UNIVERSITI PUTRA MALAYSIA

VIRAL INHIBITORY EFFECT OF CAT LITTER ON FELINE CORONAVIRUS INFECTION IN VITRO

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VIRAL INHIBITORY EFFECT OF CAT LITTER ON FELINE CORONAVIRUS INFECTION *IN VITRO*

NURUL JULIANA SYAFINAZ BINTI ABU SOPIAN

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A project paper submitted to the

Faculty of Veterinary Medicine, Universiti Putra Malaysia

In partial fulfilment of the requirement for the

DEGREE OF DOCTOR OF VETERINARY MEDICINE

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CERTIFICATION

It is hereby certified that we have read this project paper entitled "Viral Inhibitory Effect of Cat Litter on Feline Coronavirus Infection *In Vitro*" by Nurul Juliana Syafinaz Binti Abu Sopian, and in our opinion it is satisfactory in term of scope, quality and presentation as partial fulfillment of the requirement for the course VPD 4999 – Final Year Project.

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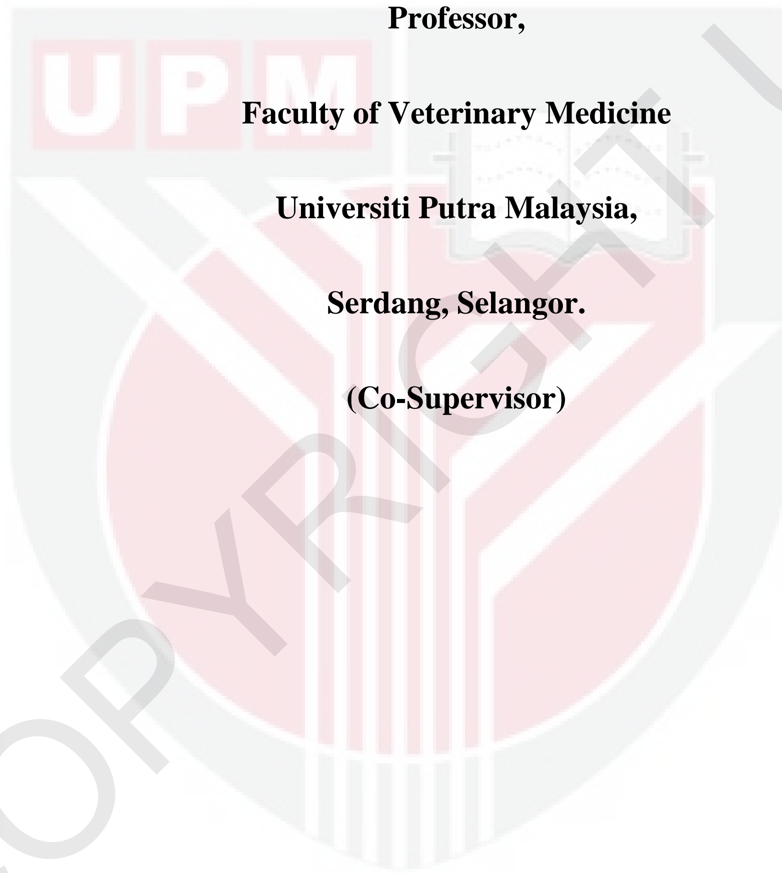

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DEDICATION

This project paper is sincerely dedicated to:

Allah the Almighty, my creator

My beloved mother (late), the source of my strength and perseverance

My supervisors for making this possible

And

My cats, Sephora & Robin, who lost their lives to FIP

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"In the name of Allah S.W.T., the Most Benevolent and Most Merciful"

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ABSTRAK

Abstrak daripada kertas projek yang dikemukakan kepada Fakulti Perubatan Veterinar untuk memenuhi sebahagian daripada keperluan kursus VPD 4999 – Projek.

KESAN PERENCATAN PASIR KUCING TERHADAP JANGKITAN KORONAVIRUS FELIN *IN VITRO*

Oleh

Nurul Juliana Syafinaz Binti Abu Sopian

2020

Penyelia: Dr Farina Mustaffa Kamal

Penyelia bersama: Prof. Dr Siti Suri Arshad

Koronavirus felin (FCoV) secara amnya boleh menyebabkan spektrum gejala daripada tiada simptom kepada cirit-birit yang ringan tetapi sekali-sekala FCoV boleh menjurus kepada penyakit maut iaitu peritonitis berjangkit felin (FIP). Koronavirus enteritis felin (FECV) sering menjadi masalah di kalangan isi rumah yang mempunyai banyak kucing terutamanya di kateri dan pusat perlindungan haiwan di mana laluan penyebaran berlaku melalui tinja-oral daripada perkongsian kotak pasir kucing. Objektif kajian *in vitro* ini dijalankan adalah untuk menentukan jika pasir kucing komersial yang didapati di Malaysia mempunyai kesan perencatan terhadap FCoV. Selain itu, objektif kajian ini juga adalah untuk mengesyorkan pasir kucing yang terbaik berpandukan hasil penemuan *in vitro* dan analisa kos. Sebanyak tujuh jenis pasir kucing telah dipilih berdasarkan status ketersediaan di kalangan sepuluh kedai haiwan peliharaan di sekitar Lembah Klang. Analisis kos pasir kucing dikendalikan

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dengan menggunakan kaedah pengukuran jumlah pasir kucing yang dibuang dalam sehari lalu kos bagi penggunaan setiap jenis pasir kucing selama tempoh sebulan dikira. Bagi menentukan kesan perencatan virus oleh pasir kucing, setiap jenis daripada tujuh jenis pasir kucing tersebut telah dicampurkan bersama FIPV 79-1146, pengeraman dijalankan selama dua jam dan supernatan yang diperoleh ditapis dan inokulasi dijalankan ke dalam sel Crandell Rees feline kidney (CRFK) yang telah dikulturkan menggunakan plat 24 lubang. Kesan sitopati diperhatikan selepas 48 jam. Kaedah dos terinfeksi kultur tisu median (TCID50) dan kaedah *Spearman-Karber* digunakan bagi menentukan titer virus. Analisa statistik menggunakan ujian *Kruskal-Wallis* menunjukkan keputusan yang signifikan ($p<0.05$) dan analisa post-hoc menggunakan ujian perbandingan berganda Dunn menunjukkan keputusan signifikan bagi pasir kucing jenis pelet kayu, kenaf dan bentonit berbanding kawalan. Namun begitu, hanya pelet kayu yang mampu mencegah jangkitan virus di dalam sel kultur sepenuhnya, manakala lima pasir kucing selebihnya sedikit sebanyak dapat mengurangkan titer virus daripada dua kali ganda sehingga enam kali ganda. Kemungkinan pasir kucing tersebut berupaya untuk mengurangkan titer virus dengan proses menjerap virus dan mengikat virion tersebut. Walaupun pelet kayu berjaya merencat jangkitan virus di dalam sel kultur, namun ianya lebih mahal daripada empat jenis pasir kucing yang lain. Kajian *in vivo* yang selanjutnya perlu dilakukan bagi menguji kesan perencatan pasir kucing di persekitaaran yang semula jadi.

Kata kunci: pasir kucing, jangkitan koronavirus felin, kesan perencatan virus, TCID50, sel CRFK

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ABSTRACT

An abstract of the project paper presented to the Faculty of Veterinary Medicine in partial fulfilment of the course VPD 4999 – Project.

VIRAL INHIBITORY EFFECT OF CAT LITTER ON FELINE CORONAVIRUS INFECTION *IN VITRO*

By

Nurul Juliana Syafinaz Binti Abu Sopian

2020

Supervisor: Dr Farina Mustaffa Kamal

Co-supervisor: Prof. Dr Siti Suri Arshad

Feline coronavirus (FCoV) generally causes asymptomatic infection to mild diarrhoea, but sporadically may develop into the highly fatal feline infectious peritonitis (FIP). Feline enteric coronavirus (FECV) is a common problem in multi-cat households especially catteries and shelters as the route of transmission is via faecal-oral route through sharing of litter boxes. The aim of this *in vitro* study was to determine whether commercial cat litters in Malaysia has antiviral properties against FCoV. Another objective was to recommend best cat litter with reference to *in vitro* results and cost analysis. A total of seven types of cat litters were chosen based on their availability in ten pet shops surveyed within Klang Valley. The cost analysis of each litter type was determined by measuring the amount of litter removed in a day and the cost of litter per month was then calculated. To determine the viral inhibitory effect of the cat litter, each of the seven types of cat litter were mixed with FIPV 79-

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1146 strain, incubated for 2 hours and the supernatants were filtered and inoculated into 24-well plates of Crandell Rees feline kidney (CRFK) cells. Cytopathic effects (CPE) were observed after 48 hours. Median tissue culture infectious dose (TCID50) method was used to determine the viral titre using Spearman-Karber method. Statistical analysis using Kruskal-Wallis test showed significant result ($p<0.05$) and post-hoc analysis of Dunn's multiple comparisons test revealed significant result for wood pellet, kenaf and bentonite *versus* control. However, only wood pellet managed to completely prevent virus infection in cell culture, while the other five cat litters reduced virus load to varying extent from 2-fold to 6-fold reduction. Cat litters presumably reduced viral titre by adsorbing virus and binding the virions. Although wood pellet successfully inhibited virus growth in CRFK cells, it was costlier than four other cat litters. Further *in vivo* study needs to be done to test the inhibitory effects of the cat litters in natural settings.

Keywords: cat litter, feline coronavirus infection, viral inhibitory effect, TCID50, CRFK cells

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1.0 INTRODUCTION

1.1 Background

Feline coronavirus (FCoV) is an enveloped single-stranded RNA virus in the *Coronaviridae* family. FCoV manifests in two biotypes which are the feline enteric coronavirus (FECV) which is ubiquitous in catteries and multi-cat households and the mutated form, feline infectious peritonitis virus (FIPV). FECV generally causes asymptomatic infection to mild gastroenteritis but mutation of FECV leads to FIPV biotype that causes a highly fatal and systemic disease. FECV transmission occurs by faecal-oral route in which the transmission of FECV could occur through sharing of cat litter especially in multi-cat environment (Drechsler et al., 2011).

Currently, there is only one commercially available intranasal vaccine to aid in the prevention of FCoV for kittens of more than 16 weeks old; however, the efficacy of this vaccine is questionable. In addition, maternally derived FCoV antibodies in kittens decline after 60 days, which leaves a window for infection before vaccination can be performed (Day, 2007). Therefore, preventing infection of FCoV at the primary level can avoid the potential occurrence of FIP. There was a recent evidence that Fuller's earth-based cat litters can reduce occurrence of infection or re-infection of FCoV, thus decreasing the chances of FIP to develop (Addie et al., 2020). However, this type of cat litter is not commonly found in pet stores in Malaysia. Hence, this study aims to identify common commercial cat litters used in Malaysia that has similar viral inhibitory properties.

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1.2 Justification

In Malaysia, prevalence of FCoV is as high as 84% in a study conducted within two catteries (Sharif S., 2009). By identifying a commercial cat litter that is capable of virus inhibition, proposed cat litter can be implemented widely in shelters and multi-cat households. Fuller's earth-based cat litters or aluminium magnesium silicate were able to inhibit FCoV infection in cell culture (Addie et al., 2020). However, composition of cat litters varies according to different regions depending on availability of raw materials. Whether or not the different types of cat litters available in Malaysia have the same viral inhibitory effect are unknown.

1.3 Objectives

Hence, the aim of this study was:

1. To determine commercial cat litters commonly found in Malaysia.
2. To investigate the effect of commercial cat litters on the inhibition of feline coronavirus infection in cell culture.
3. To recommend the best commercial cat litter(s) that can be used for the prevention of feline coronavirus transmission based on the results of *in vitro* study and cost analysis.

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1.4 Hypothesis

The expected outcome of this study would be that at least one commercial cat litter commonly found in Malaysia has viral inhibitory properties towards feline coronavirus *in vitro.*

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2.0 LITERATURE REVIEW

2.1 Feline Coronavirus

Feline coronavirus (FCoV) belongs to the family *Coronaviridae* and *Alphacoronavirus*. It is an enveloped positive-stranded RNA virus that can infect domestic and wild *Felidae* worldwide (Tekes & Thiel, 2016). The single RNA strand is made up of approximately 29,000 nucleotides consisting of 11 open reading frames (ORFs) which are two major ORFs (ORF1a and ORF1b) for non-structural proteins (nsp), four structural ORFs for envelope spike protein (S), membrane protein (M), envelope protein (E) and nucleocapsid protein (N). Additional five accessory ORFs lie between S and E ORFs (3a, 3b and 3c), and downstream of the N gene (7a, 7b) (Drechsler et al., 2011).

2.2 Classification of Feline Coronavirus

Serologically, FCoV can be classified into two serotypes, type I and type II. Type I FCoV is entirely feline-associated and it is more prevalent in clinically-infected cat population worldwide including Malaysia (Amer et al., 2012). However, isolation of Type I FCoV in cell culture is difficult compared to Type II. Type II FCoV results from a double recombination between type I FCoV and canine coronavirus (CCoV) and is adaptable to growth in cell lines such as Crandell Reese feline kidney (CRFK) cells and felis catus whole fetus 4 (Fcwf-4) cells (Jaimes & Whittaker, 2018). FCoV is widely recognized by its two biotypes, the ubiquitous enteric biotype caused by feline enteric coronavirus (FECV) and feline infectious peritonitis virus (FIPV) which is a highly virulent biotype that leads to feline infectious peritonitis (Pedersen, 2009).

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FECV typically localize at intestinal epithelial cells causing asymptomatic enteric infections to mild diarrhoea. Once FIP develops, severe systemic disease will be present in either the effusive wet form or neurological dry form (Drechsler et al., 2011).

2.3 Pathogenesis and Transmission of Feline Coronavirus

Seroprevalence of FECV are from 20% up to 100% worldwide but FIP syndrome develops in less than 10% of infected cats (Brown et al., 2009). Since the transmission is through shedding of the virus in faeces and ingestion through grooming, multi-cat environments tend to have increased occurrence of FIP due to sharing of litter trays. Faecal shedding of FCoV is presumably thought to be FECV due to its infection and replication in the gut. Constant replication of FECV in the intestine gives rise to truncating mutations in *3c* gene where it is commonly associated to FIP but it is not always the sole cause of FIP (Chang et al., 2010). FIPV which infects monocytes and macrophages is infrequently spread in a horizontal manner due to the unique mutations of 3c gene to each cat. However, FIPV with an intact 3c gene were found shed in faeces although it is very rare (Pedersen et al., 2012). Besides 3c mutation, another study by Licitra et al., (2013) concluded that mutation within spike protein gene leads to change in cellular tropism and development of FIP. Still, it is unknown whether it is the single cause of FIP.

Faecal viral shedding can appear as early as one week after infection with persistent shedding at high levels for 2 to 10 months. FCoV can either be shed consistently at varying levels up to 24 months, shed intermittently or ceased shedding

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after about 12 months (Pedersen et al., 2008). Cats with a higher dose FCoV infection tend to have earlier excretion as well as slower clearance and kittens generally shed more virus than adult cats (Vogel et al., 2010).

2.4 Diagnosis of Feline Coronavirus

The gold standard for the diagnosis of FIP is histopathology using immunohistochemical staining (IHC) of abnormal tissues and usually done via post-mortem or invasive procedures such as laparotomy or laparoscopy. Therefore, no single ante-mortem test can definitively diagnose FIP and diagnosis often involve combination of signalment, history, clinical signs and a number of clinical assays (Kennedy, 2020).

Complete blood count and serum biochemistry are not pathognomonic but routinely should be done to aid in diagnosis. Analysis of effusion, on the other hand, is helpful in wet form FIP where it is typically characterized by a highly cellular, protein and fibrin-rich modified transudate. A study by Hazuchova et al., (2017) found that α1-acid glycoprotein (AGP), one of the acute phase proteins, was useful in differentiating FIP in cats when measured in effusions. Conversely in dry form FIP, antibody titre in cerebrospinal fluid of cats can be measured and a titre of greater than 640 was indicative of FIP (Soma et al., 2018).

A common method of detecting the virus is through RT-PCR but differentiation between FECV and FIPV is difficult. An mRNA RT-PCR in accordance to the laboratory results, signifies high specificity in detection of virus

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whereby the high numbers of viral mRNA correlates with the degree of virus replication (Simons et al., 2005). FIPV may be indicated when viral copy numbers are high in samples from extraintestinal sites such as blood, effusion or tissue. Another RT-PCR specifically designed to detect FCoV S gene mutation was found to have high specificity for effusion samples but there is still uncertainty regarding role of S gene in FIP pathogenesis (Felten et al., 2017).

Tissue culture infectious dose 50 (TCID50) assay is an endpoint dilution essay used for virus quantification. TCID50 is done in serial dilutions of virus and identifying the dilution at which 50% of infected cells are showing cytopathic effects (CPE). Reed and Muench method or Spearman-Karber method are two calculations that has been commonly used for calculation. A newly proposed formula by Ramakrishnan has been evaluated and it is a much simpler method although not proposed to be used exclusively (Ramakrishnan, 2016). Since FCoV causes CPE in cell culture, TCID50 is selected as opposed to median lethal dose (LD50) where it is often used when virus does not form CPE.

2.5 Control & Prevention of Feline Coronavirus

Proper hygiene is crucial in the control and prevention of FCoV because the virus is predominantly transmitted via faecal-oral route. Ideally, a single cat should be given access to a personal litter tray, but it is not practical. Otherwise, sufficient amount of litter box should be provided with frequent cleaning and kept at a distance from the food bowls (Lloret, 2009). In FCoV-endemic catteries and shelters, providing individual cages are effective in avoiding sharing of litter trays although it requires

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bigger infrastructure and staffs. Individual cages can be used instead to isolate and monitor heavy shedders. Segregation of cats according to antibody status can prevent exposure to naïve cats while segregation by shedding status can prevent reinfection of cats; however, both requires serology or PCR testing which will be costly. By establishing a workflow for staff to manage new cats and only then move on to the long-term residents, this will reduce exposure to naïve cats (Drechsler et al., 2011). Strict sanitation must be enforced to minimise spread and stress should also be minimized.

In terms of vaccination, there is only one commercially available vaccine but the efficacy is doubtful and it is administered intranasally at 16 weeks of age. Maternal derived antibodies only protect kittens until 5-6 weeks of age, which leaves a window of infection until 16 weeks old and the vaccine is beneficial only if kittens have not been exposed to FCoV (Lloret, 2009).

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3.0 MATERIALS AND METHODS

3.1 Selection of Cat Litters

Ten pet shops in Klang Valley, Selangor was surveyed for the types of cat litters sold. Eight different types of cat litters were chosen primarily according to the material. Availability of each type of cat litters in the pet shops were also recorded to determine the common commercial cat litters found in Malaysia.

3.2 Cost Analysis

A total of 500g cat litter of each type was poured into 35 cm x 25 cm litter tray. 80 mL of tap water with food colouring added was poured onto litter. The 80 mL was calculated based on average daily urine output for an adult cat which was 20 mL per kg, assuming a 4 kg body weight. This method involved urine output due to limitations to simulate faecal output. After 24 hours, soiled and clumped cat litter was removed and remaining cat litter was weighted. Calculation for cost effectiveness of cat litter is shown as the following:

Cost of cat litter per month $= \text{Price of cat litter/g} \times \text{Amount of litter used/day (g)} \times 30 \text{ days}$

$$= \left(\frac{\text{Price of a bag of cat litter}}{\text{Weight of a bag of cat litter (g)}}\right) \times (500\text{g} - \text{remaining cat litter}) \times 30 \text{ days}$$

Example of calculation for tofu cat litter:

Cost of tofu cat litter per month $= \left(\frac{\text{RM } 20}{2800\text{g}}\right) \times (500\text{g} - 446\text{g}) \times 30 \text{ days}$

$= \text{RM } 11.60$ per month

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3.3 Cell Culture

Crandell-Reese feline kidney (CRFK) cells at more than 90% confluency were sub-cultured into T75 flasks (SPL Life Sciences, Korea). After removing used media, the cells were thoroughly rinsed with 10 mL Phosphate Buffer Saline (PBS), pH 7.2 twice to remove traces of serum that may inhibit the activity of trypsin. Next, 2 mL of 0.5% trypsin-EDTA (Gibco, United States) were added and incubated at 37ºC for 2 mins to 3 mins. The flask was removed from incubator and swirled gently to aid detachment of cells. The cells were viewed under microscope (Olympus, Japan) at low magnification (x4) to observe approximately 90% of cells detached. Then, 2 mL of pre-warmed minimum essential media (MEM) supplemented with 15% fetal bovine serum (FBS) (Gibco, United States) and 1% Penicillin-Streptomycin (HiMedia, India) were added into the flask. Subsequently, the media containing detached cells in the flask were transferred to a 15 mL centrifuge tube (SPL Life Sciences, Korea) and centrifuged at 15,000 rpm for 5 mins. Afterwards, supernatant was removed and 4 mL of growth media was added and resuspended using pipette (Eppendorf, Germany) to break any clumping of cells so the cells could attach to the flask in a uniform manner. The cells were then seeded into two T75 flasks at 2 mL with concentration of $2x10^6$ cells/mL per flask and incubated at 37’C, 5% CO_2 (Eppendorf, Germany).

CRFK cells in one T75 flask that reached more than 90% confluency were used to subculture into four 24-well plates (BioFil, India). Cell passaging was treated the same as previously described. 4 mL of cells were divided into two 50 mL centrifuge tubes (Thermo Fisher Scientific, United States) equally. Next, 22 mL of

growth media was added into each tube and resuspended. Finally, 500 uL of cells were pipetted into each well of 24-well plate and incubated at 37°C, 5% CO_2.

3.4 Cell Counting

Cells were counted using Cell Counting Chamber Improved Neubauer Hemacytometer (Marien Field, Germany). CRFK cells in T75 flask with 100% confluency was used. After rinsing with PBS twice and treated with trypsin-EDTA, 2 mL of growth media was added and cells were transferred into 15 mL centrifuge tube to be centrifuged. After discarding the supernatant, 1 mL of media was added and resuspension of media was done to avoid clumping. Then, 20 uL of cells were mixed with 20 uL of 0.4% trypan blue stain (Gibco, United States) before seeding into both chambers at 10 uL each.

Living cells appeared round and transparent while dead cells looked dark with roughened edges. Four large squares at each corner were chosen for enumeration. Total number of cells were obtained using the following formula (Absher, 1973):

$$\text{Total number of cells/mL} = \frac{\text{Total number of cells}}{\text{No. of box counted}} \times \text{dilution factor} \times 10^4 \text{ cells/mL}$$

$$= \frac{981}{4} \times 2 \times 10^4 \text{ cells/mL}$$

$$= 4.9 \times 10^6 \text{ cells/mL}$$

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3.5 Virus Propagation

CRFK cells in T25 flask at 80-90% confluency were rinsed with 5 mL PBS twice. The cells were then inoculated with 0.1 mL of FIPV 79-1146 strain and incubated for 1 hour. After incubation, 5 mL of growth media was added and flask was incubated at 37°C, 5% CO_2 (Thermo Fisher Scientific, United States) until cytopathic effect (CPE) was observed. Next, the flask was subjected to freeze-thaw cycle for three times to break the cells allowing the virus to be released into the supernatant. Then, the content was pipetted into a 15 mL centrifuge tube. The tube was centrifuged at 3000 rpm for 5 mins and the virus supernatant was kept in -20°C until further used. The FIPV 79-1146 virus propagation in CRFK cells was repeated several times until the virus stock was adequate for the subsequent analyses.

3.6 Median Tissue Culture Infectious Dose

Median tissue culture infectious dose (TCID50) method was used for virus titer quantification. CRFK cells in T75 flask that has reached >90% confluency was selected to seed into four 24-well plate at $0.05x10^6$ cells per well. The seeded 24-well plates were incubated at 37°C, 5% CO_2 for two days to obtain 90% confluency.

After 24-well plates reached 90% confluency, two 24-well plates were used for determination of FIPV stock titre. A 10-fold serial dilution was made from the virus stock. 100 µL of virus stock was pipetted into 1.5 mL centrifuge tube containing 900 µL MEM supplemented with 10% FBS and 1% Penicillin-Streptomycin. After resuspending for ten times, 100 µL was transferred from the first (1st) tube into the

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subsequent tubes (2nd-9th) and process was repeated until the tenth tube (10th). Then, 200 µL of the first dilution (10^{-1}) was added into the first well of the first column in a 24-well plate followed by 10^{-2} to 10^{-10} dilutions into the subsequent wells. The last two wells of the last two columns were added with 200 µL of MEM to serve as control (Figure 1). This process was repeated for the other three rows which served as replicates.

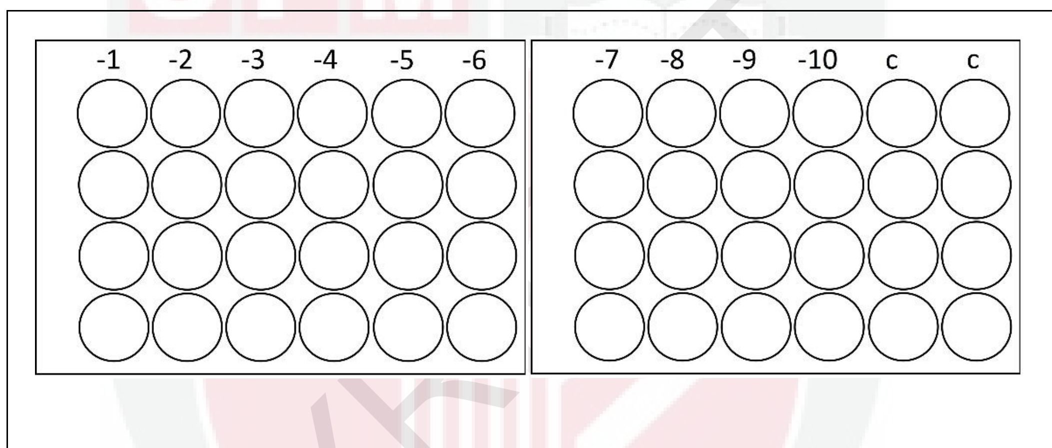

Fig. 1. Template for TCID50 inoculation in a 24-well plate

The cells were monitored daily and CPE was recorded after 48 hours. $TCID_{50}$/mL value was calculated using Spearmen (1908)–Karber (1931) method as shown below:

$\log_{10}$ 50% end point dilution = $-(x_0 - d/2 + d \sum r_i/n_i)$

$x_0 = \log_{10}$ of the reciprocal of the highest dilution (lowest concentration) at which all animals are positive;

$d = \log_{10}$ of the dilution factor;

n_i = number of animals used in each individual dilution (after discounting accidental deaths);

r_i = number of positive animals (out of n_i).

Summation is started at dilution x_0.

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Example of TCID50 calculation for determination of FIPV stock titre:

$$\log_{10} 50\% \text{ end point dilution} = -\left[7 - \frac{1}{2} + 1\left(\frac{6}{4}\right)\right] \times 1$$

$$\log_{10} 50\% \text{ end point dilution} = -8$$

$$50\% \text{ end point dilution} = 10^{-8} \text{ for } 0.2 \text{ mL}$$

$$\text{For 1 mL} = \text{TCID}_{50}/\text{mL} = 10^{8} \times 5$$

$$= 10^{8.7}$$

$$= 5 \times 10^{8} \text{ TCID}_{50}/\text{mL}$$

3.7 Virus Dilution

The virus stock was diluted to obtain 3.75×10^{7} TCID_{50}/mL by using the following common dilution equation:

$$C_1V_1 = C_2V_2$$

where

C_1 = initial concentration or molarity

V_1 = initial volume

C_2 = final concentration or molarity

V_2 = final volume

Example of calculation for dilution of virus stock:

$$(5 \times 10^{8})V_1 = (3.75\times 10^{7})(1 \text{ mL})$$

$$V_1 = \frac{3.75\times10^{7}}{5 \text{ x } 10^{8}}$$

$$V_1 = 0.075 \text{ mL}$$

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Therefore, 75 μL of virus stock was added to 925 μL of media to get 1 mL of FIPV at concentration of 3.75×10^7 $TCID_{50}$/mL.

3.8 Sample Processing and Residual Virus Titration

A total of 4 mL of FIPV 79-1146 strain at 3.75×10^7 $TCID_{50}$/mL were mixed with 1 g of cat litter sample in a 15 mL centrifuge tube and rotated (Stuart Scientific, United Kingdom) at room temperature for 2 hrs. Then, the tube was spun at 3000 rpm for 10 mins (Kubota, Japan) and supernatant was transferred into 1.5 mL centrifuge tube (Eppendorf, Germany). Next, the tube was spun at 14000 rpm for 5 mins and the virus supernatant was filtered through a 0.45 μM filter (Sartorius Stedim, France). A total of 100 uL virus supernatant was subjected to a 10-fold serial dilution and the dilution series was made in triplicates. Then, TCID50 method of viral quantification was performed as described earlier to determine the viral titre upon treatment with the cat litters. The plates were incubated at 37°C, 5% CO_2 and CPE was examined and recorded for 48 hours.

As a positive control, 1 mL of FIPV at 3.75×10^7 $TCID_{50}$/mL without cat litter was added into 15 mL centrifuge tube and treated as above. As for negative control, 4 mL of MEM without FIPV was mixed with 1 g cat litter and treated as above to check for possible cytotoxic effects of the litter towards the cells.

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3.9 Statistical Analysis

Statistical analysis with Kruskal-Wallis test was carried out using GraphPad Prism (Version 8.4.3, USA) with p-value set at 95% confidence interval. If significant, post-hoc analysis of Dunn's multiple comparisons test was done to identify which cat litter was significant.

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4.0 RESULTS

4.1 Identification of Common Commercial Cat Litters

A survey of 10 pet stores in Klang Valley, Selangor revealed seven common types of cat litters. Silica was the most common cat litter where it was found in nine out of ten stores, followed by bentonite and wood pellet at eight out of ten stores (Figure 2). The least common cat litter was non-clumping clay whereby only two stores carried that litter type.

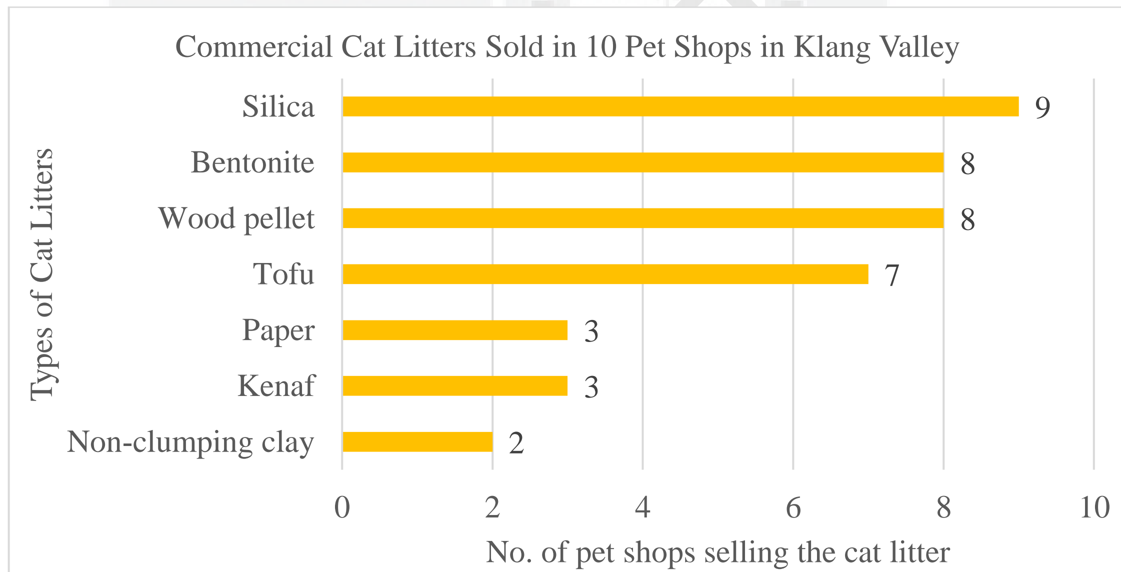

Fig. 2. Number of pet stores carrying a particular litter type in descending order

4.2 Cost Analysis

Based on the seven cat litters, recycled paper was the most cost effective in terms of amount used in a day as well as cost of litter per month. Recycled paper used the least amount at 20 g per day, while wood pellet used up the most at 214 g of litter removed per day (Table 1). Recycled paper costed the lowest at RM3.30 per month and kenaf costed the highest at RM29.70 per month (Figure 3).

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Table 1. Cost analysis of seven types of cat litters in increasing amount

Type	Price (RM)	Weight (g)	Price of litter/g (RM)	Amount used/day (g)	Cost of litter/day (RM)	Cost of litter/month (RM)
Recycled paper	8.40	1500	0.0056	20	0.11	3.40
Bentonite	20.00	9000	0.0022	131	0.29	8.70
Tofu	20.00	2800	0.0071	54	0.38	11.60
Non-clumping clay	12.90	4500	0.0029	143	0.41	12.30
Wood pellet	31.00	9000	0.0034	214	0.73	22.10
Silica	17.80	2500	0.0071	119	0.84	25.40
Kenaf	18.90	2000	0.0095	104	0.99	29.50

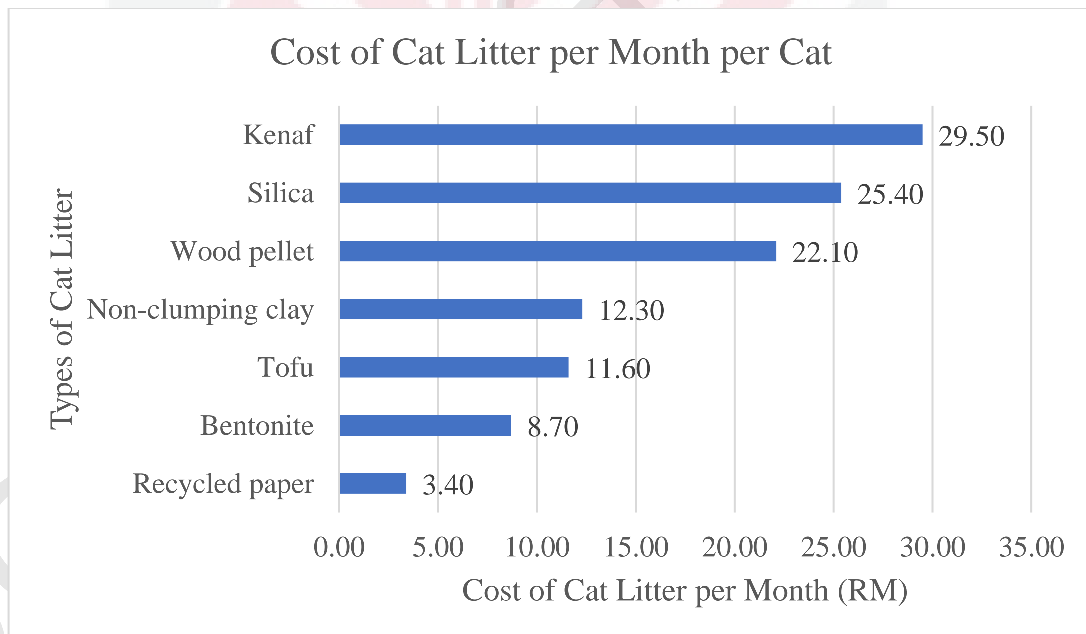

Fig. 3. Cost of seven types of cat litters per month per cat in decreasing amount.

4.3 *In Vitro* Effect of Cat Litter Against FCoV

The results of effects of cat litter on FCoV infectivity are calculated (Appendix A) and summarized in Table 2. None of the seven cat litters cause cytotoxicity to CRFK cells. However, paper litter was excluded from the final result due to unhealthy CRFK cells at the start of experiment, leaving six cat litter left for final analyses.

All six cat litters reduced virus titre up to a varying extent (Figure 4). Only one cat litter which was wood pellet, managed to fully inhibit FCoV infection significantly in cell culture. Non-clumping clay and tofu litter were able to reduce virus titre by 2-fold. Silica and bentonite reduced FCoV load by 4-fold and 5-fold respectively. Subsequently, kenaf almost fully inhibited FCoV infectivity demonstrated by 6-fold reduction of viral titre. Among the six cat litters tested, wood pellet, kenaf and bentonite showed significant result when compared to virus control (p=0.0077). However, comparison between types of cat litters showed no significant outcome. Hence, the null hypothesis was rejected and there was viral inhibitory effect of at least one type of cat litter.

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Table 2. Reduction of FCoV titre for cell culture infection in decreasing effect

Cat Litters	Cytotoxicity	$TCID_{50}$/mL Before	$TCID_{50}$/mL After	Fold Reduction
Wood pellet	No	5×10^7	0	Complete reduction
Paper*	No	5×10^7	Invalid	-
Kenaf	No	5×10^7	0.9×10	6 – fold
Bentonite	No	5×10^7	1.7×10^2	5 – fold
Silica	No	5×10^7	1.9×10^3	4 – fold
Tofu	No	5×10^7	2.9×10^5	2 – fold
Non-clumping clay	No	5×10^7	3.9×10^5	2 – fold

*Excluded from final result due to unhealthy CRFK cells at start of experiment.

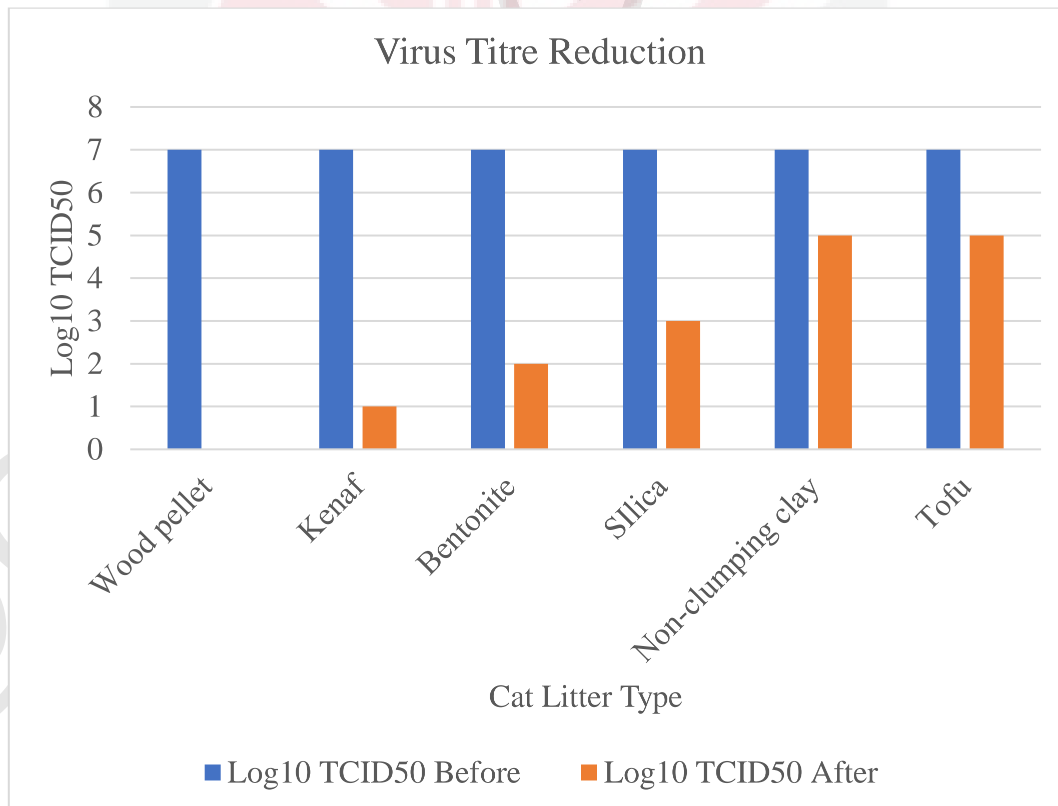

Fig. 4. FCoV titre reduction in six cat litters in decreasing effect.

5.0 DISCUSSION

5.1 Properties of Cat Litter

A variety of cat litters made from different types of materials can be found in the market today to meet pets' and owners' demands. Each type of litter comes with its own advantages and disadvantages in terms of cost, dustiness, tracking ability, clumping ability and ease of disposal. From this study, another factor may be taken into consideration before choosing a cat litter and that will be the inhibitory effect against viral pathogens specifically FCoV.

Based on the results from this *in vitro* study, all six cat litters excluding paper has viral inhibitory properties up to a varying extent. Unfortunately, paper had to be excluded from the final analyses due to the unhealthy CRFK cells prior to sample inoculation. Three cat litters were identified to be potentially used as litters that have viral inhibitory effects based on the significant result of the viral titres which were the wood pellet, kenaf and bentonite. However, only wood pellet was able to completely reduce viral titre to zero, and this is the ideal situation because even with low viral load or infectious dose, there is possibility of successful viral replication within individual cats especially those with no protective immunity. The exact mechanism of wood pellet inhibitory effect on viral infection in cell culture is not presently known. However, a study by Lupini et al., (2009), showed that chestnut and quebracho wood extracts have *in vitro* antiviral activity against avian reovirus (ARV) and avian metapneumovirus (AMPV). The antiviral activity was presumed to be due to an interaction of tannins extracted from the plant and viral proteins that leads to inhibition of viral attachment and penetration of cell membrane. Tannins are a class of

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polyphenolic compounds that can bind to and precipitate proteins from a solution (Combs, 2016). The wood pellet litter used in the study was made from pine wood (*Pinus spp.*) where condensed tannins can be found (Filgueira et al., 2017; Li & Maplesden, 1998). Further research needs to be conducted to determine whether the tannins are the active compounds responsible for viral inhibitory effect of wood pellet on FCoV. On the other hand, kenaf (*Hibiscus cannabinus*) was almost able to completely reduce viral titre load (6-fold reduction). As previously stated, the mechanism of this plant against FCoV infection in cell culture is unknown, although there was evidence of phytotoxic and fungitoxic properties of kenaf essential oil (Kobaisy et al., 2001).

Bentonite, the third cat litter with significant viral titre reduction by 5-fold, presumably reduced cell culture infection through good adsorbent property. A study by Clark et al. (1998) has proven excellent capability (>90% adsorption) of sodium bentonite to adsorb bovine coronavirus. According to the study, the virions have larger diameters than the pores of the adsorbent substance particles causing the virions to be attached to the external surface of the particles. In other words, the material can only bind the virus together but infectivity of the virus remains. Addie at al. (2020) also stated that Fuller's earth-based cat litters which has similar properties to bentonite, possibly prevented cell culture infection by binding the virus rather than actually killing it.

Another observation made throughout the study is the high liquid absorption property of wood pellet and kenaf. Absorption refers to the phenomenon where molecules of a substance enters the bulk or volume of the absorbent, in contrast with

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adsorption which is a process of adhesion of molecules onto the surface of solid particle. There are two hypotheses derived from this where the first one being wood pellet and kenaf presumably have the ability to absorb fluids from the virus leading the virus particles to dry up and become inactivated. The other theory is the cat litters absorb the liquid in the supernatant, leading to ease of adsorption of virion particles onto the litter material, and forms a tight cat litter-to-virion complex and sediments in the centrifuge tube. This allows for less free-flowing virions in the supernatant, where viral titre reduction is observed after inoculation.

In summary, wood pellet, kenaf and bentonite each has certain chemical or physical properties that may contribute to the inhibition of virus in cell culture.

5.2 Limitations

There were several limitations in the study that must be taken into consideration in future studies. The first one was the usage of virus supernatant did not fully translate to natural setting, whereby virus was protected by faecal material. FIPV strain was used in this study instead of FECV which are more commonly shed in faeces due to the unavailability of FECV in our laboratory. Nevertheless, FECV and FIPV are morphologically similar and it was assumed that both of the virus will act similarly in cell culture infection.

Determination of TCID50 requires careful observation of CPE, whereby a single CPE denotes a positive well that ultimately affects calculation of the end-point titre. Therefore, the observation skill is subjective to each person, which can affect the result greatly if a person is inexperienced.

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6.0 CONCLUSION AND RECOMMENDATION

Overall, all six cat litters (wood pellet, kenaf, bentonite, silica, tofu, non-clumping clay) were able to reduce FCoV titre in CRFK cell culture. Only wood pellet cat litter was able to completely prevent virus infection in cell culture but it is not as cost-effective. Although kenaf and bentonite cat litters also have significant viral titre reduction, virions are still present whereby even a single virus particle could still infect a cat. Thus, kenaf and bentonite could not be recommended as a viral inhibitory cat litter despite bentonite also having another benefit of being cost-effective.

In future studies, an *in vivo* study using FCoV positive cats can be done to test effectiveness of wood pellet since several important variables can be tested in field study which are otherwise not done in this experiment. A further improvement that can be done from this study is to test for virus infectivity. The cat litter-virus complex can be processed and re-inoculated into cell culture to observe for presence of infection, which would indicate viability of the virus. If virus is viable, it could lead to infection in cats, therefore litters that can successfully deactivate virus are ideal to be discovered or created. Next recommendation is to test for different brands of each type of litter. For example, wood pellet cat litters are manufactured by several brands, and each brand should be tested because different brands may have different raw material composition such as pine, oak or cedar. Lastly, an *in vitro* or *in vivo* study can be carried out for other important feline pathogens that transmit through excretion including feline parvovirus and toxoplasma.

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APPENDICES

APPENDIX A

TCID50 CALCULATION FOR CAT LITTERS AND POSITIVE CONTROL

Silica

Dilution	Replicate 1		Replicate 2		Replicate 3	
	Positive wells	Inoculated wells	Positive wells	Inoculated wells	Positive wells	Inoculated wells
10^{-1}	4	4	4	4	4	4
10^{-2}	1	4	2	4	4	4
10^{-3}	0	4	0	4	2	4
10^{-4}	0	4	0	4	0	4
10^{-5}	0	4	0	4	0	4
10^{-6}	0	4	0	4	0	4
10^{-7}	0	4	0	4	0	4
10^{-8}	0	4	0	4	0	4
10^{-9}	0	4	0	4	0	4
10^{-10}	0	4	0	4	0	4

Replicate 1

$\log_{10}$ 50% end point dilution $= -\left[1 - \frac{1}{2} + 1\left(\frac{5}{4}\right)\right] \times 1$

$\log_{10}$ 50% end point dilution $= -1.75$

50% end point dilution $= 10^{-1.75}$ for 0.2 mL

For 1 mL = TCID50/mL $= 10^{1.75} \times 5$

$= 10^{2.45}$

$= 2.8 \times 10^{2}$ TCID50/mL

Replicate 2

$\log_{10}$ 50% end point dilution $= -\left[1 - \frac{1}{2} + 1\left(\frac{6}{4}\right)\right] \times 1$

$\log_{10}$ 50% end point dilution $= -2$

50% end point dilution $= 10^{-2}$ for 0.2 mL

For 1 mL = TCID50/mL $= 10^{-2} \times 5$

$= 10^{2.7}$

$= 5.0 \times 10^{2}$ TCID50/mL

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Replicate 3

log$_{10}$ 50% end point dilution $= -\left[2 - \frac{1}{2} + 1\left(\frac{6}{4}\right)\right] \times 1$

log$_{10}$ 50% end point dilution $= -3$

50% end point dilution $= 10^{-3}$ for 0.2 mL

For 1 mL = TCID50/mL $= 10^{3} \times 5$

$= 10^{3.7}$

$= 5.0 \times 10^{3}$ TCID50/mL

BENTONITE

Dilution	Replicate 1		Replicate 2		Replicate 3	
	Positive wells	Inoculated wells	Positive wells	Inoculated wells	Positive wells	Inoculated wells
10^{-1}	0	4	0	4	4	4
10^{-2}	0	4	0	4	2	4
10^{-3}	0	4	0	4	0	4
10^{-4}	0	4	0	4	0	4
10^{-5}	0	4	0	4	0	4
10^{-6}	0	4	0	4	0	4
10^{-7}	0	4	0	4	0	4
10^{-8}	0	4	0	4	0	4
10^{-9}	0	4	0	4	0	4
10^{-10}	0	4	0	4	0	4

Replicate 3

log$_{10}$ 50% end point dilution $= -\left[1 - \frac{1}{2} + 1\left(\frac{6}{4}\right)\right] \times 1$

log$_{10}$ 50% end point dilution $= -2$

50% end point dilution $= 10^{-2}$ for 0.2 mL

For 1 mL = TCID50/mL $= 10^{-2} \times 5$

$= 10^{2.7}$

$= 5.0 \times 10^{2}$ TCID50/mL

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KENAF

Dilution	Replicate 1		Replicate 2		Replicate 3	
	Positive wells	Inoculated wells	Positive wells	Inoculated wells	Positive wells	Inoculated wells
10^{-1}	0	4	0	4	1	4
10^{-2}	0	4	0	4	0	4
10^{-3}	0	4	0	4	0	4
10^{-4}	0	4	0	4	0	4
10^{-5}	0	4	0	4	0	4
10^{-6}	0	4	0	4	0	4
10^{-7}	0	4	0	4	0	4
10^{-8}	0	4	0	4	0	4
10^{-9}	0	4	0	4	0	4
10^{-10}	0	4	0	4	0	4

Replicate 3

$$\log_{10} 50\% \text{ end point dilution} = -\left[1 - \frac{1}{2} + 1\left(\frac{1}{4}\right)\right] \times 1$$

$$\log_{10} 50\% \text{ end point dilution} = -0.75$$

$$50\% \text{ end point dilution} = 10^{-0.75} \text{ for } 0.2 \text{ mL}$$

$$\text{For } 1 \text{ mL} = \text{TCID50/mL} = 10^{0.75} \times 5$$

$$= 10^{1.45}$$

$$= 2.8 \times 10 \text{ TCID50/mL}$$

WOOD

Dilution	Replicate 1		Replicate 2		Replicate 3	
	Positive wells	Inoculated wells	Positive wells	Inoculated wells	Positive wells	Inoculated wells
10^{-1}	0	4	0	4	0	4
10^{-2}	0	4	0	4	0	4
10^{-3}	0	4	0	4	0	4
10^{-4}	0	4	0	4	0	4
10^{-5}	0	4	0	4	0	4
10^{-6}	0	4	0	4	0	4
10^{-7}	0	4	0	4	0	4
10^{-8}	0	4	0	4	0	4
10^{-9}	0	4	0	4	0	4
10^{-10}	0	4	0	4	0	4

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PAPER*

Dilution	Replicate 1		Replicate 2		Replicate 3	
	Positive wells	Inoculated wells	Positive wells	Inoculated wells	Positive wells	Inoculated wells
10^{-1}	*Invalid*		*Invalid*		*Invalid*	
10^{-2}						
10^{-3}						
10^{-4}						
10^{-5}						
10^{-6}						
10^{-7}						
10^{-8}						
10^{-9}						
10^{-10}						

*Excluded from final result due to unhealthy CRFK cells at start of experiment.

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CLAY

Dilution	Replicate 1		Replicate 2		Replicate 3	
	Positive wells	Inoculated wells	Positive wells	Inoculated wells	Positive wells	Inoculated wells
10^{-1}	4	4	4	4	*Invalid*	
10^{-2}	4	4	4	4		
10^{-3}	4	4	4	4		
10^{-4}	4	4	4	4		
10^{-5}	1	4	2	4		
10^{-6}	0	4	0	4		
10^{-7}	0	4	0	4		
10^{-8}	0	4	0	4		
10^{-9}	0	4	0	4		
10^{-10}	0	4	0	4		

Replicate 1

$\log_{10}$ 50% end point dilution $= -\left[4 - \frac{1}{2} + 1\left(\frac{5}{4}\right)\right] \times 1$

$\log_{10}$ 50% end point dilution $= -4.75$

50% end point dilution $= 10^{-4.75}$ for 0.2 mL

For 1 mL = TCID50/mL $= 10^{4.75} \times 5$

$= 10^{5.45}$

$= 2.8 \times 10^{5}$ TCID50/mL

Replicate 2

$\log_{10}$ 50% end point dilution $= -\left[4 - \frac{1}{2} + 1\left(\frac{6}{4}\right)\right] \times 1$

$\log_{10}$ 50% end point dilution $= -5$

50% end point dilution $= 10^{-5}$ for 0.2 mL

For 1 mL = TCID50/mL $= 10^{5} \times 5$

$= 10^{5.7}$

$= 5.0 \times 10^{5}$ TCID50/mL

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TOFU

Dilution	Replicate 1		Replicate 2		Replicate 3	
	Positive wells	Inoculated wells	Positive wells	Inoculated wells	Positive wells	Inoculated wells
10^{-1}	4	4	4	4	4	4
10^{-2}	4	4	4	4	4	4
10^{-3}	4	4	4	4	4	4
10^{-4}	4	4	4	4	3	4
10^{-5}	2	4	1	4	0	4
10^{-6}	0	4	0	4	0	4
10^{-7}	0	4	0	4	0	4
10^{-8}	0	4	0	4	0	4
10^{-9}	0	4	0	4	0	4
10^{-10}	0	4	0	4	0	4

Replicate 1

$\log_{10}$ 50% end point dilution $= -\left[4 - \frac{1}{2} + 1\left(\frac{6}{4}\right)\right] \times 1$

$\log_{10}$ 50% end point dilution $= -5$

50% end point dilution $= 10^{-5}$ for 0.2 mL

For 1 mL = TCID50/mL $= 10^{5} \times 5$

$= 10^{5.7}$

$= 5.0 \times 10^{5}$ TCID50/mL

Replicate 2

$\log_{10}$ 50% end point dilution $= -\left[4 - \frac{1}{2} + 1\left(\frac{5}{4}\right)\right] \times 1$

$\log_{10}$ 50% end point dilution $= -4.75$

50% end point dilution $= 10^{-4.75}$ for 0.2 mL

For 1 mL = TCID50/mL $= 10^{4.75} \times 5$

$= 10^{5.45}$

$= 2.8 \times 10^{5}$ TCID50/mL

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Replicate 3

$\log_{10}$ 50% end point dilution $= -\left[3 - \frac{1}{2} + 1\left(\frac{7}{4}\right)\right] \times 1$

$\log_{10}$ 50% end point dilution $= -4.25$

50% end point dilution $= 10^{-4.25}$ for 0.2 mL

For 1 mL = TCID50/mL $= 10^{4.25} \times 5$

$= 10^{4.95}$

$= 8.9 \times 10^{4}$ TCID50/mL

POSITIVE CONTROL

Dilution	Positive wells	Inoculated wells
10^{-1}	4	4
10^{-2}	4	4
10^{-3}	4	4
10^{-4}	4	4
10^{-5}	4	4
10^{-6}	2	4
10^{-7}	0	4
10^{-8}	0	4
10^{-9}	0	4
10^{-10}	0	4

$\log_{10}$ 50% end point dilution $= -\left[6 - \frac{1}{2} + 1\left(\frac{6}{4}\right)\right] \times 1$

$\log_{10}$ 50% end point dilution $= -7$

50% end point dilution $= 10^{-7}$ for 0.2 mL

For 1 mL = TCID50/mL $= 10^{7} \times 5$

$= 10^{7.7}$

$= 5.0 \times 10^{7}$ TCID50/mL

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AVERAGE TCID50 CALCULATION

	Litter type	Cyto-toxicity	Replica	Virus titre (TCID-50/mL)	Average (TCID50/mL)
	Virus control	-	-	-	5.0×10^7
1	Non-clumping clay	No	1	2.8×10^5	$\left[\frac{(2.8 \times 10^5) + (5.0 \times 10^5)}{2}\right] = 3.9 \times 10^5$
			2	5.0×10^5	
			3	Invalid	
2	Tofu	No	1	5.0×10^5	$\left[\frac{(5.0 \times 10^5) + (2.8 \times 10^5) + (8.9 \times 10^4)}{3}\right] = 2.9 \times 10^5$
			2	2.8×10^5	
			3	8.9×10^4	
3	Silica	No	1	2.8×10^2	$\left[\frac{(2.8 \times 10^2) + (5.0 \times 10^2) + (5.0 \times 10^3)}{3}\right] = 1.9 \times 10^3$
			2	5.0×10^2	
			3	5.0×10^3	
4	Bentonite	No	1	0	$\left[\frac{0 + 0 + (5.0 \times 10^2)}{3}\right] = 1.7 \times 10^2$
			2	0	
			3	5.0×10^2	
5	Kenaf	No	1	0	$\left[\frac{0 + 0 + (2.8 \times 10)}{3}\right] = 0.9 \times 10$
			2	0	
			3	2.8×10	
6	Paper	No	1	Invalid	Invalid
			2		
			3		
7	Wood pellet	No	1	0	0
			2	0	
			3	0	

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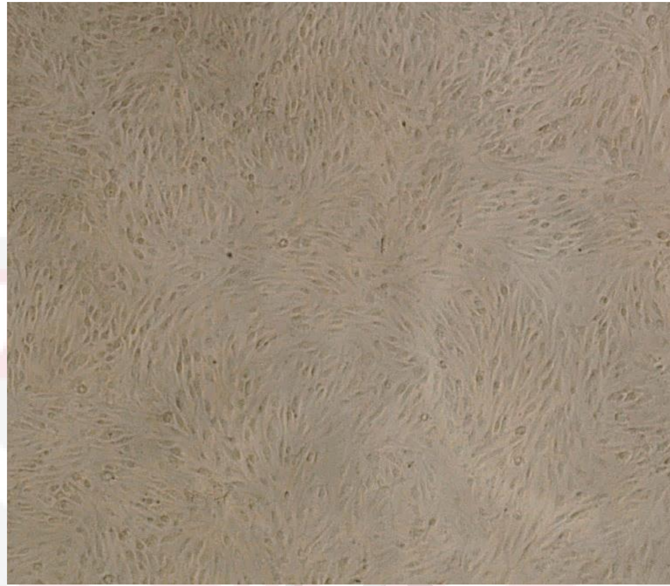
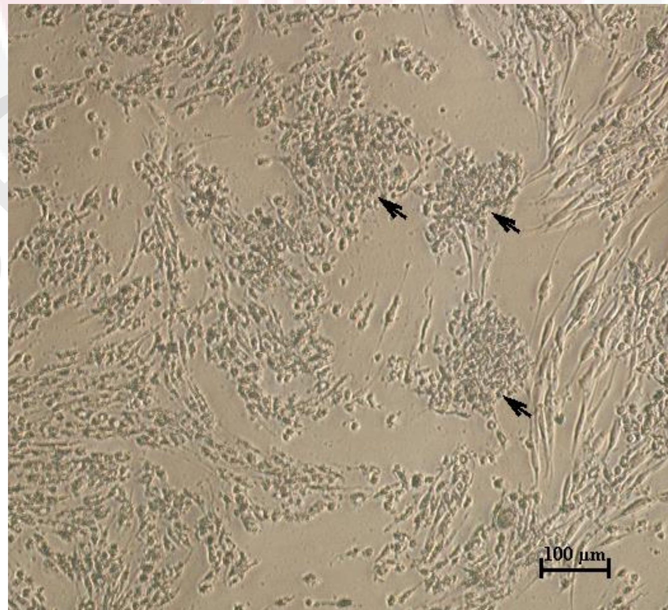
APPENDIX B

OBSERVATION OF CYTOPATHIC EFFECT IN CRFK CELLS



CRFK cells which are uninfected at 10x magnification after 48 hrs.



CRFK cells inoculated with silica mixed with FIPV 79-1146 at 10x magnification after 48 hrs. Cytopathic effects are indicated by the arrowhead.

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