

Full Length Research Paper

Molecular and pathological identification of feline coronavirus type I

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The coronavirus in cats has been described as feline infectious peritonitis (FIPV) and feline enteric coronavirus (FECV). FIPV is highly fatal and caused immune-mediated pyogranulomatous disease, whereas FECV causes mild enteric infection. In this study, we described the isolation and molecular characterization of naturally occurring feline coronavirus from domestic cat in Malaysia. Additionally, the resultant pathological conditions observed in the infected cat were reported. Ascitic fluid sample was collected from 3-year-old domestic cat clinically suspected of effusive (wet form) FIP and subjected to virus isolation in *Felis catus* whole fetus cell cultures (Fcwf-4). The result of virus isolation was confirmed using one step reverse transcription polymerase chain reaction (RT-PCR). To gain insight on the genetic variant of FCoV, the S-gene sequence was amplified using type 1 specific primers. Virus isolation showed cytopathic effect (CPE) characterized by giant cells, ballooning and cells detachment. Ultrastructural findings showed virus particles attached to plasma membrane and later invaginated from the cell membrane. Virus-like particles were also observed in the vacuoles, likely as a result of spillage of mature virus-like particles into the cytoplasmic matrix. Histopathological examination of kidney, spleen and intestine organ samples revealed coagulative necrosis with infiltration of neutrophils and mononuclear cells. In conclusion, this study reports the first isolation of feline coronavirus in Malaysian cats and importantly the isolated virus was confirmed to be type I using S-gene amplification.

Key words: Feline coronavirus, Fcwf-4, RT-PCR, effusive FIP, ultrastructure, histopathology.

INTRODUCTION

Feline coronavirus (FCoV) is an envelope, positive-stranded large RNA virus belonging to the family Coronaviridae within the order *Nidovirales* (Cavanagh, 1997; Gorbalenya et al., 2006). Feline coronaviruses

(FCoVs) infection is distributed worldwide in domestic cat population. Natural infection of cats is usually transient, although significant percentage of cats may become persistently infected (Addie and Jarrett, 2001). Feline coronavirus has been divided into two biotypes; feline enteric coronavirus (FECV) and feline infectious peritonitis (FIPV). Infection with FECV is usually sub-clinical except in young kittens where it may cause mild to severe diarrhea (Pedersen et al., 1981), while the FIPV causes lethal conditions characterized by vasculitis and/or pyogranulomatous lesions in different organs. It is the most infectious cause of mortality in domestic and

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Abbreviations: FIPV, Feline infectious peritonitis virus; FECV, feline enteric coronavirus; RT-PCR, reverse transcription polymerase chain reaction; CPE, cytopathic effect.

wild felids (Simons et al., 2005; Shiba et al., 2007; Pedersen, 2009). Furthermore, while FECV replicates locally in the enterocytes of the intestine, FIPV infects bloodmonocytes where it spreads systemically (Boyle et al., 1984; Fiscus and Teramoto, 1987). Despite the high prevalence of FCoV in cat populations around the world (Pedersen, 1976; Kiss et al., 2000; Bell et al., 2006), only a small percentage of about 1 to 5% of the seropositive cats developed FIP (Addie and Jarrett, 1990; Rottier, 1999). FIP is a fatal multi-systemic and immune-mediated disease with the high incidence mainly in kittens and to some extent, the old cats (Pedersen, 1995; Rottier, 1999). Based on their antigenic relatedness with canine coronavirus (CCoV), sequence analyses of S gene and growth characteristics *in vitro*; FECV and FIPV can be classified into two distinct serotypes I and II. Serotype I FCoV infection is the most prevalent and accounts for approximately 80% of all naturally occurring cases. However, this serotype is difficult to be isolated and propagated in cell culture (Pedersen et al., 1984; Hohdatsu et al., 1992; Addie et al., 2003). On the other hand, type II FCoV which appeared to be more closely related to canine coronavirus and transmissible gastro-enteritis virus (TGEV) is the least common but however, replicate well in cell culture.

The difficulty in isolation of FCoVs is largely associated with poor adaption of the virus *in vitro* (Woods, 1982; Pedersen, 2009). Diagnosis of FIP by serologic test is virtually impossible as available antibody detection tests are unable to distinguish between antibodies generated by FECV exposure from those induced by FIP (Fiscus and Teramoto, 1987). Difficulty in arriving at definitive diagnosis of FIP largely resulted from lack of specific clinical signs, pathognomonic, hematological and biochemical abnormalities as well as low sensitivity and specificity of diagnostic tests (Hartmann et al., 2003). Thus, histopathological confirmation of FIP has been used to define cases and it is generally regarded as the "gold standard" for diagnostic test comparison (Addie and Jarrett, 1990). Recently, molecular assays such as the polymerase chain reaction (PCR) have been developed and used to detect FCoV. PCR assays were found highly sensitive and specific compared with serology as they provide obvious advantage of directly detecting FCoV genome using primers targeting one or more regions of viral genome (Herrewegh et al., 1995).

Since the first FIP case report in 1983 in Malaysia (Wong et al., 1983), there is lack of information about the disease. Although a more recent follow up studies involving cats kept in catteries revealed that almost all the tested cats had antibodies to FCoV (Arshad et al., 2004) with 84% showing positive response when RT-PCR assay was applied as diagnostic test (Sharif et al., 2010a), no study was carried out to isolate and characterized FCoV in Malaysia. Hence, the aims of this study were to isolate and molecularly characterized FCoV among Malaysian domestic cats. It also described related pathological findings in effusive FIP case.

MATERIALS AND METHODS

A 3-year-old cat suspected of FCoV infection was obtained from the University Veterinary Hospital (UVH), Universiti Putra Malaysia (UPM), and used for this study. The animal was sacrificed following approval by Institutional Animal Care and Use Committee, Faculty of Veterinary Medicine (FPV/PU/B901). Ascites fluid and tissue specimens from kidney, spleen and intestine were collected after post mortem examination. The tissues specimens were fixed with the 10% neutral buffered formalin, dehydrated in a graded ethanol series, cleared in xylene, embedded in paraffin, cut into 5- μ m thickness, and the sections stained with hematoxylin and eosin (H&E). Stained sections were viewed under an Olympus image analysis microscope (Olympic, BX 51 TF, Japan with attached CC 12 camera). The sample was designated as FCoV UPM11C/08, where the last 2 digits refer to the year of sampling.

PCR amplification

The viral RNA was extracted from infected cell cultures using Trizol reagent (Invitrogen, USA) according to the manufacturer's instructions. The primers (p 205 and p 211) were selected from a highly conserved region of 3'untranslated region (UTR) of the virus genome (Herrewegh et al., 1995), whereas the primers (fec1b, fecv2b) designed in the regions of S-protein gene were used to differentiate between FCoV type I and II (Benetka et al., 2004). The RT-PCR protocol was performed using a master cycler gradient (Eppendorf, Hamburg, Germany). The amplification program consisted of reverse transcription, followed by an initial 2 min denaturation at 94°C, denaturation at 94°C for 30 s, annealing at 62 and 60°C for 1 min, extension at 72°C for 2 min, 35 cycles of repetitions and the final extension was achieved at 72°C for 7 min. All steps were conducted under strict hygiene to avoid the contamination. To differentiate FCoV serotype, published primers (fecv1bf and fecv1br) were used to amplify S gene region of FCoV isolate. The positive RNA of type I (FIPV NWI) reference virus was kindly provided by Dr. Vivian Benetka of University of Veterinary Medicine Vienna, Austria.

Ascites fluid and cell culture

About 5 ml of ascites fluid was collected using 20 ml syringe (TERUMO[®] SYRINGE, Tokyo, Japan) and kept into a sterile tube (Nunc[®], Denmark). The fluid was clarified by centrifugation at 1000 g for 10 min at 4°C (Hettich, Germany) and supernatant was collected. The specimen was aliquot and stored at -80°C (Sanyo, Japan) until further used. The Fcwf-4 cells were obtained commercially (ATCC[®] No. CRL-2787) and maintained in Eagle's minimal essential media supplemented with 15% heat-inactivated fetal bovine serum (FBS) (Gibco, UK), 100 IU of penicillin/ml, 100 μ g of streptomycin/ml, and 2.5 μ g of amphotericin/ml. Cultures were maintained in a humidified incubator at 37°C with 5% CO₂ (Galaxy, UK). The processed ascites was adapted and propagated in Fcwf-4 cells for the purposes of viral isolation, viral nucleic acid detection and to study the ultrastructural changes *in vitro*.

Haematoxylin and eosin staining of infected Fcwf-4 cell cultures

Monolayer of Fcwf-4 cell tissue was grown on two well chamber slides (Lab-Tek[®], USA). The slide culture was infected with 0.1 ml of 100 TCID₅₀ of FCoV sample. Haematoxylin and eosin (H&E) staining of infected monolayer of Fcwf-4 was carried out according to the method of Hsiung (1973).

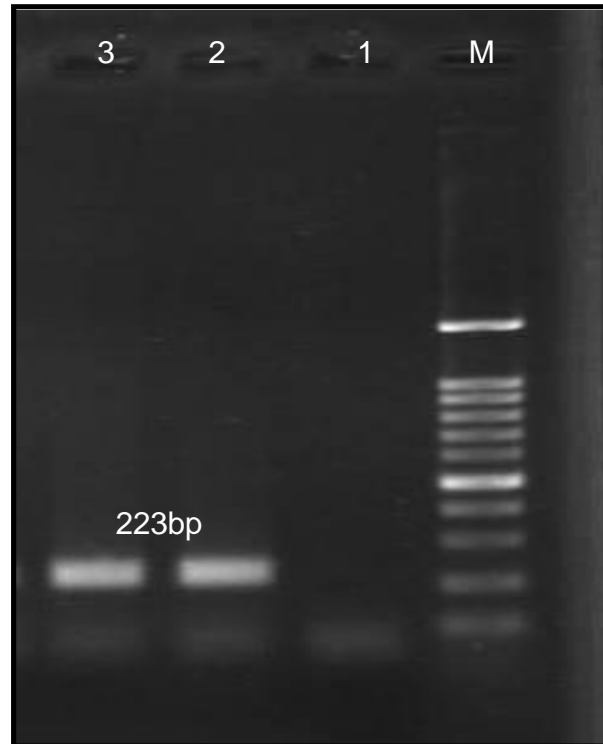


Figure 1. Screening ascites fluid and tissue samples of cats suspected of FIP for FCoV using RT-PCR and 3'UTR primers pair P205/P211 produced the target band of 223b. The 1.5% agarose gel electrophoresis stained with 0.5 µg/ml ethidium bromide. Lane M: 100 marker; NTC: non-template control; lane 1: UPM11C/08; lane 2 positive control FIPV 79-1146.

Transmission electron microscope (TEM)

Control (uninfected cells culture) and infected Fcwf-4 cells were processed for TEM as described by Hayat (1986) with little modification. Briefly, the confluent monolayers of 3 days-old Fcwf-4 cells were infected with 100 µL of FCoV sample. The infected and control flasks were incubated at 37°C for 1 h to allow virus adsorption before adding the maintenance media that containing 2% FBS. The cell cultures were further incubated and examined daily for CPE. Two infected cell culture flasks and one non-infected culture flask as control were removed consecutively at 1, 2, 3, 4, 6, 15, 24, 36, 48 and 72 h post infection (PI) for the TEM processing.

RESULTS

Detection of FCoV in ascites fluid using RT-PCR

Extracted RNA samples from FCoV suspected case UPM11C/ 08 and reference FIP 79-1146 (ATCC number VR-990), were screened by RT-PCR assay to detect FCoV. The oligonucleotide primer sequences were chosen from a highly conserved region of 3' untranslated region (3'UTR) of FCoV. All the amplified cDNA showed identical mobility on 2% agarose gel. Positive samples generated specific DNA band of 223 bp (Figure 1).

Amplification of S gene by the outer primer of type I (fecv1bf and fecv1br) for the sample in this study showed expected DNA product of 275 bp in length for type I FCoV isolate (Figure 2). The virus isolate in this study was molecularly confirmed as type 1 FCoV (Genbank, with accession number HM 628778).

Virus adaptation and propagation

The infected cell cultures showed moderate to diffuse cytopathic effect after second viral passage. The CPE pattern of infected Fcwf-4 cell cultures were characterized by an increase in opacity, refractile cells appearance initially and formation of syncytial cells at 36 h (post infection) PI (Figure 3), followed by rounded and ballooned cells at 48 h PI (Figure 4) and finally detachment of infected monolayer from the culture flask at 72 h PI.

Histopathology findings

The histopathological examination of all organs that were collected from naturally infected cat showed necrosis with

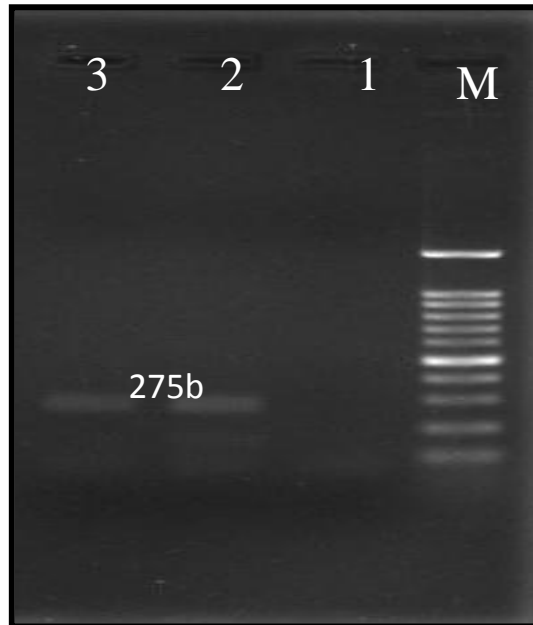


Figure 2. Screening ascites fluid and tissue samples of cats suspected of FIP for detected FCoV type I using RT-PCR assay of the S region. Primer sets fecv1bf/fecv1br detects the outer region of the S gene with product size of 275 bp. The 1.5% agarose gel was electrophoresed and stained with 0.5 µg/ml ethidium bromide. Lane M: 100 bp DNA marker; NTC: non-template control; lane 1: UPM11C/08 type I; lane 2 positive control reference virus type I FIPV NW1.

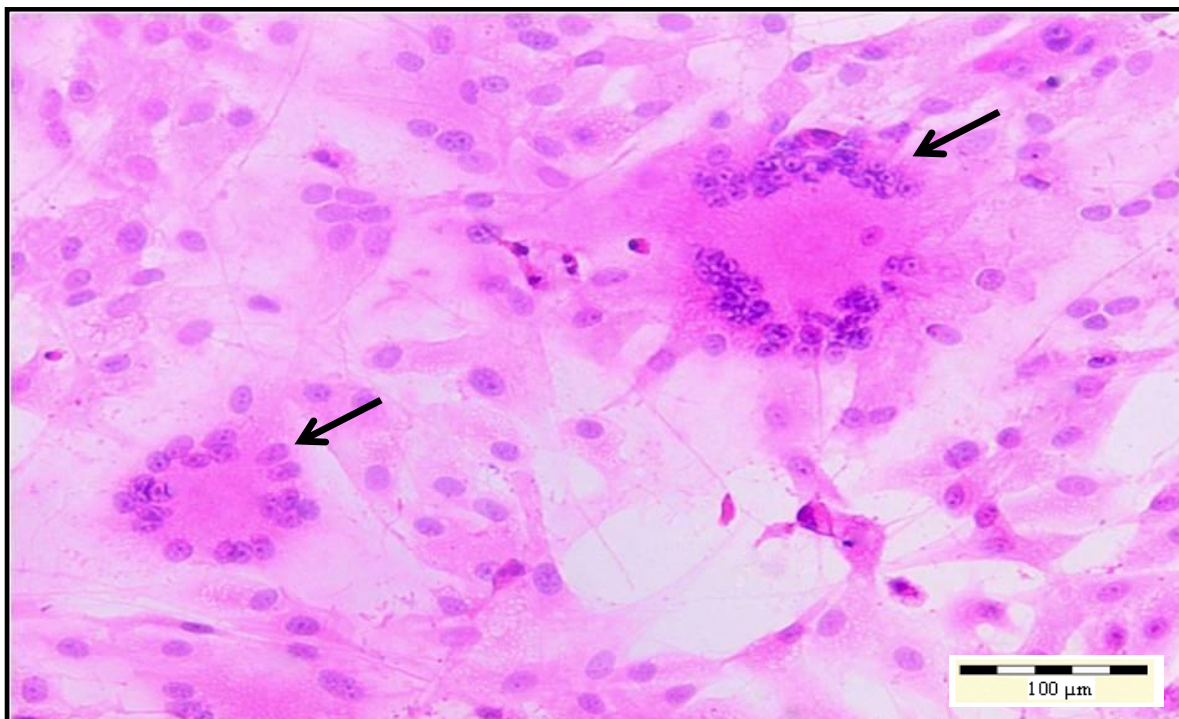


Figure 3. Fcwf-4 cell culture infected with clone purified FCoV UPM11C/08. Infected cells lost their plasma membrane and aggregate nuclei formed syncytial cells (arrows) at 36 h. H&E staining Scale bar 100.

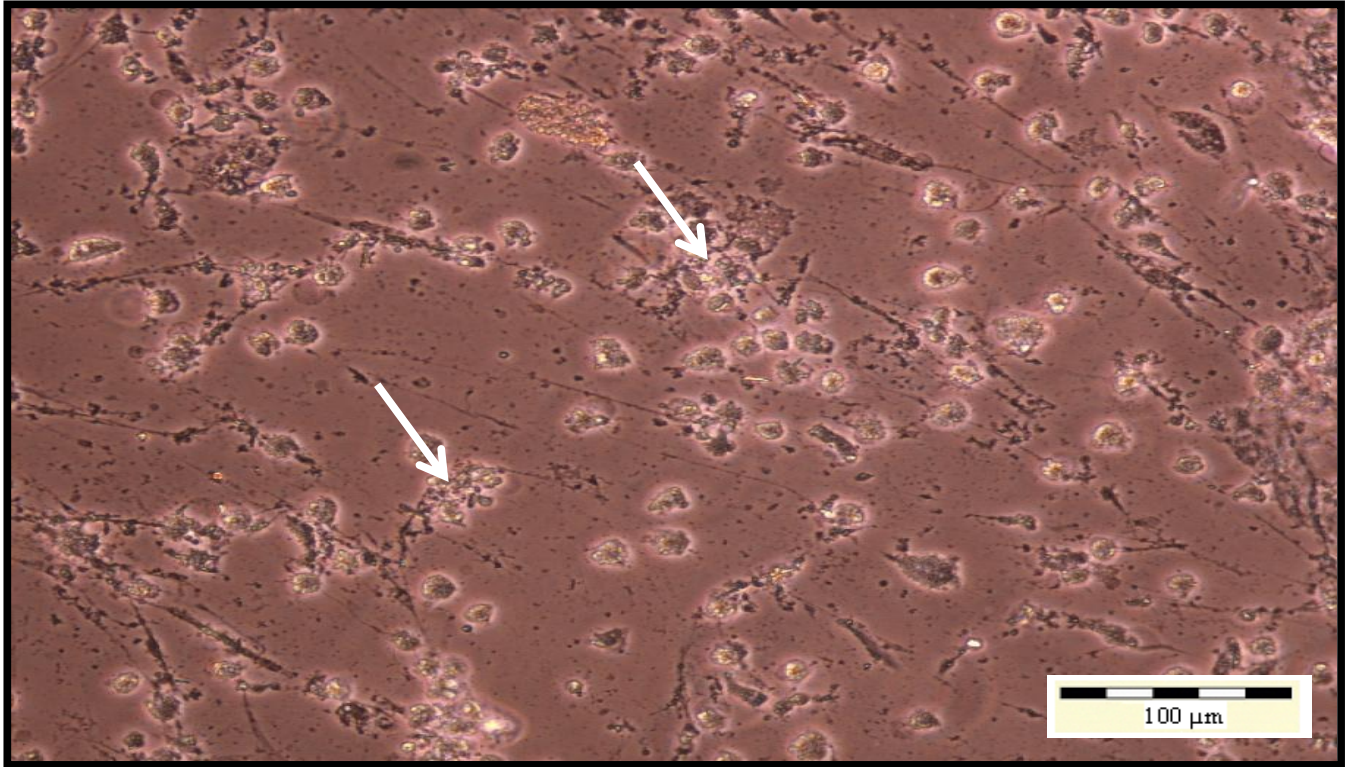


Figure 4. The unstained Fcwf-4 cell culture infected with clone UPM11C/08, showing CPE characterized by multinuclear giant cells formation and cell rounding (arrows). Scale bar 100 μ .

various degrees of infiltration of neutrophils, macrophages, lymphocytes and plasma cells. The main changes in the kidney of infected cat with effusive FIP (Figure 5) appeared that most of the renal tubular epithelium undergo either acute cellular swelling (ballooning), hydrodegeneration due to the accumulation of multiple clear water vacuoles with the cytoplasm leading to the narrowing of the lumen and giving the tubules star like shape appearance. Other tubules showed advance coagulative type necrosis characterized by increased in acidophilia of the cytoplasm with absence of nuclear staining. In the spleen (Figure 6), white pulp showed overlapping between subacute to chronic inflammatory type cells mostly plasma cells, macrophage with the existing lymphocytes, also the red pulp were inflamed by these cells. The most important finding in red pulp of the spleen was the sever congestion of the venous sinus.

In the intestine (Figure 7), most of the blood vessels, specially medium and smaller venules undergo severe congestion with pavingmenting of the polymorphonuclear leukocytes (PMNs). The surrounded loss connective tissue especially near the congested blood vessels undergoes focal aggregation or diffuse sub acute to chronic inflammatory cells mostly lymphocytes, plasma cells and macrophages. Most of the inflamed blood vessels were surrounded by infiltration of sub acute to chronic of inflammatory cells, which we called vasculitis.

The loss of connective tissues of lamina propria showed irregular edematous areas containing excessive amount of fluid.

Transmission electron microscope (TEM)

Infected Fcwf-4 cell cultures were examined after being infected with clone purified FCoV UPM11C/08. The infected cell cultures showed numerous virus particles closely opposed to the host cell plasma membrane after 2 h PI (Figure 8). At the same time, virus particles were observed to penetrate the cell by invaginating the plasma membrane and entering into the cytoplasm. Invaginated viruses were observed to have double membrane vacuole (DMV) which appears to bind the virus particles through invagination from cell membrane. In the infected cells, the virus-like particles appeared to be present in vacuoles of two types. Both types of vacuoles contained variable number of particles, ranging from a single to numerous particles (Figure 9), but there was no evidence of replication and budding of virus from this vacuoles membrane. After 48 h PI or at later stage of infection, the number of degenerated cells increased with hardly any intact cells remaining and the cytoplasm of each cell was filled with swollen vacuoles consisting mostly of mature virus particles (Figure 10).

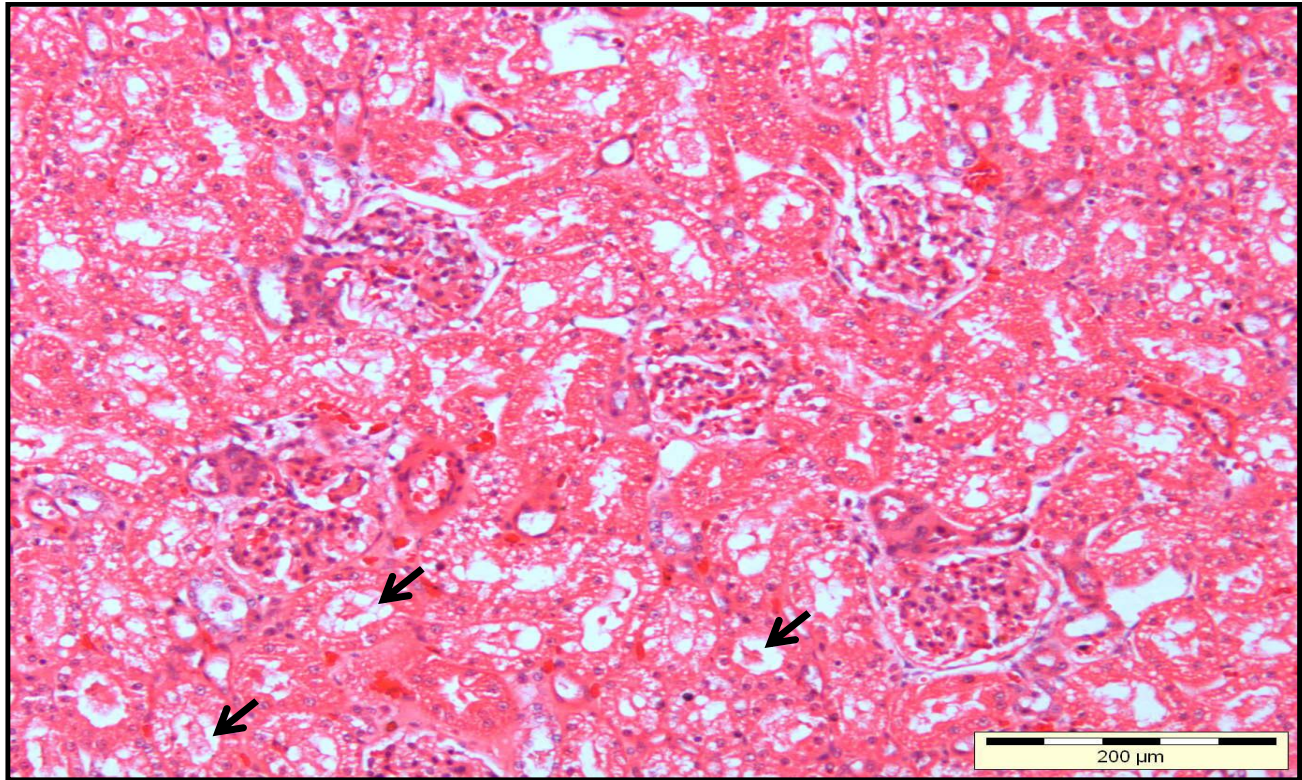


Figure 5. Kidney of cat infected naturally with effusive FIP, showing swelling (ballooning) hydropic degeneration of renal tubular epithelium leading to narrowing of the lumen give the tubules star like (arrow). H&E scale bar, 200 μ M.

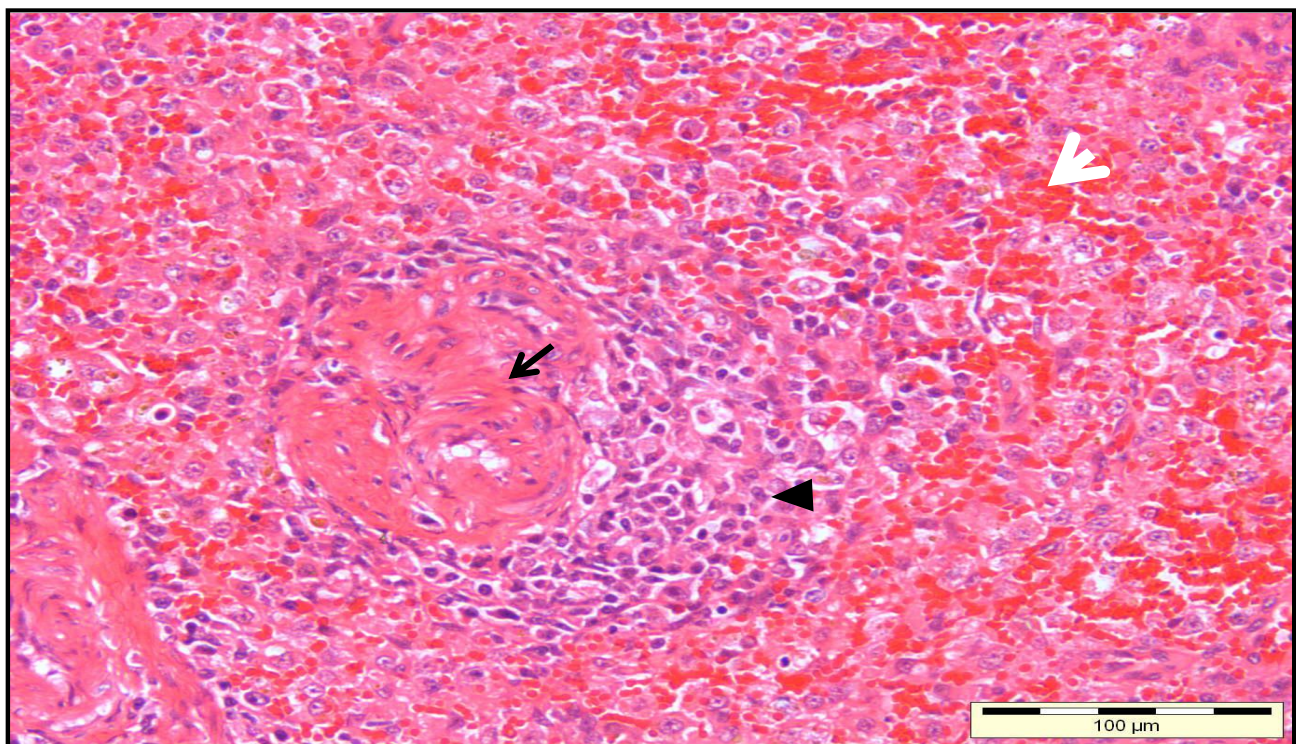


Figure 6. Intestine of infected cat with effusive FIP, showing pavementation of PMNs cells surrounding blood vessel (black arrow head). Most of the inflamed blood vessels are surrounded by infiltration of subacute to chronic type cells (vasculitis) (black arrow). H&E scale bar, 100 μ M.

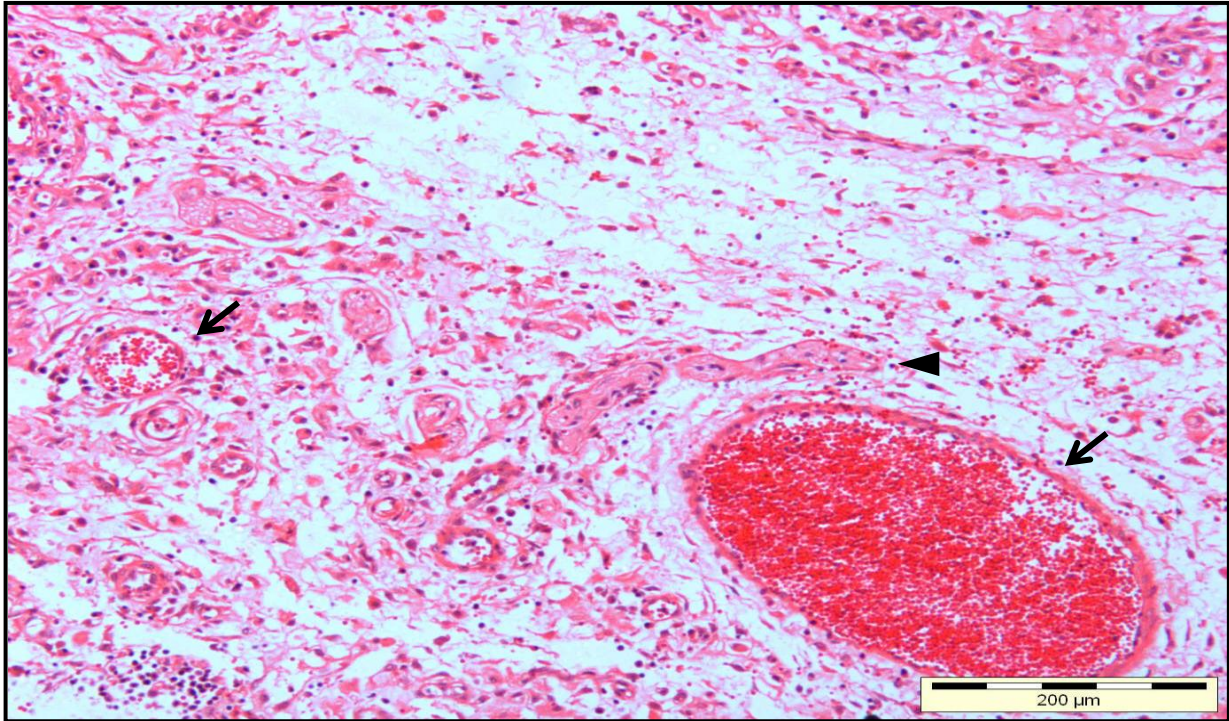


Figure 7. Intestine of infected cat with effusive FIP, shows pavementing of PMNs cells surrounded blood vessel (black arrow head) most of inflamed blood vessel surrounded by infiltration of subacute to chronic type cells (vasculitis) (black arrow). H&E Scale bar, 200 μ m.

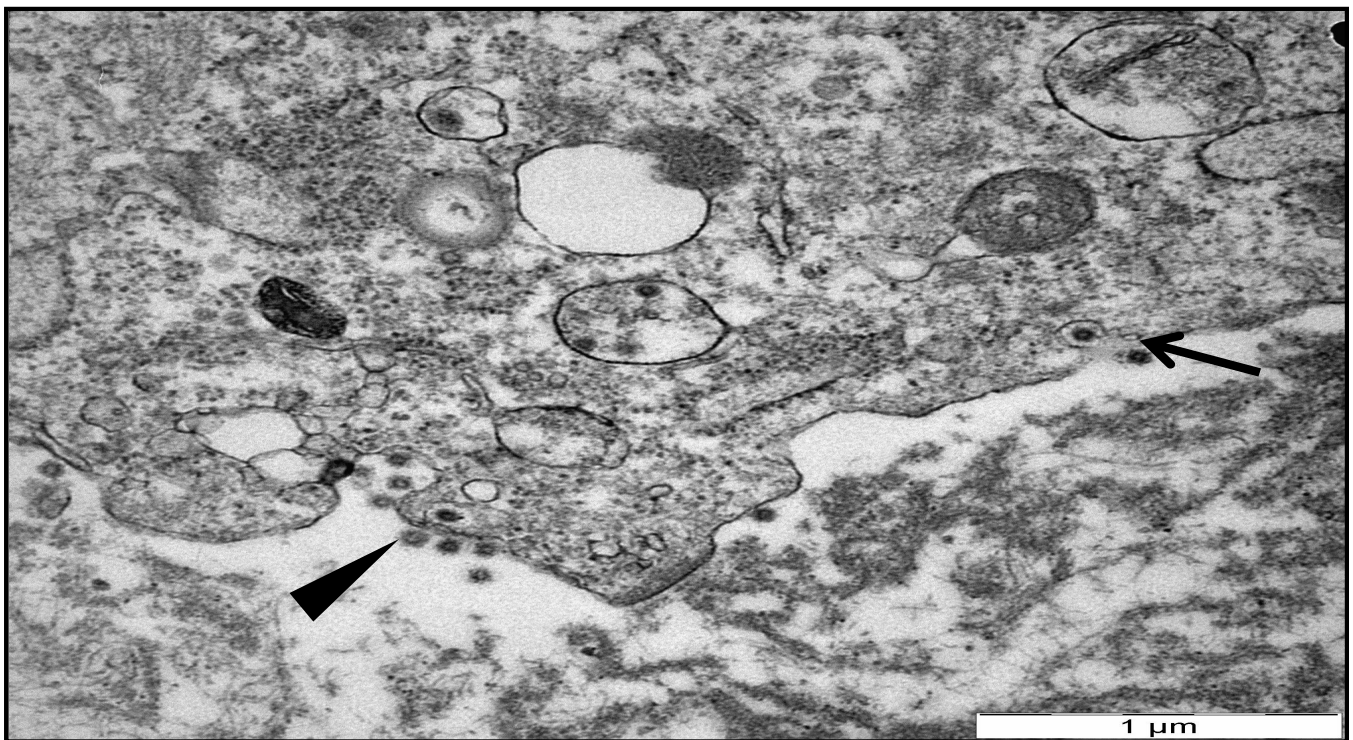


Figure 8. Micrograph of Fcwf-4 cell culture infected with FCoV UPM11C/08 showing virus-like particles invagination of host cell membrane coated with double membrane (arrow) and other virus particles attached to the plasma membrane (arrow head). Scale bar, 1 μ m.

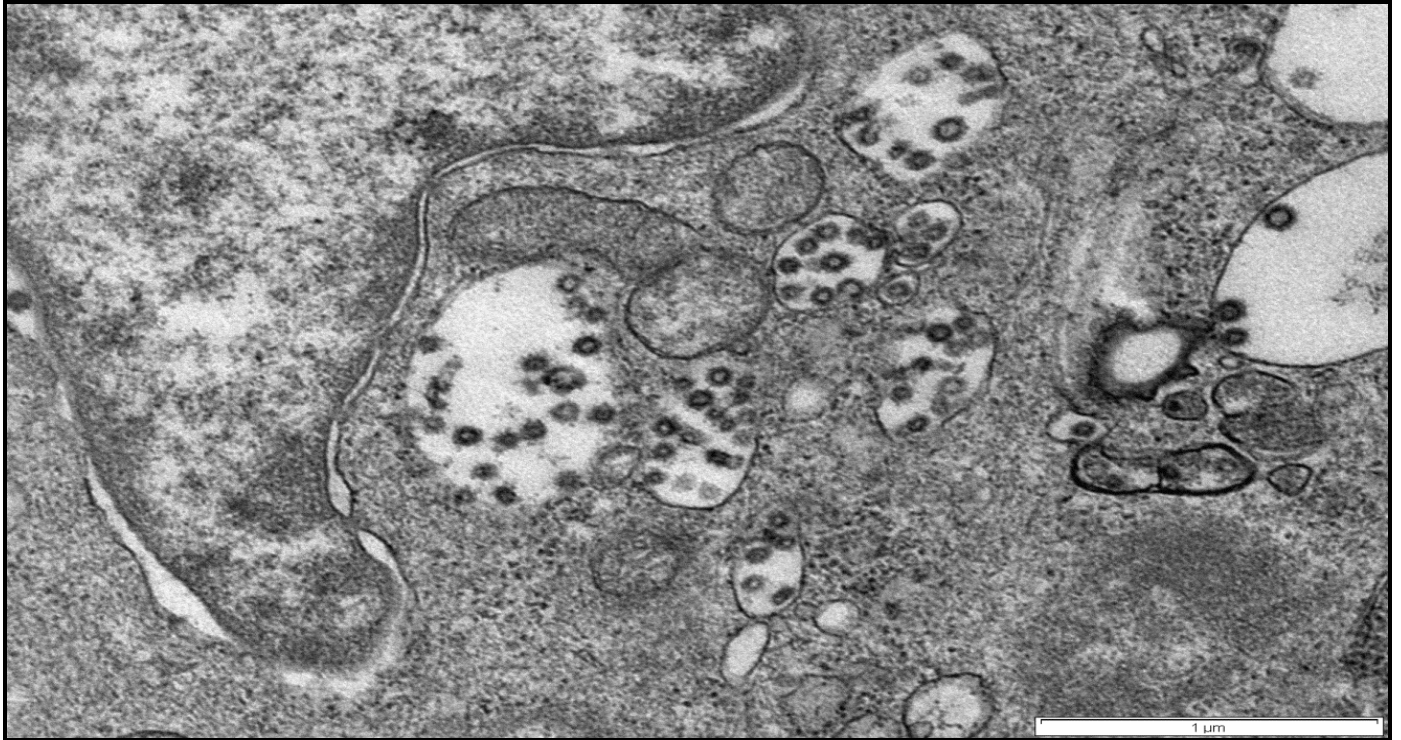


Figure 9. Micrograph showing small vacuoles containing 1 to 3 virus-like particles (white arrow), and larger vacuole containing 20 to 24 particles (black arrow). Scale bar 1 μM.

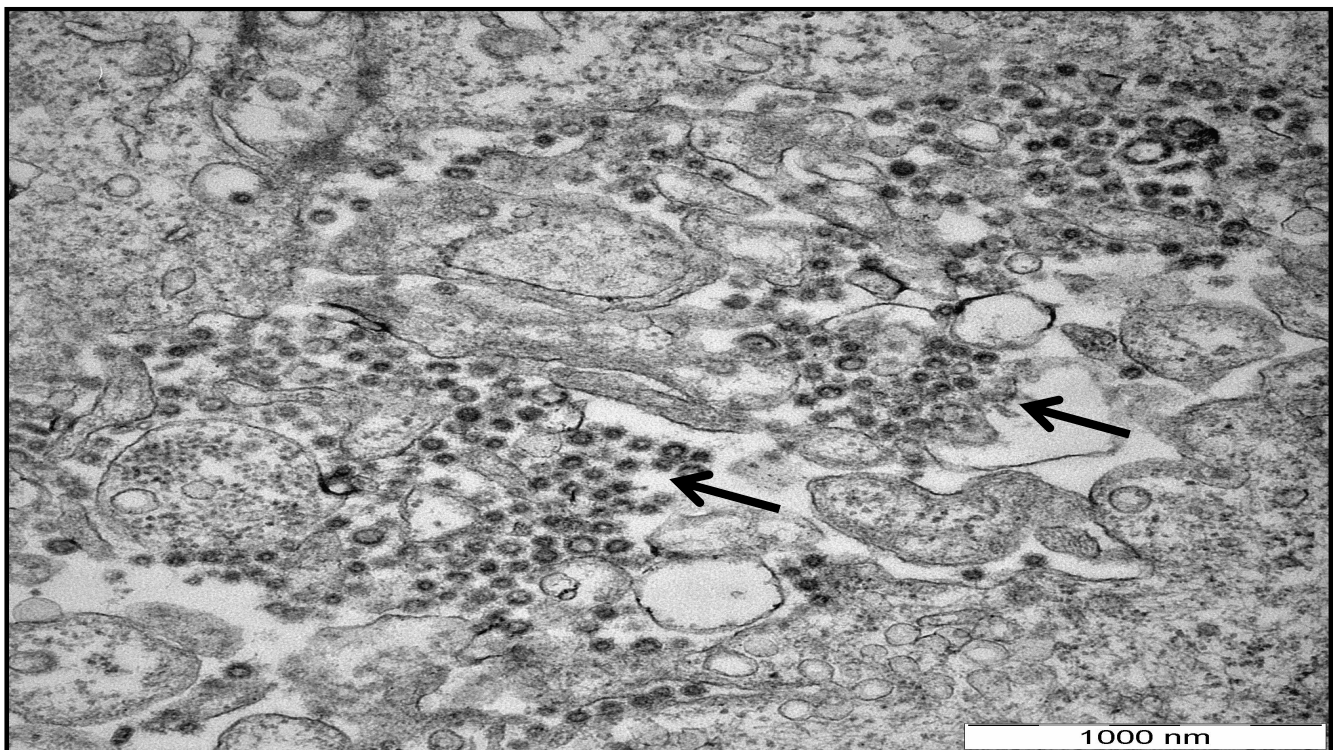


Figure 10. Micrograph of infected Fcwf-4 cell culture with FCoV UPM11C/08 after 48 h PI showing aggregation of virus particles within the membrane of vacuoles. Some parts of the vacuoles membrane were no longer intact, resulting in spillage of mature virus particles into cytoplasmic matrix (arrow). Scale bar 1000 nm.

DISCUSSION

This study describes the isolation and characterization of FCoV from cat presented with clinical evidences suggestive of FIP. Growth of local isolate FCoV UPM11C/08 in Fcwf-4 cell cultures was determined by CPE detected in the second viral passage. The CPE was characterized by an increase in opacity and refractile appearance of the infected cells at 24 h PI. Infected cells were shown to clump together, forming a giant cell known as syncytium cell which increased in size and number as the incubation period is extended. Syncytium cells was also shown to contain up to 20 to 30 nuclei per cell after 36 to 48 h PI, although 8 to 10 nuclei per cell was most common (Alazawy et al., 2011). The pathological changes observed in Fcwf-4 cells in this study are in agreement with previous reports (Jacobs and Horzinek, 1983; Pedersen et al., 1984; Pedersen, 1995). As the CPE becomes more pronounced after 48 h PI, the infected Fcwf-4 cells became rounded and detached from the surface of tissue culture flask at 72 h PI. Similar finding of CPE description that became more evident as virus passage is increased, which consisted of cell rounding, cells death and syncytial formation has been reported by many workers (Woods and Wesley, 1988; Evermann et al., 1989; Ksiazek et al., 2003). The preference of the isolates to grow in selected type of cell culture might reflect the biological properties of the virus origin. Reports have shown that FCoVs type I is difficult to grow in cell culture and that Fcwf-4 cell is more permissive (Pedersen, 1995). Similar to this assertion, our finding shows that FCoV type I has more tropism towards the monocytes/macrophages lineage such as the Fcwf-4 cells (Jacobs and Horzinek, 1983).

To further confirm our findings, RT-PCR assay was used with primers targeted to highly conserved region of the viral genome (3' UTR) (Horsburgh et al., 1992; Herrewegh et al., 1995; Bell et al., 2006). To further elucidate on the genetic characteristic of FCoV, both RNA samples from ascetic fluid and infected cell cultures were subjected to RT-PCR and the spike genes were amplified to differentiate between type I and II viruses (Addie et al., 2003; Benetka et al., 2004). The result obtained from S-gene analyses revealed the presence of type-1 FCoV based on the resultant DNA size of 275 bp as compared with reference FCoV (Figure 2). Thus, based on RT-PCR and virus isolation, the suspected sample was confirmed to be positive for type I FCoV. Our findings is in concordance with the report from other countries in which type I is the endemic FCoV strain (Addie et al., 2003; Benetka et al., Shiba et al., 2007; Lin et al., 2009).

In this study, histopathological findings on kidney, spleen and intestine were similar with those reported by other workers (Hayashi et al., 1978; Weiss and Scott, 1981; Lewis and O'Brien, 2010). The FCoV might disrupt the site of infection leading to the release of chemotactic factor, attraction of antibodies, complement, neutrophils

and macrophages surrounding the inflamed blood vessels and consequently vasculitis (Pedersen, 1995). The degenerative changes and necrotic lesions observed in kidney and other organs might be induced by disruption of the sodium- potassium- adenosine triphosphatase pump (Na⁺/K⁺ -ATPase pump) which led to hypokalemia, and subsequently retention of the sodium inside the cells led to increased osmolarity and induced hydrodegeneration which if prolonged induces irreversible necrotic changes (Ewart and Klip, 1995). Ultra-structural analysis of the FCoV UPM11C/08 following infection of Fcwf-4 cell cultures showed viral particles within cytoplasmic vacuoles and no virus particles were observed in the cytoplasm of infected cells. Our observations are in agreement with Wilhelmsen et al. (1981) and Ng et al. (2003) both of whom detected that the site of replication for coronavirus occurred in the cytoplasm of infected cells. Initially, viral particles were closely opposed to the host plasma membrane at 2 h PI, and thereafter the virions invaginated the cell membrane at different depths into the cytoplasmic matrix. Similar results were reported by previous researchers (Pedersen and Floyd, 1985; Ng et al., 2003) who detected that the attachment of virus particles to the cell membrane was observed at 1 h PI. In contrast, severe acute respiratory syndrome (SARS) coronavirus infecting Vero E6 cell cultures were found to attach, enter and un-coat its nucleocapsid at 30 minutes PI (Ng et al., 2003).

Invaginated viruses are seen to be in the form of double membrane vacuole (DMV) which appears to bound the virus particles through invagination of cell membrane. The DMV is commonly observed in positive-stranded RNA such as coronavirus (Froshauer et al., 1988; Pedersen et al., 1999). Deeply invaginated virions were seen to carry along with it parts of the cell membrane that it invaginates. The sites where the virus enters showed absence of the plasma membrane and similar observation has been reported previously (Pedersen, 1976; Evermann et al., 1989). In the infected cells, the virus-like particles appeared in two forms and can be present in vacuoles of two types. Both types of vacuoles contained variable number of particles, ranging from a single to numerous particles but there is no evidence of replication and budding of virus from this vacuoles membrane. The DMV is believed to play a role for viral defences against cellular autophagy and the membrane offering suitable microenvironment for viral synthesis and served to bind as medium to the virus particles through invagination from cell membrane (Lai, 1998; Kirkegaard et al., 2004). The vacuole membranes did not remain intact as spillages of the virus were detected in the cytoplasmic matrix or fuse directly with plasma membrane through exocytosis to infect neighbouring cells after 48 PI.

In conclusion, this study for the first time described isolation of FCoV designated as UPM11C/08 in Malaysia. The virus was found to be well adaptable in Fcwf-4 cell cultures and confirmed to belong to type I serotype by

S-gene amplification. This study provides baseline information which could be used to further understand the pathogenesis and genetic diversity of FCoV in domestic cat population.

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