Occurence of Feline Coronavirus and Feline Infectious Peritonitis Virus in Thailand

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ABSTRACT

Feline infectious peritonitis virus (FIPV), a mutant of feline coronavirus (FCoV), is a member of the family *Coronaviridae*. FIPV induces an Arthus-type immune response and causes feline infectious peritonitis (FIP). To date, there has been no scientific report of FCoV and FIPV in Thailand, although cats with clinical signs suggesting FIP have been sporadically observed. In this study, 190 serum and body fluid samples were collected from solitary cats and multi-cat households residing in the central and eastern parts of Thailand. Nine out of 174 serum samples and 12 out of 13 abdominal or thoracic fluid samples were from cats with clinical signs suggesting FIP. In addition, three thoracic fluid samples were obtained from cats that visited a small animal hospital in Thailand. Detection of FCoV and FIPV RNAs was carried out on 184 samples using recently developed nested reverse transcriptase-polymerase chain reactions (RT-nPCR). The results demonstrated that 57 of 184 (30.97%) and 3 of 184 (1.63%) samples contained FCoV and FIPV RNAs, respectively. In addition, a parallel study tested for the presence of the antibodies to FCoV using a dot blot ELISA with the same samples. The results indicated that 55 out of 98 (56.12%) cats had antibodies to the FCoV. This was the first report demonstrating the occurrence of FCoV and FIPV in Thailand.

Key words: feline infectious peritonitis virus**,** feline coronavirus**,** RT-nPCRs, Thailand

INTRODUCTION

Feline infectious peritonitis virus (FIPV), a mutant of feline enteric coronavirus (FECV), infects domestic and wild felines of all species (Horzinek and Osterhaus, 1979; Poland *et al.*, 1996). FIPV causes a fatal systemic disease in cats called feline infectious peritonitis (FIP). The disease is characterized by an Arthus-type immune response. FIP can be divided into two major forms: effusive and non effusive (Pedersen, 1995). The most common clinical signs are non-specific including: fluctuating fever, inappetence, lethargy and weight loss. FIPV and FECV are examples of feline coronavirus (FCoV) of the family *Coronaviridae* (Lai and Holmes, 2001). The FCoV genome is a single-stranded RNA consisting of five major open reading frames (ORFs) which encode polymerase (*pol*), peplomer (S), envelope (E), matrix (M) and nucleocapsid (N) gene

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products, respectively (Hohdatsu *et al*., 1998; Rottier, 1999). The S gene is divided into S1 and S2 regions. The S1 region is more variable, containing various degrees of deletion and substitutions in different coronavirus strains or isolates (Pedersen *et al*., 1981).

FCoV is classified into two serotypes according to its antigenicity of the peplomer glycoprotein. Both serotypes I and II contained both virulent (FIPV) and non-virulent (FECV) isolates (Ficus *et al*., 1987). FCoV serotype I is predominant in Europe, Japan and USA whereas serotype II is rarely found (Hohdatsu *et al*., 1992; Rottier, 1999; Kennedy *et al*., 2002). The serotype II virus is closely related to canine coronavirus (CCoV) (Ficus *et al*., 1987) and may emerge from a double recombination between the FCoV serotype I and CCoV. The first recombination occurs in the E and M genes and the other presents in the *pol* gene. Thus, CCoV sera can neutralize FCoV serotype II but weakly neutralize serotype I (Horzinek and Herrewegh, 1995; Herrewegh *et al*., 1998). Both FECV and FIPV are very closely related so that they are indistinguishable by serological methods (Pedersen, 1976; Pedersen *et al*., 1981).

FIPV infection in cats has been reported in European countries (Pedersen, 1995; Cave *et al*., 2004; Holst *et al*., 2006), Australia (Bell *et al*., 2006), the United State of America (Kennedy *et al*., 2002) and Japan (Hohdatsu *et al*., 1992). Serological studies of FCoV in European countries showed that 25% of household cats and up to 80 to 90% of cats in multi-cat households and catteries had FCoV antibodies (Pedersen, 1995). The prevalence of antibodies against FCoV was 31% in Swedish cats (Holst *et al*., 2006) and 25.6% in British cats (Cave *et al*., 2004). Similarly, a serological survey of antibodies to FCoV in cats in Sydney, Australia indicated 34% were seropositive (Bell *et al*., 2006). To date in Thailand, no scientific report of FCoV including FIPV has been documented. Thus, the objective of this study was to survey the occurrence of FCoV and FIPV in central and eastern Thailand using RT-nPCR and a dot blot ELISA.

MATERIALS AND METHODS

Sampling

During May to September 2003, 174 serum and 13 abdominal and thoracic fluid samples were randomly collected from both solitary- and multi-cat households in central and eastern Thailand including Bangkok, Pathum Thani, Nakhon Pathom, Ratchaburi, Suphan Buri, Samut Sakhon and Chon Buri. One-hundred and two samples were from animals living in multi-cat households. The samples were from cats whose age ranged from one month to 18 years. The majority of samples was collected from 164 domestic short-hair breeds (including 2 Siamese, 1 Korat and 1 Kaomanee) and 10 Persian cats. Nine of the 174 serum samples and 12 of the 13 thoracic and abdominal fluid samples were from cats with clinical signs suggesting FIP (Table 1). In addition, thoracic fluids were collected from three sick cats that visited the Small Animal Teaching Hospital at Kasetsart University. Two of the cats had FIP-positive signs, while the third cat did not match the clinical criteria. The two FIP-suspected cats showed clinical signs of abdominal and/or pleural fluid, uveitis and icterus. Additionally, laboratory findings of these cats included hyperproteinemia, hypergammaglobulinemia and an albumin to globulin ratio of less than 0.4.

Table 1   Number of samples separated into healthy and FIP-suspected cats.

	Serum	Thoracic/abdominal fluids	Purebred cats
Healthy cats	165	1	14
FIP suspected cats	9	12	-

The FCoV and FIPV reference strains were obtained from commercially available, modified-live intranasal FIP vaccine (Premucell FIP®).

RT-nPCR

RNA was isolated from 184 samples while 98 serum samples were examined for the presence of antibodies to FCoV. Ninety-two samples were detected by both assays. The remaining samples were not tested by both assays because of limited sample amounts. Viral RNA was extracted from the sera and effusive fluids of the cats, using the RNeasy Total RNA Kit (Qiagen GmbH Germany) according to the manufacturer's instructions. The cDNA was synthesized in a 20 µl total reaction volume containing 7 µl of RNA, 10XRT buffer (37.5 mM KCl, 25.0 mM Tri-HCl, pH 8.3), 5 mM MgCl$_2$, 10 mM of each dNTP, 0.01 M DTT, 0.5 µg random primer, 20U RNaseOUT Recombinant RNase Inhibitor (Invitrogen), 50 U SuperScript™ II RT (Invitrogen). The RNA was denatured at 70°C for 10 min. The cDNA was synthesized at 42°C for 50 min followed by incubation at 70°C for 15 min.

Two sets of four primers reported previously (Herrewegh *et al.*, 1995; Gamble *et al.*, 1997) were exploited in two nPCR assays for the detection of FCoV and FIPV nucleic acids. Firstly, the primers for FCoV nPCR are targeted to the 3′-untranslated region (3′-UTR) of the viral genome, which are 97 to 100% homologous among FCoV isolates (Herrewegh *et al.*, 1995). The sequences of the outer primers are FIP205 5′-GGCAA CCCGATGTTTAAAACTGG-3′ located between nucleotides 1 and 23 and FIP211 5′-CACTAGAT CCAGACGTTAGCTC-3′ spanning nucleotides 211 to 192. The internal primers include FIPg276 5′-CCGAGGAATTACTGGTCA TCGCG-3′ located from nucleotides 29 to 51 and FIPg204 5′-GCTCTTCCATTGTTGGCTCG TC-3′, which is between nucleotides 205 to 184.

Secondly, the target sequences for the FIPV nPCR are in the 5′ UTR and S1 region of the S gene (Gamble *et al.*, 1997). These primers consist of outer primers: FIP251 5′-CTACAGA GGTGTGGTACAAC-3′ located between nucleotides 251 and 271 and FIP621 5′-TTCCACTCAAGACCATAGAT-3′ spanning nucleotides 621 to 601. The internal primers include FIPs361 5′-GGTAATGCACGTGGT AAACC-3′ located from nucleotides 361 to 381 and FIPs530 5′-CACTGGTTGGAGGTGAA TTG-3′, which are between nucleotides 530 to 510. The primers have sufficient sequence homology among FIPV strains including serotype I and II and are able to differentiate FIPV from FECV presenting in body fluids (Gamble *et al.*, 1997).

In both nPCRs, 20 µl of the RT reaction mixture was added to 80 µl of the PCR mixture containing 10XPCR buffer (50.0 mM KCl, 10.0 mM Tris-HCl, pH 8.3), 2 mM MgCl$_2$, 5 mM each dNTP, 5 pmol of each outer primer and 1 U of Taq DNA polymerase (Invitrogen). The temperature cycling protocol consisted of 94°C for 10 min and 35 cycles of 94°C for 30 seconds, 53°C for 30 seconds and 72°C for 30 seconds. A second round of amplification used 4 µl of the first amplification reaction mixture with the nested pair of primers in a 100 µl reaction volume using similar conditions and a similar cycling procedure. The modified live FIP vaccine was processed similarly to the samples and served as positive control for both RNA isolation and RT-nPCR.

Detection of antibodies to FCoV

Antibodies against FCoV were detected by a dot blot ELISA, using the Immuno Comb® FCoV Antibody Test Kit (Biogal Galed Laboratories, Israel). Briefly, 5 µl of each serum sample were allowed to incubate with the diluents in the well for 60 min. Then, the comb containing spotted antigen was incubated with the diluted serum for 10 min. The comb was washed once in the next well for 2 min prior to incubation with secondary antibodies for 10 min. The color was developed in the well containing substrates for 10 min. The color was fixed and compared to the comb scale.
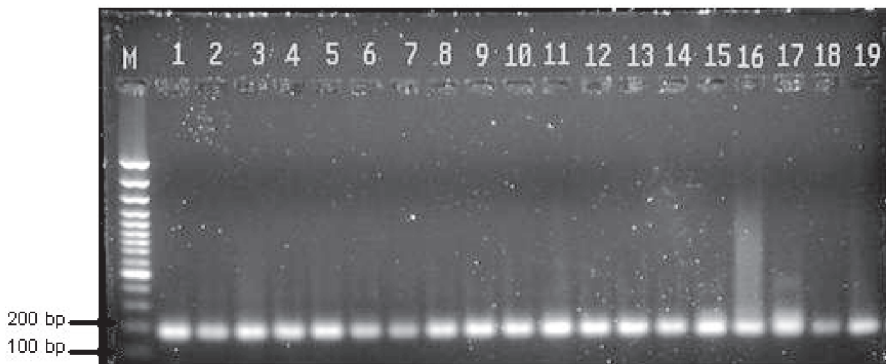
RESULTS

The proportion of cats harboring FCoV and/or FIPV was determined from the presence of either viral nucleic acids or antibodies to the virus. The results showed that the 177 basepair RT-nPCR product, specific for FCoV (Figure 1) was present in 57 out of 184 (30.97%) samples. The RT-nPCR product specific to FIPV is the 170 basepair (Figure 2) and was detected in three samples (1.63%) (Table 2), including the effusive fluids from the two cats clinically diagnosed as FIP. However, another fluid sample from a cat suffering from other diseases was negative. Three samples containing FIPV RNA were also positive for FCoV. The FCoV RT-nPCR products from the two FIP cats were sequenced using the dideoxynucleotide termination method. The results showed that they were 97% identical to the 3-UTR sequences of FCoV and FIPV deposited in the GenBank database (Accession # EU186072). The sequencing results confirmed that the amplified PCR products were FCoV cDNA.

In addition, the serum samples were tested for the presence of FCoV antibodies in a dot blot ELISA technique using the Immunocomb FCoV Antibody Test Kit (Biogal Galed Laboratories, Israel). Six out of 104 samples were invalid since the dark blue color did not develop in the internal control slot. Therefore, 98 samples were determined for the presence of FCoV antibodies. The results demonstrated that antibodies to FCoV were detected in 55 of 98 sera (56.12%). Numbers of FCoV-seropositive cats were equally distributed between males and females. The majority of the sera were from mixed-breed cats living together in big groups.
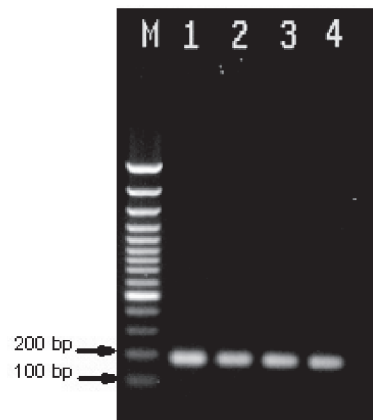
Figure 2 Electrophoresis photograph of PCR products from FIPV RT-nPCR positive samples. M represents the 100 bp DNA ladder. Lane 1 is FIPV vaccine strain and lanes 2-4 are samples positive for FIPV RT-nPCR.

Figure 1 Electrophoresis photograph of PCR products from FCoV RT-nPCR positive samples. M represents the 100 bp DNA ladder. Lane 1 is the FIPV vaccine strain and lanes 2-19 are examples of FCoV RT-nPCR positive samples.

The results examined by FCoV RT-nPCR and dot blot ELISA were compared (Table 3). Out of 92 samples, 14 sera (15.2%) contained both FCoV RNAs and antibodies against FCoV. Twenty-seven cats (29.3%) were negative for both FCoV RNAs and its antibodies. Thirty-six out of 92 cats (39.1%) contained antibodies to FCoV but not FCoV RNA. This suggested that these cats might have been previously exposed to FCoV. FCoV RNAs were detected in the sera of 15 healthy cats (16.3%) without the presence of antibodies to FCoV. It was possible that these cats were infected with FCoV and were viremia when sampled.

DISCUSSION

This is the first report of the occurrence of FCoV and FIPV infection in cats in Thailand. The prevalence of FCoV found by RT-nPCR was in accordance with various studies that used serological methods to detect antibodies to FCoV (Pedersen, 1995; Cave *et al.*, 2004; Bell *et al.*, 2006; Holst *et al.*, 2006). The previous studies revealed that the number of cats with anti-FCoV antibodies was higher in purebred cats than in mixed-breed cats and in multi- rather than single-cat households (Cave *et al.*, 2004; Bell *et al.*, 2006; Holst *et al.*, 2006). However, the sero-prevalence of FCoV infection reported in this study is common for multiple-cat households. The current study confirmed that cats living in big groups may promote FCoV transmission. The fecal-oral route of transmission is a possible mean of infection since these cats utilized similar litter areas. The results of one study of cats with different breeds, management, sex, health and age (one month to eighteen years) (Pesteanu-Somogyi *et al.*, 2006), suggested that purebred young cats from three months to three years of age were at high risk of developing FIP.

The comparison between RT-nPCR and the dot blot ELISA demonstrated that there was no correlation in both assays due to different target detection. The discrepancy between serology and nucleic acid detection has been previously documented (Kennedy *et al.*, 2002 and 2003). For example, a survey of the prevalence of FCoV from 75 samples in the USA using immunofluorescence assay found that the prevalence of FCoV detected by RT-PCR was 24 (32.0%), of which 13 (17.33%) samples were seronegative to FCoV serotype I and serotype II. In addition, 20 (26.66%) seropositive samples tested negative for FCoV RT-PCR. The

Table 2 Proportion of cats from central and eastern Thailand that were positive for FCoV and FIPV RNAs, as tested by RT-nPCR or antibodies to FCoV, as examined by dot blot ELISA.

Number of samples / Assays	Positive (%)	Negative	Total
FIPV nPCR	3 (1.63%)	181	184
FCoV nPCR	57 (30.97%)	127	184
ELISA	55 (56.12%)	43	98

Table 3 Relationship between detection of FCoV RNA by RT-nPCR and detection of antibodies to FCoV by ImmunoComb.

FCoV- nPCR / Immuno-Comb	Negative	Positive	Total
Negative	27	15	42
Positive	36	14	50
Total	63	29	92

results showed that the serological method may not correlate with viremia or virus shedding since ELISA detects the appearance of antibodies but RT-PCR detects the presence of viral nucleic acids. The higher prevalence observed by the serological method compared with the absence of viral nucleic acids determined by RT-nPCR suggests the presence of coronavirus antibodies in non-viremic cats (Kennedy *et al.*, 2002). Cats that are serological-positive represent cats previously exposed to the coronavirus, which may not necessarily shed or carry FCoV. A seropositive test result only indicates that a cat has been previously infected with FCoV prior to taking the serological test (Kennedy *et al.*, 2002).

Thus far, there is no individual test or criteria that can distinguish FIPV from other feline coronaviruses (Addie *et al.*, 2004). RT-nPCR may be a better diagnostic tool. One of the studies showed that the FIPV RT-nPCR possessed sensitivity and specificity as high as 91.6% and 94%, respectively, when the tested samples were from FIP cats confirmed by protein electrophoresis and histopathology (Gamble *et al.*, 1997). FIPV, but not FECV, causes abnormal body fluids such as abdominal and thoracic fluids. Therefore, the FCoV detected in these samples by RT-nPCR is most likely to be FIPV. In the current study, FIPV RT-nPCR may have been less sensitive when compared with FCoV RT-nPCR, due to various factors including variation within the S gene. The selected primers were reported to detect both serotypes of FCoV (Gamble *et al.*, 1997); however, nucleotide comparison of the primers and the FIPV-S gene sequences revealed that these primers have high homology to the serotype II, strain 11-1146, but not the serotype I, such as strains Black and UDC1. Therefore, the low prevalence of FIPV in this study may in part have resulted from the limitation of the primers used in the FIPV RT-nPCR assay. New primers specific to the serotype I of FIPV should be considered for further study to reveal the true prevalence of FIPV in Thailand.

CONCLUSION

This is the first report of the appearance of both FCoV and FIPV in Thailand. The results from this study indicated that FCoV RT-nPCR is likely to be the test of choice for FIPV diagnosis of samples from body fluids. The contradiction between the RT-nPCR and serological assays suggests that clinical laboratory and physical findings are also indispensable for definitive diagnosis, especially for the non-effusive form of FIP.

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LITERATURE CITED

Addie, D. D., S. Paltrinieri and N. C. Pedersen. 2004. Recommendations from workshops of the second international feline coronavirus/ feline infectious peritonitis symposium. **J. Feline Med. Sur.** 6: 125-130.

Bell, E. T., J. A. Toribio, J. D. White, R. Malik and J. M. Norris. 2006. Seroprevalence study of feline coronavirus in owned and feral cats in Sydney, Australia. **Aust. Vet. J.** 84(3): 74-81.

Cave, T. A., M. C. Golder, J. Simpson and A. D. Addie. 2004. Risk factors for feline coronavirus seropositivity in cats relinquished to a UK rescue charity. **J.Feline Med. Sur.** 6: 53-58.

Ficus, S. A., B. L. Rivoire and Y. A. Teramoto. 1987. Epitope-Sepecific antibody responses to virulent and avirulent feline infectious peritonitis virus isolates. **J. Clin. Microbiol.** 25: 1529-1534.

Gamble, D. A., A. Lobbiani, M. Gramegna, L. M. Moore and G. Colucci. 1997. Development of a nested PCR assay for detection of feline infectious peritonitis virus in clinical specimens. **J. Clin. Microbiol.** 35: 673-675.

Herrewegh, A.P., I. Smeenk, M. C. Horzinek, P. J. Rottier and R. J. Groot. 1998. Feline coronavirus type II strain 79-1683 and 79-1146 originate from a double recombination between feline coronavirus type I and canine coronavirus. **J.Virol.** 72: 4508-4514.

Herrewegh, A. P., R. J. Groot, A. Cerpica, H. F. Egberink, M. C. Horzinek and P.J. Rottier. 1995. Detection of feline coronavirus RNA in feces, tissues, and body fluids of naturally infected cats by reverse transcriptase PCR. **J. Clin. Microbiol.** 33: 684-689.

Hohdatsu, T., M. Yamada, R. Tominaga, K. Makino, K. Kida and H.Koyama. 1998. Antibody-dependent enhancement of feline infectious peritonitis virus infection in feline alveolar macrophages and human monocyte cell line U937 by serum of cats experimentally or naturally infected with feline coronavirus. **J. Vet. Med. Sci.** 60: 49-55.

Hohdatsu, T., S. Okada, Y. Ishizuka, H. Yamada and H. Koyama. 1992. The prevalence of type I and II feline coronavirus infections in cats. **J. Vet. Med. Sci.** 3: 557-562.

Holst, B. S., L. Englund, S. Palacios, L. Renstrom and L. T. Berndtsson. 2006. Prevalence of antibodies against feline coronavirus and *Chlamydophila felis* in Swedish cats. **J. Feline Med. Sur.** 8: 207-211.

Horzinek, M. C. and A. Herrewegh. 1995. Perspectives on feline coronavirus evolution. **Feline Pract.** 23: 34-39.

Horzinek, M. C. and A. D. Osterhaus. 1979. The virology and pathogenesis of feline infectious peritonitis. **Arch. Virol.** 59: 1-15.

Kennedy, M., S. Citino, A. H. McNabb, A. S. Moffatt, K. Gertz and S. Kania. 2002. Detection of feline coronavirus in captive felidae in the USA. **J. Vet. Diagn. Invest.** 14(6): 520-522.

Kennedy, M., S. Kania, E. Stylianides, H. Bertschinger, D. Keet and M. van Vuuren. 2003. Detection of feline coronavirus infection in southern African nondomestic felids. **J. Wildl. Dis.** 39(3): 529-35.

Lai, M. C. and K. V. Holmes. 2001. *Coronaviridae:* The viruses and their replication, pp. 1163-1185. *In* D. M. Knipe, P. M. Howley, D. E. Griffin, R. A. Lamb and M. A. Martin (eds.). **Fields Virology.** 4th ed. Lippincott Williams and Wilkins. Philadelphia.

Pedersen, N. C. 1976. Serologic studies of naturally occurring feline infectious peritonitis. **Am. J. Vet. Res.** 37: 1449-1453.

Pedersen, N. C. 1995. An overview of feline enteric coronavirus and infectious peritonitis virus infection. **Feline Pract.** 23: 7-12.

Pedersen, N. C., J. F. Boyle, K. Floyd, A. Fudge and J. Barker. 1981. An enteric coronavirus infection of cats, and its relationship to feline infectious peritonitis. **Am. J. Vet. Res.** 42: 368-377.

Pesteanu-Somogyi, L. D., C. Radzai and B. M. Pressler. 2006. Prevalence of feline infectious peritonitis in specific cat breeds. **J. Feline Med. Sur.** 8: 1-5.

Poland, A. M., H. Vennema, J. E. Foley and N. C. Pedersen. 1996. Two related strains of feline infectious peritonitis virus isolated from immunocompromised cats infected with a feline enteric coronavirus. **J. Clin. Microbiol.** 34: 3180-3184.

Rottier, P. J. 1999. The molecular dynamics of feline coronaviruses. **Vet. Microbiol.** 69: 117-125.