Original Article

Molecular Detection and Genotype Differentiation of Feline Coronavirus Isolates from Clinical Specimens in Thailand

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Abstract

Feline coronavirus (FCoV) infection manifests a wide magnitude of clinical symptoms from nonpathogenic mild enteric infection to pathogenic fatal feline infectious peritonitis (FIP). Based on the *in vitro* properties and antigenic relationship to canine coronavirus (CCoV), FCoVs are classified into type I and II with different continent preference. To investigate the incidence of FCoV infection among cat populations in Thailand during 2010-2011, clinical specimens (103 bodily fluids from 95 FIP clinically suspected cats and 17 feces from 7 healthy cats living in groups and 10 healthy cats living singly) were analyzed by amplification of the 3′UTR gene. Among them, 46% (47/103), 100% (7/7) and 50% (5/10) were positive, respectively. After that, the amplification of the 3′ end of S gene was performed to differentiate the genotypes of FCoV. Among the 3′UTR positive cats, genotype I predominated with the percentage of 29-60%. Genotype II was 15-29% and mixed genotype was 0-13%. Phylogenetic analysis of S gene revealed that type I FCoV were more genetically divergent (80-100%) than type II FCoV (100%) and closely related to Malaysia and Taiwan isolates based on 3′UTR analysis. Taken together, the 3′UTR RT-PCR could be applicable to confirm FIP infection in addition with the S RT-PCR to differentiate its genotype. Feces is a suitable specimen for monitoring the FCoV carrier condition in healthy cat.

Keywords: clinical specimen, feline coronavirus, genotype, phylogenetic analysis, Thailand

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บทคัดย่อ

การตรวจทางชีวโมเลกุลและจำแนกจีโนไทป์เชื้อโคโรนาไวรัสในแมวที่แยกได้จากสิ่งส่งตรวจทางคลินิกในประเทศไทย

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อาการในแมวที่ติดเชื้อโคโรนาไวรัส (FCoV) สามารถแสดงอาการได้หลากหลายตั้งแต่ไม่แสดงอาการ ท้องเสียอย่างอ่อน จนถึงเป็นโรคเยื่อบุช่องท้องอักเสบติดต่อ เชื้อโคโรนาไวรัสในแมวสามารถจำแนกได้เป็น 2 จีโนไทป์ (I และ II) ตามคุณสมบัติที่พบในห้องปฏิบัติการและความสัมพันธ์กับแอนติเจนของเชื้อโคโรนาไวรัสในสุนัข (CCoV) ซึ่งพบอุบัติการณ์แตกต่างกันตามแต่ละภูมิภาค การศึกษาครั้งนี้มีวัตถุประสงค์เพื่อสำรวจความชุกของการติดเชื้อโคโรนาไวรัสในประชากรแมวในประเทศไทยในช่วงปี พ.ศ. 2553-2554 ทำการวิเคราะห์ตัวอย่างของเหลวจากช่องว่างในร่างกาย จำนวน 103 ตัวอย่าง (แมวป่วยที่สงสัยว่าเป็น FIP 95 ตัว) และอุจจาระจำนวน 17 ตัวอย่างทั้งจากแมวปกติที่อยู่ตัวเดียว (10 ตัว) และที่อยู่หลายตัว (7 ตัว) ด้วยเทคนิค RT-PCR ต่อจีน 3'-untranslated region (3'UTR) ผลการศึกษาพบว่าให้ผลบวกร้อยละ 46 (47/103), 50 (5/10) และ 100 (7/7) ตามลำดับ หลังจากนั้นทำการทดสอบแยกชนิดจีโนไทป์ด้วยเทคนิค RT-PCR ต่อจีน S พบว่าในกลุ่มแมวที่ให้ผลบวกต่อจีน 3'UTR เป็นจีโนไทป์ชนิด I ร้อยละ 29-60 จีโนไทป์ชนิด II ร้อยละ 15-29 และพบทั้ง 2 จีโนไทป์ร้อยละ 0-13 เมื่อศึกษาเปรียบเทียบความหลากหลายทางพันธุกรรม พบความหลากหลายในจีโนไทป์ I (ร้อยละ 80-100) มากกว่าจีโนไทป์ II (ร้อยละ 100) และมีความใกล้เคียงมากที่สุดกับบางสายพันธุ์ที่แยกได้จากประเทศมาเลเซียและไต้หวัน ดังนั้นจึงสรุปได้ว่า การตรวจด้วยวิธี RT-PCR ต่อจีน 3'UTR สามารถใช้ในการวินิจฉัยยืนยันการเป็นโรคเยื่อบุช่องท้องอักเสบติดต่อ ร่วมกับการตรวจด้วยวิธี RT-PCR ต่อจีน S เพื่อใช้ในการจำแนกจีโนไทป์ นอกจากนี้ อุจจาระยังเป็นตัวอย่างที่มีความเหมาะสมที่จะใช้ในการตรวจหาภาวะพาหะของการนำเชื้อไวรัสโคโรนาในแมวที่ไม่แสดงอาการได้

คำสำคัญ: ตัวอย่างทางคลินิก ไวรัสโคโรนาในแมว จีโนไทป์ การวิเคราะห์แผนภูมิต้นไม้ ประเทศไทย

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Introduction

Feline infectious peritonitis (FIP) is a fatal, immune-mediated, pyogranulomatous disease of domestic and wild cats. This disease is caused by an infection of mutated feline coronavirus (FCoV) belonging to the order Nidovirales and family *Coronaviridae*. In fact, FCoV exists in 2 biological types: feline enteric coronavirus (FECV) and feline infectious peritonitis virus (FIPV). FECV is usually nonpathogenic causing subclinical or mild enteric infection, whereas FIPV is always pathogenic leading to an invariable mortality. Clinical signs following FECV infection generally show a short episode of upper respiratory tract signs, transient and clinically mild diarrhea or vomiting and occasionally of stunted growth in kittens (Addie and Jarrett, 1992). However, all FECV carriers have the potential to develop either enteritis or peritonitis, even though only about 5% of infections develop into FIP.

In domestic cats, the susceptible ages to FIP range from 3 months to 3 years and older than 13 years. Intact males and purebred cats such as Abyssinian and Himalayan have a higher incidence of FIP (Rohrbach et al., 2001). Cats infected with FIPV display 2 distinct clinical forms; effusive (wet) and non-effusive (dry). Approximately, the occurrence of wet form presents as many as three times than that of dry form.

FCoVs are divided into 2 types, I and II, based on their *in vitro* growth ability, neutralization reactivity with S-protein-specific monoclonal antibodies, antigenic relationship and S-gene sequence analysis to canine coronavirus (CCoV) (Hohdatsu et al., 1991; Motokawa et al., 1995; Addie and Jarrett, 2006). The majority of FCoV type I infection is documented in Austria and Japan (Hohdatsu et al., 1992; Posch et al., 2001). Lack of effective treatment strategies due to the accurate ante-mortem diagnosis has frustrated veterinarians for decades since FIP was discovered more than 40 years ago. So far, there is no sensitive and specific diagnostic test for FIP. Veterinarians must make a tentative diagnosis based on the history and clinical

signs which are usually non-specific. Several laboratory methods have been implemented for a more precise FIP diagnosis. Complete blood cell count often shows lymphopenia, neutrophilia, non-regenerative anemia and thrombocytopenia. Hyperglobulinemia, serum albumin:globulin ratio (A:G), or serum protein electrophoresis are also insensitive that they do not help distinguish FIP from other diseases causing hyperglobulinemia (Hartmann, 2005). Alpha-1-acid glycoprotein (AGP) and serum amyloid A (SAA) may be useful as a biomarker in the future since no validated commercial test is currently available for routine evaluation of AGP and SAA levels (Bence et al., 2005; Giordano et al., 2004). Although serology is widely used and assumed to be indicative of ante-mortem FIP diagnosis, results need to be interpreted carefully due to high percentages of healthy cat population are FCoV seropositive and high antibody titers are frequently found in asymptomatic cats. In addition, FECVs and FIPVs remain indistinguishable by serology. A reverse transcriptase polymerase chain reaction (RT-PCR) was developed for the detection of FCoV RNA in feces, tissues, and body fluids of infected cats targeting to the highly conserved 3′-untranslated region (3′UTR) (Herrewegh et al., 1995). This 3′UTR-PCR reaction will help detect apparently healthy FCoV carriers and screen new cats before introducing them into FCoV-free catteries. In addition, the RT-PCR targeting the 3′ end of S gene and using type-specific primer pairs to generate different sizes of PCR products will provide the prevalence of FCoV type I and II in cat populations in Thailand. The aims of this study were to investigate the incidence of FCoV infection among cat populations by detecting the 3′UTR gene, and to differentiate the genotypes of FCoV by targeting a portion of S gene particularly from clinical specimens.
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Materials and Methods

Animals and clinical specimens: Cats were classified into 3 groups according to their health status. Group I consisted of sick cats with the presence of pleural/abdominal effusion on the first date of hospital visit during 2010-2011. Total 103 fluid samples were collected from 95 FIP clinically suspected cats. Of them, 4 cats showed both left and right sides of pleural effusion and 4 cats contained both pleural and abdominal effusions. Body fluids were harvested for routine fluid analysis and submitted to Department of Veterinary Pathology, Faculty of Veterinary Science, Chulalongkorn University, Thailand. The fluid analyses including specific gravity, total protein and total nucleated cell count (TNCC) were done. Types of fluid were classified as transudate, modified transudate and exudates according to their properties. The significant exclusion criteria was performed based on cytological evaluation to rule out lymphoma-induced bodily effusion. After centrifugation, the supernatant were harvested and kept at -80^{0}C until used for molecular study.

Group II consisted of 7 cats that had a history of multicat crowded environments and spend part of their lives living with FIP-suspected ill cats. Group III consisted of 10 clinically healthy cats that had no history of previous FIP exposure. Fecal swabs were collected in sterile phosphate buffer saline (PBS) and the supernatants were collected following centrifugation. They were kept at -80^{0}C until used.

RNA extraction: Viral RNA was extracted from 150 µl fluid using NucleoSpin® RNA Virus (Macherey-Nagel, DÜren, Germany) according to the manufacturer's instruction. Briefly, fluid was homogeneously mixed with RAV1 and heated at 70^{0}C for 5 min. Ethanol was added and filled into the NucleoSpin column. After multiple washing steps with RAW and RAV3, the RNA was eluted with RNase-free H_2O, quantified using NanoDrop spectrophotometer, and kept at -80^{0}C until used.

Reverse transcription polymerase chain reaction (RT-PCR) for 3′UTR detection: Reverse transcriptase (RT) reaction was carried out using 0.5 µg RNA, random primers (Promega, Mannheim, Germany) and the Omniscript RT-PCR kit (Qiagen, Hilden, Germany). The reaction mixtures were incubated at 60^{0}C for 1 hour. Primers for feline cDNA qualification (feline GAPDH) and FCoV detection (FCoV-3′UTR) were chosen from previous publications (Table 1) (Herrewegh et al., 1995; Penning et al., 2007).

Following cDNA amplification, PCR reaction was performed consisting of 2 µl cDNA, 0.4 µM (each) primers, 1.25 U Taq polymerase (Invitrogen), 1 mM $MgSO_4$, 0.2 mM (each) dNTPs with GoTaq Flexi buffer (Promega, Mannheim, Germany). The temperature cycling protocol consisted of 5 min of preheating at 95^{0}C followed by 39 cycles of 30 sec of denaturation at 95^{0}C, 1 min of primer annealing at 50^{0}C (feline GAPDH) or 56^{0}C (FCoV-3′UTR), and 2 min 50 sec of primer extension at 72^{0}C. The amplicons were visualized on 1.5% agarose gels after staining with ethidium bromide. The positive control was FIP histopathologically diagnosed tissue collected from a necrosied cat while the negative control was without genomic RNA.

Reverse transcription-Touchdown PCR for S-gene detection: The RT reaction was performed using the above protocol with minor modifications. Common reverse primer of 3′ end of S gene (Table 1) and 1µg RNA were used for cDNA synthesis.

Table 1 List of primer used: sequences, annealing temperatures (Ta) and the length of PCR products

	Forward primer (5′→3′)	Reverse primer (5′→3′)	Amplicon length (base pairs)
3′UTR	GGCAACCCGATGTTTAAAACTGG	CACTAGATCCAGACGTTAGCTC	223
3′ end of S	(Genotype I)		
	GTTTCAACCTAGAAAGCCTCAGAT	(Common reverse primer)	376
	(Genotype II)	CCACACATACCAAGGCC	
	GCCTAGTATTATACCTGACTA		283
Feline GAPDH	AGTATGATTCCACCCACGGCA	GATCTCGCTCCTGGAAGATGGT	102

For Touchdown PCR reaction, the mixture comprised 2 µl cDNA, 20 pmol (each) forward primers, 15 pmol common reverse primer, 1 mM $MgSO_4$ with GoTaq HotStart buffer (Promega, Mannheim, Germany). Thermal cycling conditions were as follows: 94^0C, 2 min followed by 30 cycles of 94^0C, 1 min; annealing temperature stepdowns every 2 cycles of 1.5^0C (from 65^0C to 50^0C); 72^0C, 1 min. The annealing temperature for the final 35 cycles was 50^0C with denaturation and extension phases as above. PCR products were visualized on 1.5% agarose gels after staining with ethidium bromide. The positive controls were FIP histopathologically diagnosed tissue collected from a necrosied cat (for genotype I) and feces from a necropsied dog which was test-kit positive for canine coronavirus (for genotype II). The negative control was reaction without genomic RNA.

Phylogenetic analysis: Positive PCR products were purified with Nucleospin® extract II kit (Macherey-Nagel, DÜren, Germany) and submitted for sequence analysis. Data were analyzed by Bioedit Sequence Alignment Editor version 7.0.5.3 (Hall, 1999) and compared with previously reported sequences available in GenBank.

Results

Clinical data and fluid analysis: During 2010-2011, a total of 103 body effusions from cats were submitted for routine fluid analysis. Following the criteria that classified cats into 3 groups, most FIP-suspected cats (Group I) were young cats (66%) with age ranging from 4 months - 2 years, intact male cats (61%) and domestic short hair breed (87%). The effusions were frequently found in the thoracic cavity (70%) and classified as modified transudate (71%). The healthy cats (Group II and III) were mostly young cats and domestic short hair breed (Table 2).

Detection of 3'UTR gene of FCoV in naturally infected and asymptomatic cats: The detection of FCoV RNA in the effusion and feces of various groups of cats (Group I-III) targeting the highly conserved 3'-untranslated region (3'UTR) by RT-PCR was thoroughly performed. The specific band was observed at 223 bp on 1.5% agarose gel (Table 3, Fig 1). Of the 95 clinically sick cats with FIP suspicions (Group I), 47 out of 103 fluid samples (46%) were positive with accumulative body effusions. From these 3'UTR-positive cats, 62% (29/47) were young cats (4 months-2 years), while 38% (18/47) were adult cats (> 2-7 years). The intact males and domestic short hair breed showed the highest frequency at 60% (28/47) and 87% (41/47), respectively, among the infected cats. The type of FCoV-positive effusion was mostly modified transudate (74%, 35/47), exudates (24%, 11/47) and transudate (2%, 1/47). Moreover, among total 8 FIP-suspected cats presenting either left and right pleural effusions or thoracic and abdominal effusions, five cats were positive for 3'UTR RT-PCR.

For the cats that were raised in crowded environments and exposed to FIP-suspected cats (Group II), they strikingly displayed 100% (7/7) positivity with 3'UTR RT-PCR without any clinical signs. All of them were young cats (3 months-1 year). For the cats that had no history of previous FIP exposure (Group III), they demonstrated 50% (5/10) positive with 3'UTR RT-PCR.

Table 2 Clinical signalment and fluid analysis

Group	**I** (n=95)	**II** (n=7)	**III** (n=10)
Age			
≤ 2 yr	63 (66%)	7 (100%)	7 (70%)
> 2 yr	32 (34%)	0	3 (30%)
Sex			
- Male	58 (61%)	1 (14%)	3 (30%)
- Male, castrated	9 (10%)	1 (14%)	3 (30%)
- Female	22 (23%)	4 (58%)	3 (30%)
- Female, steriled	6 (6%)	1 (14%)	1 (10%)
Breed			
- Domestic short hair	82 (87%)	3 (43%)	9 (90%)
- Persia	5 (5%)	3 (43%)	0
- Siamese	8 (8%)	1 (14%)	1 (10%)
Site of fluid accumulation (103 total fluid samples)			
- Pleural effusion	72 (70%)	-	-
- Abdominal effusion	31 (30%)	-	-
Type of fluid (103 total fluid samples)			
- Transudate	2 (2%)	-	-
- Modified t ransudate	73 (71%)	-	-
- Exudate	28 (27%)	-	-

FCoV genotype differentiation of S gene in naturally infected and asymptomatic cats: The specific region of the S gene, located within the 3' end region, was amplified by RT-PCR. Aim to differentiate FCoV genotype I and II strains, different forward primers were used with the common reverse primer resulting in different length of amplicons; 376 (genotype I) and 283 bp (genotype II) (Table 3, Fig 1). Only the 3'-UTR positive samples were further analyzed.

Using S RT-PCR, forty-seven samples of 3'UTR-positive cats in Group I showed 47% (22/47)

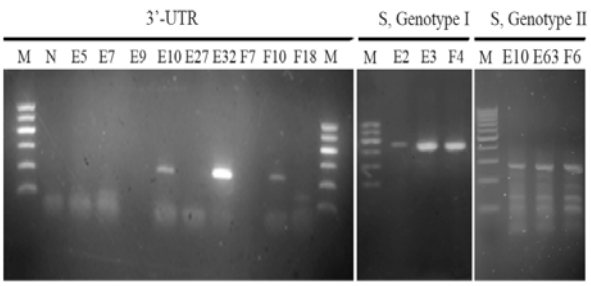

Figure 1 RT-PCR analysis of 3'UTR and S genes of FCoV. The length of specific amplicons were 223 (3'UTR), 376 (S genotype I) and 283 bp (S genotype II). M = 100 bp DNA ladder marker, N = negative, E = effusion, F = feces

genotype I, 15% (7/47) genotype II and 11% (5/47) mixed genotype, while 23% (11/47) were negative. Seven samples of cats in Group II displayed about 29% genotype I (2/7), II (2/7) and negative result (2/7), whereas mixed genotype was 13% (1/7). Similar to Group I, cats in Group III were positive for genotype I (60%, 3/5), II (20%, 1/5) and negative for all (20%, 1/5).

Phylogenetic analysis: Out of 47 3'UTR-PCR positive cases in Group I, 18 field isolates were selected for sequencing analysis. All sequences were aligned with published sequences of FCoV and other coronaviruses such as canine (CCoV), bovine (BCoV) (Table 4). Homology matrix was calculated using ClustalW Multiple alignment and Sequence Identity Matrix (Bioedit version 7.0.5.3). Neighbor phylogenetic tree was constructed uisng tool available on http://www.ebi.ac.uk/Tools/msa/clustalw2/.

Part of 3'UTR sequences of 18 Thai isolates showed 91-100% homology among them and such high similarity when compared to Malaysia and Taiwan FCoV strains (Fig 2). Except for the E76 strain, it was more closely related to the USA strain (91%, marked by dash frame) than Malaysia and Taiwan strains (83-91%, marked by solid frame). In addition, FCoV Thai strains were highly identical with CCoV, Taiwan strain (91-100%, orange box) and contrary to the CCoV, Ital strains (33-70%, Green box).

The partial S genes from 22 sequences of genotype I and 2 sequences of genotype II of Thai isolates showed 81-100% and 100% homology, respectively (Fig 3). However, they displayed only 47-52% when compared between genotypes (purple box). Accordingly, the identity of genotype I increased to 83-94% when comparatively analyzed with known genotype I FCoV/FIPV, and decreased to 49-64% compared with published genotype II FCoV/genotype I and II CCoV. Similarly, genotype II local strains increased their nucleotide identity to 91-96% (compared with known genotype II FCoV/genotype II CCoV (orange boxes)) and decreased to 63-66% (compared with published genotype I CCoV). As expected, the vaccine strain (AY452031, blue box) showed close identity to genotype II (74%, marked by dash frame) rather than genotype I FCoV local strains (57-64%, marked by solid frame).

Multiple sequence alignment of 3'UTR and S gene revealed a few point mutations with a closely related nucleotide sequence of genotype II Thai isolates to vaccine against FIP strain, known genotype II FCoV and genotype II CCoV (data not shown).

Phylogenetic tree revealed that FCoV Thai isolates were genetically divided into two main clusters; genotype I and II, based on S gene analysis and closely related to Malaysia and Taiwan isolates based on 3'UTR analysis (Fig 4-5).

Discussion

In this study, we revealed the frequency of age, sex and breed of FIP suspected clinical cats as well as the type of fluid accumulated from body cavities. Due to the fact that 13% of naturally FCoV-infected cats become lifelong carriers and they can shed virus via secretions (saliva, nasal discharge, urine, feces) for 2-3 months or longer, monitoring is needed for FCoV shedding by healthy cats as well (Addie and Jarrett, 2001). Therefore, apparently healthy cats that had a history of multicat crowded environments and experience of FIP exposure and cats that were kept indoors and were not exposed to FIP were included. Normally, when cats suffering from fluid accumulation in body cavity come to hospital, the first tentative diagnosis is FIP. However, our results demonstrated that the FCoV-positivity rate was 46% of totally submitted fluid. Veterinarians need to take history and physical examination carefully with other diagnosis including suppurative pleurisy/peritonitis, hemothorax/chylothorax from trauma, cardiomyopathy, nephritic syndrome, and neoplasms such as lymphoma, thymoma or even idiopathic disease (Sharif et al., 2010[a]). In addition, we showed that the frequency of FCoV-positive cats younger than 2 years old (62%, 29/47) was higher than adult cats. This finding is consistent with previous investigations (Benetka et al., 2004; Hartmann, 2005) indicating a higher incidence of FIP in cats below 2 years of age and agreed with the fact that FIP is a disease of young cats. The relation of gender and breed of FCoV-infected cats could not be concluded from this study due to a limited variation in cat breeds presented at the Small Animal Hospital, Chulalongkorn University. However, our data were in agreement with previous studies showing more prevalence in males and DSH breed (Sharif et al., 2010[b]).

Table 3 RT-PCR results of 3'UTR and S gene of FcoV

Group	3'UTR gene			Genotype of S gene[1]					
	n	Positive	Negative	*n*	I	II	I + II	Negative	ND[2]
I	*103*	47 (46%)	56 (54%)	*47*	22 (47%)	7 (15%)	5 (11%)	11 (23%)	2 (4%)
II	*7*	7 (100%)	0	*7*	2 (29%)	2 (29%)	1 (13%)	2 (29%)	0
III	*10*	5 (50%)	5 (50%)	*5*	3 (60%)	1 (20%)	0	1 (20%)	0

[1]Detection of S gene by RT-PCR was performed in 3'UTR positive sample
[2]ND means 'not done' due to inadequate sample

Figure 2 Comparison of homology matrix of partial 3'UTR sequences of FCoV between Thai isolates and published various coronavirus strains using Sequence Identity Matrix (Bioedit version 7.0.5.3). Eighteen local isolates showed 91-100% homology (yellow box). In addition, they displayed high identity with CCoV, Taiwan strain (orange box) and contrary to the CCoV, Italy strains (Green box). The exception was evident in E76 Thai strain that was similar to the FCoV, USA strain (marked by dash frame), instead of other FCoV, Asia strains (marked by solid frame).

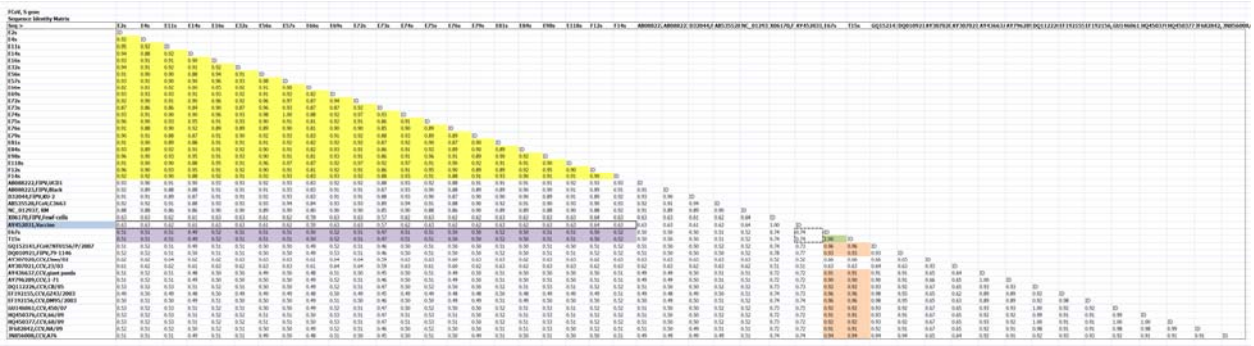

Figure 3 Comparison of homology matrix of partial S gene sequences of FCoV between Thai isolates and published various feline and canine coronavirus (CCoV) strains using Sequence Identity Matrix (Bioedit version 7.0.5.3). Twenty-two genotype I and 2 genotype II of Thai isolates showed 81-100% homology (yellow box) and 100% homology (green box), respectively. When comparing between each genotype, the identity decreased to 47-52% (purple box). Moreover, the similarity of genotype II Thai strains were as high as 91-96% when compared with known genotype II FCoV and genotype II CCoV (orange boxes). In addition, the vaccine strain (blue box) showed close identity to genotype II (74%, marked by dash frame) rather than genotype I FCoV local strains (57-64%, marked by solid frame).

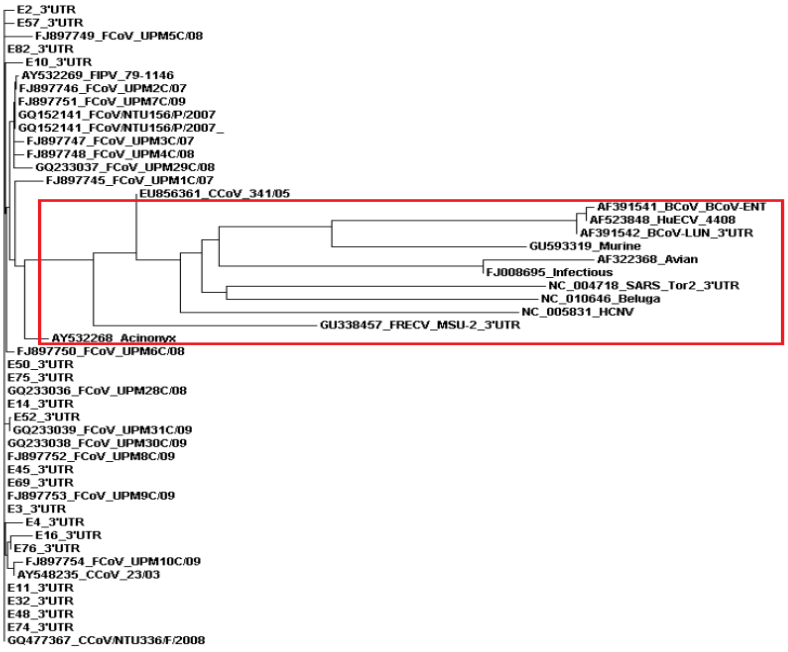

Figure 4 Phylogram of 3'UTR FCoV Thai strains and published coronavirus isolates from various species. Thai isolates were closely related to Malaysia strain. Other coronaviruses from human, bovine and avian were used as outgroup and marked by frame.

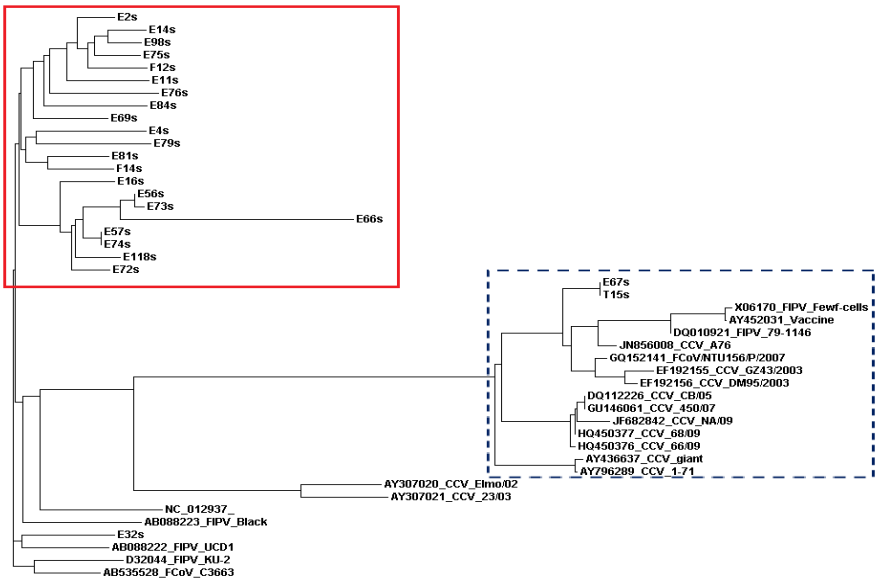

Figure 5 Phylogram of S gene FCoV Thai strains and published feline and canine coronavirus (CCoV) isolates. Genotype I Thai isolates were categorized in one main cluster (marked by solid frame) and more closely identical to other known genotype I FCoV and genotype I CCoV, while genotype II isolates were closely related to other known genotype II FCoV and genotype II CCoV (marked by dash frame).

One of the most useful and simple ante-mortem diagnosis is fluid analysis. The most likely FIP-suspected fluid type is modified transudate appearing clear, straw colored and viscous or froth when shaken due to high protein content. Cytology for FIP-suspected diagnosis usually demonstrates neutrophils, macrophages and lymphocytes (Addie and Jarrett, 2006). We found that the most FCoV-positive fluid type was modified transudate (71%) and consisted of neutrophils and macrophages on cytology (data not shown). Hence, the fluid analysis including specific gravity, total protein concentration, total nucleated cell count and cytology could be considered as a primary screening test for FIP.

The advantage of using RT-PCR assay emphasizing on the highly conserved 3'-untranslated region (3'UTR) is not only to additionally confirm the status of FCoV infection in FIP-suspected cats, but also to detect apparently healthy FCoV carriers and to screen new cats before introducing them into FCoV-free catteries. Interestingly, the FCoV positivity rate among submitted fluid samples from sick cats was 46%, while the rate among apparently healthy cats were 100% in multicat crowded environment and 50% in single cat household. Additionally, feces could be considered as a suitable specimen for FCoV detection in non-effusive cats by RT-PCR. This strongly indicates the mode of natural transmission and persistence of FCoV that is shed by healthy FCoV carriers (Addie et al., 2003).

Due to the poor clarification regarding the correlation between FCoV genotype and outcome of infection in terms of different clinical forms (wet and dry), becoming a carrier either transiently or lifelong, the greater possibility of developing FIP (a mutated FCoV) and histopathological changes have not yet been clearly elucidated. The elementary incidence of FCoV genotype is a prerequisite. Several attempts have been done to verify those questions, however the findings remained questionable and controversial (Addie et al., 2003; Benetka et al., 2004; Kummrow et al., 2005; Lin et al., 2009). The predominate genotype among FCoV-positive Thai cats either clinically ill or healthy without history of previous FIP exposure was genotype I. This observation was in agreement with incidence in USA, Austria, Taiwan and Japan. Moreover, the incidence of genotype II and co-infection of both genotypes were also evident in other regions with lower percentage. This might be explained by previous studies that genotype I is able to persist in cats living in multicat households for more than 6 years (Addie et al., 2003) and a single experimentally infected cat for at least 7 months (Herrewegh et al., 1997). In contrary, genotype II is more likely to cause acute infection and does not persist when recovered (Lin et al., 2009). Despite the use of 3'UTR RT-PCR for FCoV screening, up to 30% of the samples in this study remained negative for S RT-touchdown PCR. This was probably due to the variability in the S gene of FCoV which act as the structural spike coding protein gene. Therefore, to gain more accuracy of FCoV infection, the use of 3'UTR RT-PCR is recommended rather than of S RT-PCR.

Table 4 List of coronavirus isolates and strains from various hosts included in the phylogenetic analysis of 3'UTR and S gene

No.	Accession No.	Isolate/Strain	Host	Origin	Reference
1	AB088222	FIPV,UCD1	Feline	Japan	Motokawa et al., 1996
2	AB088223	FIPV,Black	Feline	Japan	Motokawa et al., 1996
3	AB535528	FCoV,C3663	Feline	Japan	Unpublished
4	AY452031	Vaccine	modified live vaccine; attenuated; temperature-sensitive strain		Unpublished
5	AY532269	FCoV,79-1146	Feline	USA	Dye and Siddell, 2005
6	D32044	FIPV,KU-2	Feline	Japan	Motokawa et al., 1996
7	DQ010921	FIPV,79-1146	Feline	USA	Dye and Siddell, 2005
8	FJ897745	FCoV,UPM1C/07	Feline	Malaysia	Sharif et al., 2010[b]
9	FJ897746	FCoV,UPM2C/07	Feline	Malaysia	Sharif et al., 2010[b]
10	FJ897747	FCoV,UPM3C/07	Feline	Malaysia	Sharif et al., 2010[b]
11	FJ897748	FCoV,UPM4C/08	Feline	Malaysia	Sharif et al., 2010[b]
12	FJ897749	FCoV,UPM5C/08	Feline	Malaysia	Sharif et al., 2010[b]
13	FJ897750	FCoV,UPM6C/08	Feline	Malaysia	Sharif et al., 2010[b]
14	FJ897751	FCoV,UPM7C/09	Feline	Malaysia	Sharif et al., 2010[b]
15	FJ897752	FCoV,UPM8C/09	Feline	Malaysia	Sharif et al., 2010[b]
16	FJ897753	FCoV,UPM9C/09	Feline	Malaysia	Sharif et al., 2010[b]
17	FJ897754	FCoV,UPM10C/09	Feline	Malaysia	Sharif et al., 2010[b]
18	GQ152141	FCoV,NTU156/P/2007	Feline	Taiwan	Unpublished
19	GQ233036	FCoV,UPM28C/08	Feline	Malaysia	Sharif et al., 2010[b]
20	GQ233037	FCoV,UPM29C/08	Feline	Malaysia	Sharif et al., 2010[b]
21	GQ233038	FCoV,UPM30C/09	Feline	Malaysia	Sharif et al., 2010[b]
22	GQ233039	FCoV,UPM31C/09	Feline	Malaysia	Sharif et al., 2010[b]
23	NC_012937	FCoV,RM	Feline	USA	Unpublished
24	X06170	FIPV,Fewf-cells	Cell line	The Netherlands	de Groot et al., 1987
25	AY307020	CCV,Elmo/02	Canine	Italy	Unpublished
26	AY307021	CCV,23/03	Canine	Italy	Unpublished
27	AY436637	CCV,giant panda	Giant panda	China	Unpublished
28	AY548235	CCV,23/03	Canine	Italy	Unpublished
29	AY796289	CCV,1-71	Canine	China	Ma et al., 2008
30	DQ112226	CCV,CB/05	Canine	Italy	Buonavoglia et al., 2006
31	EF192155	CCV,GZ43/2003	Raccoon dog	China	Vijaykrishna et al., 2007
32	EF192156	CCV,DM95/2003	Chinese ferret badger	China	Vijaykrishna et al., 2007
33	EU856361	CCV,341/05	Canine	Italy	Martella et al., 2009
34	GQ477367	CCoV/NTU336/F/2008	Canine	Taiwan	Unpublished
35	GU146061	CCV,450/07	Canine	Italy	Unpublished
36	HQ450376	CCV,66/09	Canine	Italy	Unpublished
37	HQ450377	CCV,68/09	Canine	Greece	Ntafis et al., 2011
38	JF682842	CCV,NA/09	Canine	Greece	Ntafis et al., 2011
39	JN856008	CCV,A76	Canine	USA	Unpublished
40	AF391541	BCoV-ENT Bovine coronavirus	Bovine	USA	Chouljenko et al., 2001
41	AF391542	BCoV-LUN Bovine coronavirus	Bovine	USA	Chouljenko et al., 2001
42	AY532268	Acinonyx jubatus coronavirus, Aju-92	Cheetah	USA	Pearks et al.,2004
43	AF322368	Avian infectious bronchitis virus,D41	Avian	China	Unpublished
44	FJ008695	Infectious bronchitis virus,Md27	Chicken	USA	Ammayappan and Vakharia,2009
45	AF523848	Human enteric coronavirus, 4408	Human	USA	Wu et al., 2003
46	NC_004718	SARS,Tor2 (Severe acute respiratory syndrome)	Human	Canada	Marra et al., 2003
47	NC_005831	HCNV NL63,Amsterdam I (Human coronavirus)	Human	The Netherlands	van der Hoek et al., 2004
48	GU338457	FRECV,MSU-2 (Ferret enteric coronavirus)	*Mustela putorius furo* (ferret)	USA	Wise et al., 2010
49	GU593319	Murine hepatitis virus, S	Mouse	USA	Koetzner et al., 2010
50	NC_010646	Beluga Whale coronavirus,SW1	*Delphinapterus leucas* (beluga whale)	USA	Mihindukulasuriya et al., 2008

For more accurate diagnosis, the 3′UTR RT-PCR could be applicable to confirm FIP infection in addition with the S RT-PCR to differentiate its genotype. To monitor FCoV carrier condition in healthy cat, feces is recommended as a suitable specimen to submit for FCoV detection. All Thai FCoV isolates are genetically divided into two main clusters; genotype I and II based on S gene analysis and closely related to Malaysia and Taiwan isolates based on 3′UTR analysis.

Acknowledgements

This study was financially supported by Chulalongkorn University-Veterinary Science Research Fund RG 2/2554 and Grants for the Development of New Faculty Staff (GDNS 55-011-31-003), Chulalongkorn University.

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