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# 3C protein of feline coronavirus inhibits viral replication independently of the autophagy pathway

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ABSTRACT

Feline coronavirus (FCoV) can cause either asymptomatic enteric infection or fatal peritonitis in cats. Although the mutation of FCoV accessory gene 3c has been suggested to be related to the occurrence of feline infectious peritonitis (FIP), how the 3C protein is involved in this phenomenon remains unknown. To investigate the role of the 3C protein, a full-length 3c gene was transiently expressed and the cytoplasmic distribution of the protein was found to be primarily in the perinuclear region. Using 3c-stable expression cells, the replication of a 3c-defective FCoV strain was titrated and a significant decrease in replication ($p < 0.05$) was observed. The mechanism underlying the decreased FIPV replication caused by the 3C protein was further investigated; neither the induction nor inhibition of autophagy rescued the viral replication. Taken together, our data suggest that the 3C protein might have a virulence-suppressing effect in FCoV-infected cats. Deletion of the 3c gene could therefore cause more efficient viral replication, which leads to a fatal infection.



## 1. Introduction

Feline coronavirus (FCoV) is an enveloped virus belonging to genus *Alphacoronavirus*, family *Coronaviridae*, order *Nidovirales*. Other closely related group 1a CoV members include canine coronavirus, transmissible gastroenteritis virus and porcine respiratory coronavirus. The genome size of FCoV is approximately 28.9 kb and encodes 1 replicase gene, 4 structural genes, the S, E, M and N genes and 5 group-specific accessory genes, including 3abc and 7ab (Lai et al., 2007).

FCoV is an important pathogen in domestic and non-domestic cats and can be further categorized into 2 biotypes: FECV, which accounts for enteric infections, and FIPV, which leads to the highly lethal disease feline infectious peritonitis (FIP) (Pedersen, 2009). In addition to variation in the *in vivo* pathogenicity, the two viruses replicate differently *in vitro* as well (Pedersen, 2009). Infection of feline peritoneal macrophages by FIPV results in more infected cells, a higher titer of viral progeny, more efficient spreading to other susceptible cells and greater sustained infection than FECV (Stoddart and Scott, 1989). Similar findings have also been demonstrated in feline peripheral blood monocytes (fPBM) (Dewerchin et al., 2005).

Several genes have been suggested to be related to the difference in virulence, including S (Chang et al., 2012; Regan et al., 2008; Rottier et al., 2005), 3abc (Balint et al., 2012; Chang et al., 2010; Pedersen et al., 2012; Vennema et al., 1998) and 7ab (Herrewegh et al., 1995; Kennedy et al., 2001). Recently, the integrity of the 3c gene has been found to be correlated with the outcome of infection (Balint et al., 2012; Chang et al., 2010; Pedersen et al., 2012; Vennema et al., 1998). Deletion of the 3c gene has been observed in many FIPV isolates (Vennema et al., 1998) and naturally FCoV-infected cats (Chang et al., 2010; Pedersen et al., 2012). In cats that are asymptomatically infected with FCoV, nearly all of the 3c genes were intact. In contrast, most of the 3c genes of FCoVs from FIP cats harbored point mutations and/or insertion/deletion mutations that led to a truncation of the 3C protein. The 3C protein has been suggested to be related to intestinal tropism (Chang et al., 2010; Pedersen et al., 2012) and the truncated version of open reading frame (ORF) 3abc has been demonstrated to play a role in the efficient macrophage/monocyte tropism of type II FIPV (Balint et al., 2012).

Autophagy is a degradation pathway that is important for the maintenance of many physiological functions and required for the growth and survival of normal cells. Recently, autophagy has also been identified as playing a role in the innate immune system, along with RNA interference and Toll-like receptors, and has been shown to be involved in either the clearance or enhancement of viral infections (Dreux and Chisari, 2010).

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Neither direct linkage between the occurrence of FIP and the truncated 3C protein, nor the function of the intact 3C protein has ever been demonstrated. In this study, the correlation of 3c gene mutation with FIP was first confirmed by analyzing local isolates. The role of the intact 3C protein in the replication of FCoV was further investigated and its possible mechanism was discussed.

## 2. Materials and methods

### 2.1. Specimens

From 2008 to 2010, a total of 93 rectal swabs or fecal samples from the quarantine ward of National Taiwan University Animal Hospital, animal hospitals in the Taipei area, or personally owned cats were collected for the surveillance of the 3c gene of FCoV from asymptomatically infected cats. For FCoV from FIP cats, organs or body effusions collected from 32 pathologically confirmed FIP cats from 2006 to 2012 were surveyed.

### 2.2. Amplification of the 3c gene

The amplification of the 3c gene from healthy and FIP cats was performed by reverse transcription-nested polymerase chain reactions (RT-nPCRs). Briefly, the total RNA of different specimens were extracted and reverse transcribed with a template-specific primer for the M gene (M154-R). cDNAs were then amplified with primers for the 3′ end of S gene and 5′ end of M gene (S4198-F and M154-R), followed by a second round of amplification with different combinations of primers specific to either the 3a or 3b gene and E gene. The sequence and position corresponding to the sequence of FCoV/NTU156/P/2007 (NTU156) (GenBank accession No.: GQ152141) are listed (Table 2). The products of the RT-nPCRs were electrophoresed and the amplicons were excised from the agarose gel, purified with a gel extraction kit (Geneaid, Taipei, Taiwan) and sequenced (Mission Biotech, Taipei, Taiwan).

### 2.3. Cells and virus

*Felis catus* whole fetus-4 (Fcwf-4) cells (kindly provided by Professor Peter J. M. Rottier, Utrecht University) were maintained in Dulbecco's modified Eagle's medium (DMEM) (Gibco, Grand Island, USA) supplemented with 10% fetal bovine serum (FBS), 100 IU/ml penicillin and 100 μg/ml streptomycin in 5% $CO_2$ at 37 °C. FCoV NTU156 is an FCoV strain recently isolated from pleural effusion of a kitten by the co-cultivation method (Lin et al., 2009). NTU156 harbors a 434-nucleotide deletion that leads to a 67 amino acid-truncated 3C protein. The complete genome sequence of strain NTU156 was deposited in GenBank under the access number GQ152141. All of the viruses used in this study for the assessment of the effect of 3C protein expression came from a stock passaged 10 times.

### 2.4. Construction of a plasmid for the expression of full-length 3C protein

The full-length 3c gene was amplified from the rectal swab of a naturally FCoV-infected cat with enteric infections (GenBank accession No.: DQ160294). Subsequently, the amplified products were cloned into mammalian expression vectors, pCi-neo (Promega, Madison, USA), and named pCi-3cHis.

### 2.5. Establishment and confirmation of the 3C protein-expressing cells

Fcwf-4 cells were transfected with either pCi-neo or pCi-3cHis using Lipofectamine™ reagent (Invitrogen, Carlsbad, USA) and selected with G418 (Gibco, Grand Island, USA) for the generation of stable expression cells. Mixed populations of G418-resistant cells generated from the transfectant of pCi-neo and pCi-3cHis will be designated as Fcwf-Ci and Fcwf-3cHis cells, respectively. The cells were than fixed and stained with mouse anti-6 histidine mAb (Invitrogen, Carlsbad, USA) as the primary antibody and FITC-conjugated goat anti-mouse IgG antibody (Leinco Technologies, St. Louis, USA) as the secondary antibody. The stained cells were observed under an inverted fluorescence microscope.

### 2.6. Effect of the expression of the 3c gene on the replication of FCoV

Fcwf-Ci and Fcwf-3cHis cells were infected with FCoV NTU156 at an MOI of 0.1. After 1 h of adsorption, the inocula were removed, cells were washed 3 times with PBS and the media was replaced with fresh DMEM containing 2% FBS. The culture supernatants were harvested at 6, 12 and 24 h after infection and titred by a plaque assay.

### 2.7. Involvement of autophagy in the effect of the 3C protein

To confirm the regulation of the autophagy activity by the treatment of chemical autophagy regulators, Fcwf-3cHis cells were treated with FBS-free DMEM containing an autophagy inhibitor, 3-methyladenine (3-MA) at 100 μM (Sigma, St. Louis, USA), or DMEM containing 10% FBS and rapamycin, an autophagy inducer, at 100 nM. After cultivation for 2 h, cells were washed with cold PBS for three times and lysed with RIPA buffer containing 20 μM Pefabloc® SC (Merck, Darmstadt, German), boiled with 4X SDS–PAGE loading buffer for 10 min and stock at −80 °C before use. For monitoring the autophagy activity by Western blotting, the cell lysates containing 15 μg total protein were resolved in 15% SDS–PAGE and transferred to the polyvinylidene difluoride membrane. LC3-II was first probed by the rabbit anti-LC3 antibody (Novus, Littleton, USA) and followed by HRP-conjugated goat anti-rabbit IgG antibody (Jackson ImmunoResearch, West Grove, USA). For the detection of β-actin, mouse anti-β-actin antibody (Novus, Littleton, USA) and HRP-conjugated goat anti-mouse IgG antibody (Jackson ImmunoResearch, West Grove, USA) was used. The signals were captured with imaging system and semi-quantified by analyzing the signal density using ImageJ 1.46 (U.S. National Institutes of Health, Bethesda, USA) (Schneider et al., 2012).

For the evaluation of the involvement of autophagy in the effect of the 3C protein, similarly, Fcwf-3cHis cells were treated with either 3-MA or rapamycin and cultured for 2 h. Subsequently, the culture supernatant was removed and the cells were infected with FCoV NTU156 at an MOI of 0.1. Subsequently, the inoculum was removed and replaced with fresh DMEM containing 2% FBS. The culture supernatant was then harvested after 12 h of cultivation and the viral titer was determined.

### 2.8. Statistical analysis

To test the correlation between the mutation of the 3c gene and the occurrence of FIP, Fisher's exact test was performed and $p$ values <0.05 were considered to be significantly correlated. The data from the cells transfected with different plasmids are presented as the mean ± standard deviation. Student's $t$-test was used for comparisons between 3c-expressing and non-expressing groups. $p$ values <0.05 were considered to be statistically significant.

## 3. Results

### 3.1. Integrity of the 3c gene of FCoV from FIP and asymptomatic cats

To confirm the correlation between the integrity of the 3c gene and occurrence of FIP in our local naturally infected cats, 93 rectal