{-21}	NS	4.109×10^{-22}	9.513×10^{-17}	4.779×10^{-20}	6.332×10^{-14}	NS







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nucleotide homology values of the whole genome sequences of AH17 and XA20 strains compared to ten selected strains ranged from 77.3% to 82.3% and 76.4%~84.2%, respectively (Supplementary Table S1). Both strains showed the highest homology with the GII-SH strain and the lowest homology with the GI vaccine strain F9. Comparative analysis of AH17 strain's ORF1, ORF2, and ORF3 sequences with reference strains, demonstrated that ORF2 had the lowest homology while ORF3 showed the highest. Furthermore, the AH17 strain's ORF1 gene sequence shared higher similarity with GII FCV isolates, while its ORF2 and ORF3 displayed higher homology to GI FCV isolates. In the E region of the VP1 protein, which includes the FCV neutralizing epitope, the nucleotide homology between AH17 and the reference strains ranged from 72.1% to 65%, significantly lower than that of the whole genome and the three ORFs. Conversely, all genomic segments of the XA20 strain showed higher similarity with GII genotype isolates, with the E region showing the lowest homology (58.5%), which is consistent with the pattern observed in AH17.

These findings suggest that the two isolates exhibit significant genetic variability and limited similarity to classical strains, which may lead to immune evasion in FCV infection. To investigate their evolutionary relationships, phylogenetic trees were constructed using nucleotide sequences of the whole genome, ORF1, ORF2, and ORF3. While ORF2 and ORF3 of AH17 clustered within the GI FCV clade, its whole genome and ORF1 sequences grouped closely with the GII FCV isolate SH (KP957265). In contrast, the XA20 strain showed consistent clustering with GII FCV strains across all genomic segments (Fig. 1B). According to the classification system proposed by Japanese scholars based on three critical amino acid positions in VP1, AH17 is classified as GI group and XA20 as GII group. However, the discordant phylogenetic placement of AH17 strain's genomic regions suggests the occurrence of recombination events in this strain. To identify potential recombination events, we analyzed isolates for recombination using the RDP5 and Simplot software packages. The AH17 strain exhibited recombination events between the XA20 (GII) and the GXNN0720 (GI) strain, with *P*-values below 1×10^{-6} in four different assays (Fig. 1C). The similarity plot identified a recombination breakpoint at the ORF1-ORF2 junction in the AH17 genome, corresponding to position 5305 in the genomic sequence. This breakpoint divides delineates the AH17 genome into two distinct regions: Region A (nt 1 to 5305), which shows 85% similarity to the XA20 strain, and Region B (nt 5305 to 7705), which exhibits 84.4% similarity to the GXNN0720 strain (Fig. 1D). By using the viral replication dynamics of three FCV strains to examine the differences in cellular tropism among the AH17, XA20, and F9 strains. The findings show that the two isolates had substantially higher replication titers at each time point than the F9 strain, the AH17 has a greater *in vitro* replication efficiency. The virus titers of AH17 and XA20 were $1 \times 10^{8.95}$ and $1 \times 10^{8.76}$ $TCID_{50}$/mL at 36 hours post infection (h.p.i.), respectively (Fig. 1E).

A serum antibody titer ≥1:32 is considered protective against FCV (Reese et al., 2008). To compare the antigenicity of the new isolates, rabbit antiserum against the classical strain F9 was produced and exhibited cross-reactivity in virus neutralization (VN) assays with multiple diverse FCV strains. The neutralizing titers of the F9 antiserum against AH17 and XA20 were 23.1 ± 2.2 and 10.4 ± 1.7, respectively, representing a significant two-fold difference ($P < 0.05$). Besides, the titer against F9 itself was 108 ± 14.25 (Fig. 1F). The above results show that the antigenic cross-reactivity of traditional vaccine strains and new epidemic strains is significantly different, especially against recombinant variants.

Recombination events are frequently observed in RNA viruses and serve as a principal mechanism for the emergence and evolution of novel variants (Wang et al., 2022). Based on key amino acid differences in the VP1 sequence, FCVs can be categorized into two genogroups: GI and GII, with the GI genogroup representing the most prevalent circulating strain in China (Sun et al., 2017). The first report of recombination between FCV strains was described in 2005 within an endemically infected cat colony, and cross-regional recombination has also been observed with isolates from Korea and the United States (Lee et al., 2021). In recent years, recombinant FCV strains have been identified in Guangxi, Qingdao, and other locations in China (Zhou et al., 2021). Additionally, recombination events between isolates from dogs and cats have been reported, demonstrating the frequency of recombination within FCV strains (Sun et al., 2023).

However, genetic evolutionary analysis indicates that all published recombination events occurred exclusively among GI strains of FCV. Currently, there is no evidence of inter-genogroup recombination in FCV isolates, although it has the potential to generate a more diverse array of viral sequences compared to intra-genogroup recombination (Li et al., 2023). For instance, the recombinant GII.4 [P16] norovirus (NoV) strain is associated with gastroenteritis outbreaks in multiple countries and has emerged as the predominant strain (Cannon et al., 2017). Similar recombination between genogroups has been documented in the rabbit hemorrhagic disease virus (RHDV), with strains GI.4eP-GI.1a and GI.3P-GI.1d shown to spread widely, resulting in epidemics in Australia and Europe, respectively (Abrantes et al., 2020). Analysis of replication capacity demonstrated that, at all time points measured, the viral titers of recombinant strains were higher than those of other FCV strains, revealing that inter-genogroup recombination may enhance viral pathogenicity.

Vaccination remains the primary method for preventing FCV infection. Preliminary findings indicated the F9-based vaccines elicited antibodies with broad cross-reactivity against diverse FCV strains (Afonso et al., 2017). However, our study revealed that employing high-titer serum from rabbits vaccinated with the F9 strain did not provide effective cross-protection against the GI-GII recombinant strain. Moreover, since all current vaccine strains belong to the GI group, limited experimental data are available to demonstrate their effectiveness against GII-FCV strains detected in China.

In conclusion, we successfully isolated and characterized two FCV strains from clinical samples. Subsequent alignment of their whole genome sequences with published data revealed that strain AH17 represents the first documented case of inter-genotype recombination

Fig. 1. Isolation, identification, genomic, and biological characterization of two FCV strains (AH17 and XA20). **A** CRFK cells inoculated with FCV isolates AH17 and XA20 exhibited typical cytopathic effects at 12 h.p.i., while negative controls remained unaffected. Following initial isolation, plaque assay-based purification (three rounds) was conducted to establish pure FCV stocks. IFA analysis using anti-VP1 mAbs showed distinct cytoplasmic fluorescence signals, whereas the control group exhibited no detectable staining. Sacle bar, 100 μm. Typical calicivirus particles (indicated by red circles), approximately 30 nm in diameter, were observed via transmission electron microscopy. **B** Phylogenetic analysis of FCVs was performed using complete genomic sequences and individual open reading frames (ORF1-3). Maximum Likelihood (ML) trees reconstructed with 1000 bootstrap replicates revealed evolutionary relationships, wherein the novel isolates AH17 and XA20 (highlighted in red) formed distinct clades. **C** Recombination analysis of AH17 using RDP5 (Recombination Detection Program version 5) revealed significant intragenetic recombination events, with clear breakpoint identification supported by 7 algorithmic methods (RDP, GENECONV, BootScan, MaxChi, Chimaera, SiScan, 3Seq). **D** Genome-scale similarity comparisons of AH17 with the XA20 isolate (purple) and FCV-GXNN07-20 isolate (blue) using a sliding window (window size: 500 bp, step size: 20 bp). Potential recombination breakpoints were marked by a red vertical line with nucleotide sites at the bottom. ML phylogenetic trees based on every recombinant fragment within AH17 and 17 reference FCV isolates are shown below the similarity plot. The isolate AH17 is labeled with a red circle, and the putative recombinant major parent isolate XA20 is marked with a purple triangle. The putative recombinant minor parent isolate FCV-GXNN07-20 is marked with a blue square. **E** The *in vitro* replicative capacities of AH17, XA20, and F9 strains were compared. The viral growth kinetics of these strains were monitored in CRFK cells over a 72 h.p.i. period. **F** Virus neutralization activity was evaluated using rabbit-derived F9 antiserum. $^{**}P < 0.01$; $^{***}P < 0.001$; $^{****}P < 0.0001$.

between GI and GII in FCV strains. Cross-serum neutralization studies indicate that vaccines based on the GI genotype may not provide sufficient protection against emerging GI-GII recombinant and GII strains. Therefore, it is essential to develop new vaccines targeting these circulating strains.

FOOTNOTES

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The genomic sequences of AH17 and XA20 have been deposited in GenBank (accession number: OR791865; OQ814183) and ScienceDB (https://doi.org/10.57760/sciencedb.26350, https://doi.org/10.57760/sciencedb.26345).

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