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Evaluation of Feline Coronavirus Viraemia in Clinically Healthy and Ill Cats with Feline Infectious Peritonitis

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Abstract: Feline Coronavirus (FCoV) comprises virulent and avirulent biotypes. While both biotypes can enter the bloodstream of a cat, only the virulent biotypes would replicate in monocytes and macrophages and develop a fatal disease known as Feline Infectious Peritonitis (FIP). In the present study, FCoV viraemia was evaluated in 50 cats consisting of 40 overtly healthy and 10 ill cats suspected of FIP. The blood samples were screened for FCoV genomic RNA by a RT-PCR assay and then followed by a duplex RT-PCR for detection of replicating viral mRNA. In the healthy cats, the virus and its replicating mRNA were detected in 67.5 and 15%, respectively. The later finding suggested that the virus was replicating in a few cats with no clinical sign shown and indicated that FCoV viraemia do not necessarily lead to FIP. Probably the avirulent virus does multiply at low level in the blood or cat can harbor the virulent virus in an early stage of FIP without clinical signs yet. In FIP-suspected cases, all of the ill cats were positive for both FCoV and the replicating viral mRNA suggesting that FCoV could have replicated in blood and produced high amount of the virus and its components which were detectable by the both assays. The duplex RT-PCR assay which has been used to detect the replicating viral mRNA in blood was more specific than the general screening RT-PCR test for the diagnosis of FIP. The RT-PCR results however, should be interpreted in conjunction with other clinical symptoms.

Key words: Feline Coronavirus (FCoV), Feline Infectious Peritonitis (FIP), cat blood, viraemia, symptom, Malaysia

INTRODUCTION

Feline Coronavirus (FCoV) is extremely common in cat populations. Antibodies against FCoV are found in 20-60% of pet cats and up to 100% of cats in catteries or multi-cat households (Addie and Jarrett, 1992; Pedersen, 1995, Arshad *et al*., 2004; Holst *et al*., 2006). FCoV comprises two biotypes; Feline Enteric Coronavirus (FECV) and Feline Infectious Peritonitis (FIPV). While infection with FECV is usually subclinical or may cause mild transient diarrhea, FIPV results in a progressive and lethal disease known as Feline Infectious Peritonitis (FIP). The pathogenesis of FIP is not fully understood. Based on widely accepted *in vivo* mutation theory, FIPV arises by mutation from parental FECV in gastrointestinal tract of infected cat, spreads systemically and leads to FIP (Pedersen *et al*., 1981; Poland *et al*., 1996; Vennema *et al*., 1998). The mutation sites are not well characterized but some accessory genes (such as 7b and 3c) are candidates for critical mutations responsible for FIP (Herrewegh *et al*., 1995a; Pedersen, 2009; Chang *et al*., 2010). An alternative hypothesis is circulating virulent/avirulent virus theory indicating that both virulent and avirulent biotypes circulate in cat populations and susceptible individuals exposed to the virulent virus, manifest the disease (Brown *et al*., 2009). In spite of controversial theories on generation of FIPV, the relationship between virulence and macrophage/monocyte tropism has been established (Pedersen, 2009). While both FIPV and FECV may cause viraemia (Herrewegh *et al*., 1995b; Fehr *et al*., 1996; Gunn-Moore *et al*., 1998) only FIPV replicate in peripheral blood mononuclear cells (PBMCs) actively and develop the disease (Stoddart and Scott 1989; Vennema *et al*., 1998).

Since specific genetic determinants of FCoV biotypes are still poorly understood and the viral genome contains various single nucleotide polymorphisms (SNPs) (Pedersen, 2009; Sharif *et al*., 2010a), it seems that it is not possible to design PCR primers to distinguish between

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mutated and non-mutated viruses (Fehr *et al*., 1996) and discriminate between FIP cases from FCoV-positive healthy cats by RT-PCR. In 2005, Simons and colleagues introduced a new PCR-based approach for FIP diagnosis.

The approach was based on the key pathogenic event of FIP which is viral replication in PBMCs. Thus, specific primers targeted to detect replicating messenger RNA (mRNA) of FCoV in blood. In this study, the PCR assay described by Simons was developed to a duplex RT-PCR and used for evaluation of FCoV viraemia in apparently healthy and sick cats with FIP.

MATERIALS AND METHODS

Viruses: Two FCoV reference strains (FECV 79-1683; ATCC® No. VR-989™ and FIPV79-1146; ATCC® No. VR-216™) were propagated in confluent Crandell Feline Kidney (CrFK) cells. The viruses were harvested when the infected CrFK cells showed 80% cytopathic effects and stored at -70°C until used.

Three feline viruses (feline parvovirus, feline calcivirus and feline herpesvirus) from a live heterogenous feline vaccine (MERIAL, USA) and also an attenuated Canine Coronavirus (CCV) from a canine vaccine (VANGUARD® PLUS, USA) were included in the assay. RNA was extracted from the infected cell culture supernatants and the vaccines using TRIZOL® Reagent (Invitrogen, USA) according to the manufacturer's instructions.

Blood samples: A total of 50 cats were evaluated for FCoV viraemia by the duplex RT-PCR assays. Blood samples were collected from 40 clinically healthy cats and 10 ill cats suspected of FIP (Table 1). The healthy cats were from multi-cat households or Kuala Lumpur SPCA (society for prevention of cruelty to animals) shelter, whereas the ill cats were diagnosed with FIP at the University Veterinary Hospital, Universiti Putra Malaysia (UVH-UPM).

About 1 mL of blood from each cat was collected in EDTA (ethylenediaminetetraacetic acid) blood collection tubes (BD Franklin, USA) and centrifuged for 10 min at 3,000 g (UNIVERSAL 32R, Hettich ZENTRIFUGEN, Germany). The buffy coat was separated carefully and subjected for RNA extraction using a high pure viral RNA kit (Roche, Germany). All procedures were performed as recommended by the manufacturer. Age, breed and gender differences were compared by calculating probability and p value. Values of $p<0.05$ were considered significant.

Table 1: Age, gender, breed distribution and clinical status of cats used in the study

		Age range		Gender		Breed	
Clinical status	No. of blood samples	<2 years	>2 years	Male	Female	Persian	Mix-breed*
Healthy	40	13	27	22	18	14	26
FIP-suspected	10	6	4	7	3	4	6

*Cross-breed and Domestic Short Hair (DSH)

Reverse Transcription Polymerase Chain Reaction (RT-PCR) assays: A general screening for FcoV genomic RNA was carried out using primers targeted to a conserved region of the viral genome (Herrewegh *et al*., 1995b) as described previously. Subsequently all the blood samples were submitted to further analysis employing two set of oligonucleotide primers which had been designed by Simons *et al*. (2005). The FCoV-specific primers were combined to the internal control primers in a duplex RT-PCR amplification. The internal control primers targeted to a housekeeping gene (glyceraldehyde-3-phosphate dehydrogenase or GAPDH) and were used to rule out any failure in RNA extraction or RT-PCR assay. Single-tube RT-PCR reaction was carried out using an Access RT-PCR system (Promega, USA). About 25 μL reaction mixture contained in final concentration of 1× AMV/Tfl reaction buffer, 2 mM $MgSO_4$, 0.4 mM dNTPs mix, 0.2 U AMV reverse transcriptase, 0.2 U Tfl DNA polymerase, 0.8 U Rnase inhibitor (Promega, USA), 0.5 μM of each primers (Research Biolabs Sdn Bhd, Malaysia), 1 μL of RNA and RNase-free water. A programmable thermal controller (MJ Research, USA) was used for RT-PCR and the amplification program consisted of reverse transcription at 48°C for 45 min, pre-denaturation at 94°C for 3 min followed by 35 cycles of denaturation at 94°C for 1 min, two annealing temperatures at 58 and 62°C for 30 sec each and extension at 72°C for 1 min. The final extension was done at 72°C for 5 min. Size of amplicons was verified by agarose gel electrophoresis in TAE (Tris-acetate EDTA) buffer using known standards.

RESULTS

General screening for FCoV genomic RNA: The extracted RNA from blood samples of 40 healthy cats and 10 FIP-suspected sick cats were tested by the RT-PCR assay for general screening for FCoV genomic RNA. The results showed that 27 of 40 healthy cats (67.5%) and all FIP-suspected cats (100%) were FCoV-positive. Amongst FCoV-positive cats, 73% (16/22), 73% (19/26) and 67% (10/27) were male, mix-breed and cats with <2 years old, respectively. However, these proportions are not significantly different ($p>0.05$).

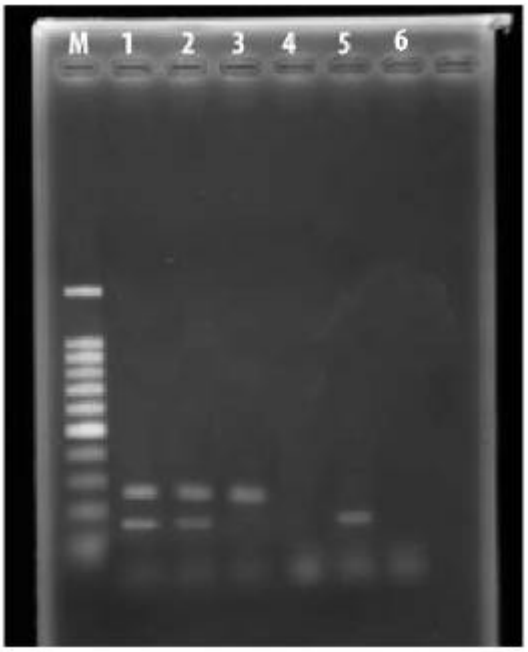

Fig. 1: Duplex RT-PCR assay for detection of replicating mRNA of FCoV. A 295 bp band generated by the detective primers was shown on two FCoVs (lanes 1 and 2) and CCV (lane 3). The internal control primers generated a band of 195 bp on FCoVs (Lanes 1 and 2) and CrFK cells (lane 5). No band was detected on the other feline viruses (lane 4) and NTC (lanes 6). M: 100 bp DNA marker; Lane 1: FECV 79-1683; Lane 2: FIPV79-1146; Lane 3: CCV; Lane 4: Feline vaccine viruses; Lane 5: Uninfected CrFK cells; Lane 6: No Template Control (NTC)

Detection of replicating FcoV mRNA: In the duplex RT-PCR assay, the FCoV-specific primers targeted to mRNA of the M gene produced the target band of 295 bp on the reference strains of FCoV and CCV. A band of 195 bp generated by the internal control primers was observed on FCoVs and uninfected CrFK cells. No band was detected on the feline vaccine viruses and the negative control (Fig. 1). After the assay setup, all the 50 blood samples (FCoV-positive as well as FCoV-negative samples) were examined for the viral replicating mRNA. Positive amplification was observed in 6 healthy cats (15%) and all 10 FIP cases (100%) and none in the FCoV-negative samples.

DISCUSSION

In the general screening test, FCoV was found in 67.5% of blood sample of healthy cats from multi-cat households. Since the previous studies on Malaysian catteries showed that 100% of cats have antibody against FCoV (Arshad *et al*., 2004) and the virus shed by 84% of animals (Sharif *et al*., 2009) such a high rate of viraemia was expected. Also this observation is in accordance with previous reports that found FCoV viraemia in apparently healthy cats (Herrewegh *et al*., 1995b; Gunn-Moore *et al*., 1998). Following general screening for FCoV genomic RNA, blood samples of the healthy cats were assessed for detection of replicating viral mRNA. The mRNA RT-PCR for detection of replicating FCoV was first described by Simons *et al*. (2005). The method was based on designing of primers for two part of the viral genome; a conserved region of the M gene and the leader sequence which were joint together during the viral replication. The researchers hypothesized that replicating mRNA in blood may be correlated with the occurrence of FIP.

In that research work, internal control primers for a housekeeping gene (GAPDH) were used to check the efficiency of the assay. However, the amplification of the internal control primers was done in separate tubes. In this study, a duplex RT-PCR for detection of replicating mRNA of FCoV was performed by combination of the primers previously described. The simultaneous amplification of the target region of the viral genome and the GAPDH gene in the duplex RT-PCR can rule out any failure in RNA extraction or RT-PCR in an identical reaction condition and increase the accuracy of the assay.

Also, one duplex RT-PCR is more cost-effective than two monoplex RT-PCR amplifications which are needed for testing each sample. The original research was done in two separate steps of RT and PCR which is time-consuming process and may prone to produce false results. The fragile viral RNA can be degraded by ubiquitous RNAses during the research and lead to false-negative results. Moreover, cross-contamination with other coronaviruses like CCV may resultant in false-positive. To improve the accuracy of the results and shorten the hands-on time, one-step RT-PCR was applied in this research. Although, Simons *et al*. (2005) reported high diagnostic specificity of the assay for the clinical diagnosis of FIP, Can-Sahna *et al*. (2007) found the specificity of the assay using same primers to be poor. Since the controversial finding of the latter study were obtained from only 26 cats (25 healthy and 1 FIP-suspected cat) while the results of the former study arose from testing >1000 cats (424 healthy and 651 sick cats suspected of FIP), it seems that more samples are needed for evaluation the specificity of this test. In the present study, 50 blood samples from 40 healthy cats and 10 cats with clinical signs suggestive of FIP were evaluated by the FCoV general screening test and the duplex RT-PCR assay. In the duplex RT-PCR assay on the 40 healthy cats, 6 samples of 27 FCoV-positive cats (22%) showed the 295 bp target band of mRNA. In other word, the virus was replicating in blood of 15% (6/40) of tested cats while they were apparently healthy.

The observation of positive results in healthy cats was also reported in the 2 previous studies which used mRNA PCR (Simons *et al.*, 2005; Can-Sahna *et al.*, 2007) and indicates that the duplex RT-PCR assay could not be used as a single diagnostic tool for FIP. Also, these findings may suggest that although, the FIPV replicates actively in blood and cause FIP, FECV could also replicate in blood of infected cats but at much lower level.

This hypothesis could be assessed by a quantitative real-time RT-PCR assay which is able to determine the amount of viral mRNA in blood and it could differentiate FCoV-positive healthy cats from FIP cases. In the study, 15% of healthy cats were positive for replicating mRNA of FcoV. While in the previous research works by Simons *et al.* (2005) and Can-Sahna *et al.* (2007), 5 and 52% of healthy tested cats are positive, respectively. The difference in positivity rates in healthy cats may due to various amount of the virus in blood samples or different stages of the viraemia caused by FCoV.

Moreover, different RNA extraction kits might affect the quality of RNA template and RT-PCR outcome (Dye *et al.*, 2008). There was no significant association between the age, breed or gender of tested cats and replicating mRNA in blood. This finding is in concordance with those of Can-Sahna *et al.* (2007) who found no significant correlation between the viral replication in blood and the age or gender of the pedigree Tekir cats which were used in that study.

All FCoV negative samples obtained from the general screening test remain negative in the duplex RT-PCR assay. This result indicates the high sensitivity of the RT-PCR assay in detecting the virus. The internal control band which was observed in all tested samples ruled out any false-negative results.

CONCLUSION

The FCoV was found in all the 10 blood samples of FIP-suspected cats. Subsequently they were also positive by duplex RT-PCR assay indicating the replicating mRNA. This finding agrees to the assumption that in FIP diseased cats, the virus multiplies in blood cells and thus, the primers designed for replicating mRNA were able to detect possible reaction in the blood and diagnose the FIP. Currently, a definitive diagnosis of FIP could be confirmed by histopathology or detection of intracellular FCoV antigen by immunofluorescent or immunohistochemical staining (Sharif *et al.*, 2010b), however, a quantitative PCR assay would be useful to determine the amount of replicating mRNA in blood of FIP cases and draw a threshold line between them and FCoV-positive healthy cats.

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