DETECTION AND ISOLATION OF FELINE CORONAVIRUS IN TISSUE OF NATURALLY INFECTED CATS

ROSLINA H., NORSHUHADA A.H., ZURAIDAH O., SITI SURAYA HANI M.S., ROSHASLINDA D., NORLINA D., MOHD HASRUL A.H. AND ZUNAIDA B.

Veterinary Research Institute, 59, Jalan Sultan Azlan Shah, 31400 Ipoh
* *Corresponding author:* roslina@dvs.gov.my

ABSTRACT. Feline coronavirus (FCoV) consists of two biotypes, the feline infectious peritonitis virus (FIPV) and the feline enteric coronavirus (FECV) which are important viral diseases of cats. The diseases are often associated with various clinical signs from asymptomatic to highly fatal depending on its infection by different biotypes. Generally, FIPV infection can be highly fatal and are associated with various clinical signs including intermittent loss of appetite, depression, rough hair coat, weight loss and fever. Whereas, FECV infection can result in various symptoms from asymptomatic to severe enteritis. This paper describes isolation and molecular detection of feline coronavirus from tissue samples of suspected FIPV submitted to the Veterinary Research Institute (VRI) from 2013 to 2017. Forty-seven (47) samples were subjected to nested reverse transcriptase PCR (nRT-PCR) targeted on 3' untranslated region (3'-UTR) and virus isolation in Crandell Rees feline kidney (CrFK) cells. Based on the results, 55.4% (26/47) samples were positive for FcoV by nRT-PCR with amplified product of 223 bp and 177 bp of primary and secondary PCR respectively. Meanwhile, 29.9% (14/47) samples were positive for virus isolation with the presence of cytopathic effect (CPE) formation characterised as rounded, granular and clumped forming of syncytial cells. The results suggest that diagnosis of FCoV in cats cannot be based on clinical signs alone and it should be confirmed by laboratory tests. However, further sequence analysis need to be conducted for determining biotypes of FCoV

Keywords: feline infectious peritonitis virus, Crandell Rees feline kidney**,** cytopathic effect

INTRODUCTION

Feline coronavirus (FCoV) is an enveloped RNA virus belonging to the family Coronaviridae (Herrewegh *et al.,* 1995) which consists of two biotypes: feline infectious peritonitis (FIPV) and feline enteric coronavirus (FECV). Both are two important coronaviruses of the domestic cat worldwide.

FIPV is one the most serious viral diseases of cats with high prevalence especially in catteries and multiple-cat households (Amer *et al*., 2012). Commonly, presumptive diagnosis of FIPV is made based on clinical signs and evaluation of abdominal fluid.

However, clinical findings in FIP are non-specific and not helpful in making a differential diagnosis (Sharif *et al.,* 2010). It was suggested to confirm FIPV infection by

other laboratory tests. There are a number of general laboratory tests for diagnosis FIP available including serology tests, histopathology and reverse transcriptase polymerase chain reaction (RT-PCR) assays.

Although evidence of FIP has been reported among cat population in Malaysia, the circulating FCoV virus has neither been isolated nor characterised (Amer *et al*., 2012). Thus, the objectives of this study are to isolate and detect the presence of FCoV from suspected FIP tissue samples submitted to VRI from 2013 to 2017.

MATERIALS AND METHOD

Samples

The pooled organ (liver, lung, kidney and intestine) samples from cat species used in this study were obtained from routine diagnostic work which were submitted for investigation of feline coronavirus suspected with FIP. All samples were processed based on the Manual on Veterinary Virology (Sharifah S.H. and Mohd Ali A.R., 1992). In this study, about 47 samples exhibiting FIP clinical signs were subjected to nRT-PCR test and then a viral isolation test.

RNA extraction

The viral RNA from pooled organs (liver, lung, kidney and intestine) was extracted using innuPREP RNA Mini Kit (AJ Innuscreen GmbH) as described by the manufacturer's protocol. The extracted RNA was suspended in 30 µl of RNase-free water.

Reverse transcription-polymerase chain reaction (RT-PCR) and nested RT-PCR (nRT-PCR) for virus detection

The nRT-PCR was performed according to the method of Herrewegh *et al*. (1995), modified and adapted to conditions of the laboratory in this study. Herrewegh *et al.* (1995) had performed RT-PCR using Taq DNA polymerase.

In order to amplify the genomic sequence, the specific primers for DNA region of FCoV (Table 1) were used at 20 pmol concentration/µl for the first and the second PCR. The nRT-PCR tests were performed in PCR thermocyler Biorad in two steps.

Briefly, the primary RT-PCR was carried out in a total volume of 25 µl as follows*:* 2.0 µl of extracted RNA were added into the reaction mixture of 23.0 µl (containing 0.5 µl of forward primer (P211/F-), 0.5 µl of reverse primer (P205/R-), 1 µl Superscript™ III RT-PCR/Platinum Taq™ (Invitrogen, Thermo Scientific), 12.5 µl of 2× reaction mix, and 8.5 µl nuclease-free water).

The PCR tube were then amplified as follows: reverse transcription at 50 °C for 30 min, 94 °C for 2 min (initial denaturation), followed by 40 cycles of heat denaturation at 94 °C for 15 sec, primer annealing at 55 °C for 30 sec, and primer extension at 68 °C for 1 min. After the last cycle, the extension step at 68°C was prolonged for 5 min.

The DNA from the first step of reaction of 2.0 µl was used as a template for the second round of amplification with the nested RT-PCR of primers in the 23.0 µl reaction volume (containing 1.0 µl of forward primer (P276/F-), 1.0 µl of reverse primer

(P204/R-), 12.5 µl Go Taq™ Green Master Mix (Promega Corporation, Madison USA), and 8.5 µl nuclease-free water), followed by 40 cycles of heat denaturation at 95 °C for 1 min, primer annealing at 55 °C for 1 min, and primer extension at 72 °C for 1 min. The final PCR product was detected by gel electrophoresis ethidium bromide staining and the UV light trans illumination.

Cell Cultures

For isolation of FCoV, the Crandell Rees feline kidney (CrFK) cell culture was used. Briefly,

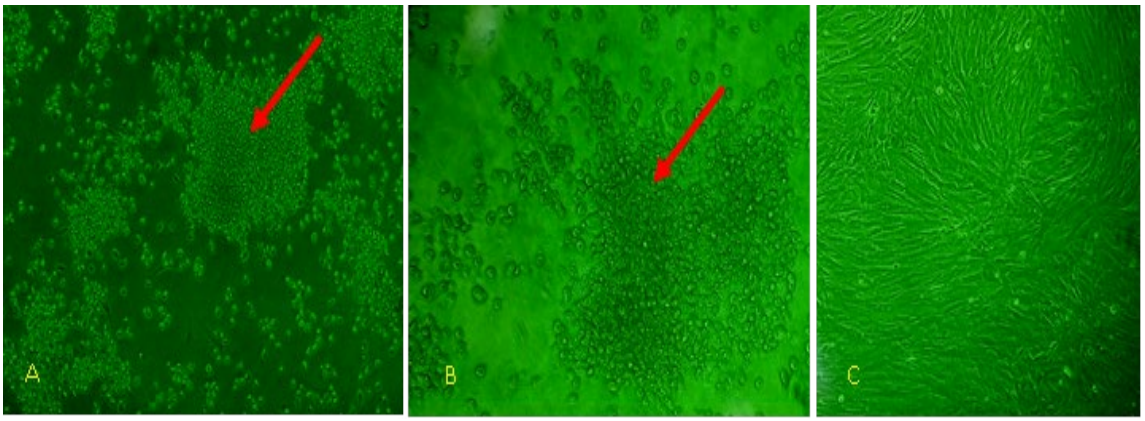

Figure 2. (A) CrFK with granular and clumped forming syncytial cells induced by Feline Coronavirus from homogenate tissue at days 1 pi, passage 1. (B) CrFK with clumping syncytial CPE were observed at days 2 pi, passage 1 (C) Normal CrFK

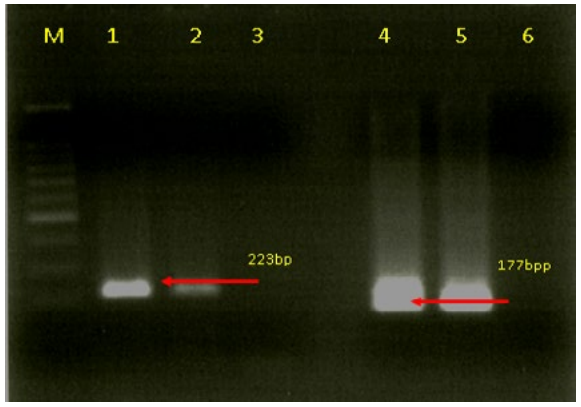

Figure 1. 1.5% Agarose gel electrophoresis of Feline Coronaviruses with amplified PCR product of 223 bp and 177 bp amplicons after the first PCR and nested PCR, respectively. Lane M: Molecular marker 100bp; Lane 1: 3605/14 (1st PCR); Lane 2: Positive control PCR of FCoV; Lane 3: Negative control PCR. Lane 4: 3605/14 (2nd PCR PCR); Lane 5: Positive control PCR of FCoV; Lane 6: Negative control PCR.

Table 1. Oligonucleotide primers for nRT-PCR amplification of FCoV according to Herrewegh *et al.*, 1995

Primer	Direction	Nucleotide Sequence (5′ to 3′)	Target gene	Fragment length
P211	F	5′ CAC TAG ATC CAG ACG TTA GCT C 3′	3′UTR	223 bp
P205	R	5′ GCC AAC CCG ATG TTT AAA ACT GG 3′		
P276	F	5′ CCG AGG AAT TAC TGG TCA TCG CG 3′	3′UTR	177 bp
P204	R	5′ GCT CTT CCA TTG TTG GCT CGT C 3′		

Table 2. The number of FCoV cases received at Veterinary Research Institute from 2013 to 2017.

No	Year	Various Clinical sign of FCoV	Types of sample	No of cases	Percentage of positive FCoV	
					Nested RT-PCR	Virus isolation
1.	2013	Can't walk, kidney not function, vomiting watery material, anaroxia, fever, ulceration, dehydration, lethargy	Pooled organ	7	5/7	5/7
2.	2014	Inflamed, flu, swallon head, conjunctivitis	Pooled organ, nasal swab	7	4/7	4/7
3.	2015	No appetite, conjunctivitis, no drinking, aneamia, loss weight	Pooled organ, intestine	7	6/7	3/7
4.	2016	Acute blindness, pyrexia	Pooled organ, blood	5	1/5	0/5
5.	2017	No appetite, vomit, panting, convulsion, dilated pupil	Pooled organ, intestine	13	6/13	1/13
6.	2014-2017	No clinical signs mention	Pooled organ	8	4/8	1/8
		Total		47	**26/47 (55.4%)**	**14/47 (29.9%)**

* Pooled organ for virus isolation including liver, lung, and kidney

Table 3: CPE observation of FCoV daily

Samples/Psg	CPE OBSERVATION											
	P1				P2				P3			
Dpi	24	48	72	96	24	48	72	96	24	48	72	96
Ctrl	-	--	-	-	--	--	--	-	--	--	--	-
Pooled organ	--	--	--	+	--	-+	++	Minor detach	-+	++	+++	detach

Standard scoring of CPE: - = No CPE, + = 25% CPE, ++ = 50% CPE, +++ = 75% CPE, ++++ = >90% CPE

the CRFK cells use in this study were seeded containing a density of 3×10^5 ml^{-1} using 24 well plates. The confluence CrFK cells were prepared 24 hours before each passage of inoculation. The growth medium added was: minimal essential medium (MEM) with Earle salts, 100 IU/mL antibiotic penicillin, 100 IU/mL streptomycin, 0.2 M/L L-glutamine, 7.5% sodium bicarbonate for pH 7.4 and 10% foetal bovine serum (FBS). The maintenance medium was the same as the growth medium but with only 2% of FBS.

The media were then removed from 24-well plate of CrFK cells. 100µl of suspension was inoculated onto CrFK cell and left to absorb for 1 hour at 37 °C in a carbon dioxide incubator. 1 ml of 2% maintenance medium was added and incubated at 37 °C in the carbon dioxide incubator for 5 days. The cytophatic effect was seen within 48 hours after inoculation at the second passage and continued at the third passage following simultaneous inoculation of virus with freshly trypsinised cells. The inoculum was harvested by freeze-thawed method.

RESULTS AND DISCUSSION

After being subjected to nRT-PCR, the results showed that the target 3' UTR gene has successfully amplified the primary and nested RT-PCR producing 223 bp and 177 bp amplicons, respectively (Figure 1). 55.4% (26/47) samples were positive for FcoV by nRT-PCR (Table 2). Most of the positive cases detect FCoV RNA in 46.8% (22/47) of cats which had clinical signs and suspected of FIPV. However, 8.5% (4/47) of cats were found with FCoV RNA without showing any clinical signs.

Furthermore, this study successfully isolated FCoV in organ tissues (liver, kidney, lung and intestine) in about 29.9% (14/47) of routine diagnostic cases. The samples were positive for virus isolation, with the presence of cytopathic effect (CPE) characterised as rounded, granular and clumped forming of syncytial cells (Figure 2). Overall, the isolation of FCoV was less sensitive compared to detection by nRT-PCR.

The initial CPE at the first passage showed moderate to diffused CPE on the 4th to 5th day of post inoculation (dpi). The appearance of CPE became rapid during the second passage. As the virus propagation reached the third passage; the onset of CPE appeared within 24 to 48 hours post inoculation (PI).

Complete CPE (more than 80%) was observed 48 to 72 hours PI (Table 3). All infected CrFK cells showed cells similarly increased in size and number.

According to Sharif *et al. (*2010), coronavirus infection in cats cannot be solely diagnosed by PCR assay. Although it is more sensitive, the PCR results suggest it has to be interpreted in conjunction with other clinical findings and intracellular detection (eg: histopathology). However, clinical findings and neurological signs in cat suspected of FCoV are non-specific and not helpful in diagnosing of FIP (David *et al.,* 1997, Sharif *et al.,* 2010).

Previous studies performing FCoV using conventional PCR on effusion samples from cats with FIP have shown promising results (Longstaff *et al.,* 2017). Recently, studies have found that cats with FIP have

higher amounts of FCoV RNA and are more likely to be shedding FCoV, (Tasker, 2018). The current RT-PCR assays are available for the detection of FCoV, however, they are not specific for FIP-associated FCoVs infection. Therefore, a diagnostic test for FIPV based on a nested PCR (nPCR) need to be established to detect FIPV in the tissue.

All the isolates in this study showed similar findings as previously reported by Amer *et al.* (2012) and Evermann *et al. (*1981), that is, morphological changes characterised by increased opacity and refractile of the infected cells. Infected cells became rounded, granular and clumped forming syncytial. Four samples were detected positive by nRT-PCR and negative for virus isolation. These could be due to the virus viability.

CONCLUSION

In conclusion, the FCoV was successfully detected by nRT-PCR and isolated in Crandell Rees feline kidney (CrFK) cells from FIP suspected samples. The results of nRT-PCR tests must be interpreted in together with other intercellular detection and should not be used as the sole criterion for determining FCoV associated with active FIPV infection.

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