Feline infectious peritonitis virus with a large deletion in the 5’-terminal region of the spike gene retains its virulence for cats

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In this study, the Japanese strain of type I feline infectious peritonitis virus (FIPV), C3663, was found to have a large deletion of 735 bp within the gene encoding the spike (S) protein, with a deduced loss of 245 aa of the N-terminal region of the S protein. This deletion is similar to that observed in porcine respiratory coronavirus (PRCoV) when compared to transmissible gastroenteritis virus, which correlates with reduced virulence. By analogy to PRCoV, we expected that the pathogenicity of C3663 may be attenuated in cats. However, two of four cats inoculated with C3663 died of FIP, and a third C3663-inoculated cat showed FIP lesions at 91 days after challenge. These results indicate that the 5′-terminal region of the S gene is not essential for the development of FIP.

Feline infectious peritonitis (FIP) is a progressive, systemic fatal disease in domestic and wild felids. The causative agent of FIP is feline coronavirus (FCoV) belonging to the order Nidovirales, family Coronaviridae, subfamily Coronavirinae, genus Alphacoronavirus, species Alphacoronavirus 1. Other members of this species include canine coronavirus (CCoV), transmissible gastroenteritis virus (TGEV) and porcine respiratory coronavirus (PRCoV).

FCoVs are classified into two biotypes based on their pathogenicity in cats. One is feline enteric coronavirus (FECV); the second is feline infectious peritonitis virus (FIPV). FECV infection is asymptomatic in cats, occasionally causing enteritis in kittens, but FIPV infection causes severe and lethal disease in cats. However, there is no clear marker to distinguish between FIPVs and FECVs (Pedersen et al., 1981; Pedersen, 1987; Kennedy et al., 2001).

FIPVs and FECVs are antigenically divided into two types (I and II) based on their reactivity with mAbs raised against the spike (S) protein (Fiscus & Teramoto, 1987; Hohdatsu et al., 1991b, 1992). This distinction relates to differences in the nucleotide sequence of the gene encoding the S protein (Motokawa et al., 1996; Herrewegh et al., 1998). There are also some differences in the biological characteristics between types I and II. Type II can grow well in vitro, while type I exhibits poor growth in cell culture (Pedersen et al., 1984). As a result, it has been difficult to isolate type I FIPVs from cats exhibiting FIP. In Japan, only three strains of type I FIPV (C3663, KU-2 and Yayoi) have been isolated severe and lethal disease in cats. However, there is no clear marker to distinguish between FIPVs and FECVs (Pedersen et al., 1981; Pedersen, 1987; Kennedy et al., 2001).

The GenBank/EMBL/DDBJ accession number for the nucleotide sequence of C3663 is AB535528.
(Hayashi et al., 1981; Hohdatsu et al., 1991b; Mochizuki et al., 1997). In this study, we describe the genetics and pathogenesis of a recent Japanese type I FIPV isolate (C3663).

Type I FIPV strains, C3663 and Yayoi, were propagated in Felis catus whole fetus (fcwf)-4 cells (Jacobse-Geels & Horzinek, 1983) as described previously (Shiba et al., 2007). C3663 was isolated from a cat with FIP from Kagoshima in 1994 (Mochizuki et al., 1997) and was classified as a type I FIPV by using FCoV type-specific mAbs, which were kindly provided by Dr Hohdatsu (Hohdatsu et al., 1991a, b).

To characterize the 3′-terminal region of the C3663 genome, RT-PCR was carried out on RNA extracted from C3663-infected fcwf-4 cells. Fcwf-4 cells grown in 35 mm dishes (Sumitomo Bakelite) were inoculated with C3663 and incubated at 37 °C until cytopathic effect was observed. RNA was isolated from the infected cells using the RNaseasy Mini kit (Qiagen) and cDNA was reverse-transcribed using the oligo(dT) M4 primer and TaKaRa RNA LA PCR kit (AMV) version 1.1. Reactions were carried out at 30 °C for 10 min, 42 °C for 30 min and 70 °C for 15 min, using a Little Gene cycler (Toyobo). PCR amplification of subgenomic RNA was performed by using the forward primer 52F (5′-ACTAGCCTTGTGCTAGATT-3′) with one of the following reverse primers: 24991R (5′-TCACCAAAACCTATACACAC-3′), 26218R (5′-CTTCATTTTGTGTAGTTCAAAC-3′), M-R (5′-TAA-GCCCATCCTGTAGCAGT-3′), NR (5′-TAATAAAATACAAGCGTGGAGGAAAAC-3′) or M4 (5′-GTTTTCCAGTTCACGAC-3′). Each amplification was performed using an initial denaturation at 94 °C for 2 min, followed by 40 cycles at 94 °C for 30 s, 55 °C for 30 s, 72 °C for 2–5 min and a final extension at 72 °C for 10 min. PCR products were cloned using the TOPO-TA cloning kit (Invitrogen) according to the manufacturer’s instructions. Plasmid DNA containing the C3663 strain genes was purified using the QIAprep Spin Miniprep kit (Qiagen) for sequencing. Sequencing was performed by using BigDye Terminator v3.1 Cycle Sequencing kit (Applied Biosystems) and the results were analysed using an ABI Prism 310 Genetic Analyzer (Applied Biosystems). Sequences were deposited in the DNA Data Bank of Japan as accession no. AB535528.

Sequence analysis and alignments for a total of 8245 bp of C3663 were used to identify the following genes: S

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**Fig. 1.** Confirmation of the S gene deletion in the Japanese strain of type I FIPV C3663. (a) Schematic of the S genes of Yayoi and C3663. The box framed by a dotted line shows the deletion (735 bp) in the S gene of C3663 compared with Yayoi (GenBank accession no. AB695067). Arrowheads indicate the position and orientation of the primers, Yayoi S 46F and Yayoi S 1058R, used to confirm the deletion. (b) Confirmation of the deletion by RT-PCR using Yayoi S 46F and Yayoi S 1058R. (c) Phylogenetic tree using amino acid sequences of the S proteins excluding the distinct N-terminal domains. The tree was constructed using MEGA5. GenBank accession numbers of the sequences used are AB695067 (Yayoi), DQ848678 (C1Je), DQ160294 (NTU2/R/2003), DQ010921 (79-1146), X80799 (79-1683), AY307021 (23/03), D13096 (INSVC-1), DQ811789 (TGEV-Purdue), DQ811785 (TGEV-Miller M6) and Z24675 (PRCoV-RM4).
(3669 bp; encoding a 1222 residue protein), ORF3a (213 bp; encoding a 70 residue protein), ORF3b (222 bp; encoding a 73 residue protein), ORF3c (714 bp; encoding a 237 residue protein), envelope (E) (249 bp; encoding a 82 residue protein), membrane (M) (786 bp; encoding a 261 residue protein), nucleocapsid (N) (1131 bp; encoding a 376 residue protein), ORF7a (306 bp; encoding a 101 residue protein) and ORF7b (621 bp; encoding a 206 residue protein). Interestingly, the alignment of the S genes indicated that C3663 has a large deletion of 735 bp (capable of encoding 245 aa) at the 5′ terminus of the S gene (Fig. 1a). To confirm this deletion, RT-PCR was performed on RNA from C3663- or Yayoi-infected cells by using the forward primer Yayoi S 46F (5′-GATGCTC-CTCATGGTGTTAC-3′) and the reverse primer Yayoi S 1058R (5′-CTCAAAACATCTGCCGTGAC-3′) that span the corresponding domain of the S gene. PCR products of 278 and 1013 bp were obtained from C3663- and Yayoi-infected cells, respectively (Fig. 1b).

A phylogenetic tree was constructed for the S proteins from the deduced amino acid sequences (residues 278–1467) using MEGA5 software (Tamura et al., 2011). The result showed that C3663 is a type I FCoV (Fig. 1c). The S protein of C3663 exhibited 89–93% aa identity to those of other type I FCoVs and a much lower identity (<50%) to those of type II FCoVs or type II CCoVs.

The pathogenicity of C3663 was investigated by infecting four specific-pathogen-free (SPF) cats (male, 6 months old; Liberty Research) intra-orally with 10 ml of a viral solution containing 3.9 × 10⁶ p.f.u. of C3663 per cat (nos 1–4). Clinical signs, body weight and temperature were recorded daily. Weekly blood samples were collected under anaesthesia with ketamine (Daiichi Sankyo). Serum amyloid A (SAA) was measured by Mitsubishi Chemical Medience. Cat sera were stored at −80 °C until use in virus-neutralization (VN) tests and viral RNA quantification. All animal experiments were approved by the Ethics Committee for Animal Experiments, Faculty of Agriculture, Yamaguchi University.

One cat (no. 2) was found dead on post-inoculation day (PID) 21 and a second cat (no. 3) was euthanized because of severe clinical signs on PID 37. The two remaining cats (nos 1 and 4) survived until PID 91 (the end of the observation period), at which time both were euthanized. At necropsy, one of the surviving cats (no. 1) exhibited FIP lesions but cat no. 4 did not show any lesions. All cats showed clinical signs after inoculation. Anorexia was observed in three cats (nos 2, 3 and 4) and vomiting in two cats (nos 3 and 4). Lethargy and weight loss were observed in two cats (nos 2 and 3) (Fig. 2a). Dyspnoea was observed in one cat (no. 2) on PID 20 and 21. In cat no. 3, jaundice was observed on PID 33–37 and melena on PID 35 and 37. Furthermore, the concentration of SAA increased in all cats (Fig. 2b). Cat nos 2, 3 and 4 showed a rapid increase during the acute phase. Cat no.1 showed intermittent increased levels of SAA and, despite a lack of clinical signs, exhibited a high concentration of 49.6 µg ml⁻¹ at the end of our observation period. In all cats, viral RNA was detected in sera and VN activity to C3663 was observed (data not shown).

Following post-mortem examination, no lesions were found in cat no. 4. However, cat nos 1 and 2 showed pleural effusion and pyogranulomatous lesions in the pleural cavities. Pleural effusion and ascites were observed in cat no. 3. Lesions were seen in the kidneys, liver, stomach, intestine, pancreas, diaphragm and lung by

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**Fig. 2.** (a) Normalized body weight among cats following inoculation with FIPV-C3663. Body weights were normalized using the weight at post-inoculation day (PID) 0 as 100%. (b) Concentration of SAA in cat sera. The limit of detection of SAA is <2.5 µg ml⁻¹.
macrophathy, and confirmed as being pyogranulomatous by histopathological examination.

In this study, it was found that C3663 has a large (735 bp) deletion in the 5′ terminus of the S gene. While C3663 has been adapted to propagation in tissue culture and the deletion may therefore have occurred in vitro, there is in fact evidence for naturally occurring FCoVs with similar deletions. The type I FCoV field variants UU16 (GenBank accession no. FJ938058) and UU21 (GenBank accession no. HQ012369) have deletions of 705 and 792 bp, respectively, in the 5′-terminal region of their S genes, whereas field variant UU3 (GenBank accession no. FJ938061) has a small 126 bp deletion. It therefore appears that C3663-like FIPVs are present and maintained under field conditions.

To examine the pathogenicity of C3663, four SPF cats were intra-orally inoculated with C3663. Three of four SPF cats exhibited typical FIP during the observation period and two died within 1 month of inoculation. Adaptation of FIPV to propagation in tissue culture often results in a loss of pathogenicity (Pedersen & Black, 1983; Pedersen & Floyd, 1985; Christianson et al., 1989; Kiss et al., 2004). Conceivably, the deletion in the S gene of C3663 might well have resulted in virus attenuation. In fact, a naturally occurring mutant of TGEV, PRCoV suffered a similar deletion in the S gene (Wesley et al., 1991), which caused a loss of virulence and a change in tissue tropism. PRCoV exhibits reduced sialic acid binding and haemagglutination activity (Schultzze et al., 1996). It replicates efficiently in the respiratory tract, but, different from TGEV, does not replicate in the small intestine (Cox et al., 1990). In contrast, we demonstrate that FIPV C3663 is highly virulent. Our findings unequivocally show that large deletions in the 5′-terminal region of the FIPV S gene are tolerated without loss of pathogenicity.

In conclusion, we succeeded in efficiently inducing FIP in cats by inoculation with tissue culture adapted type I FIPV, C3663, using a natural route of infection (oral inoculation). Furthermore, we confirmed that the 5′ terminus of the S gene is not essential for the development of FIP.

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References


