ISOLATION OF FELINE CORONAVIRUS TYPE 1 FROM CLINICAL SAMPLES

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ABSTRACT

The non-virulent feline enteric coronavirus (FECV) may cause a transient and mild enteric infection in cats but does occasionally mutate into a highly virulent form, the systemic replicating feline infectious peritonitis virus (FIPV). Feline infectious peritonitis (FIP) is after half a century of research still one of the most studied infectious diseases in cats. Because of its complex nature, FIP remains one of the leading infectious causes of death of cats all around the world for which there is no conclusive routine diagnostic test, no vaccine, no cure and no clear explanations about how virus-host interaction leads to clinical disease. Feline coronaviruses are divided into two serotypes: type I and II, with type I being the most prevalent of the two. Feline coronavirus research has been inhibited by the inability to establish cell lines susceptible for propagation of type I FCoV strains. However recently, a cell line which enabled the growth of type I feline enteric coronavirus to high titres was developed. This cell line is called fDC-SIGN Huh7, a human cell line susceptible for type I FCoV transfected with a feline DC-SIGN, a nonspecific receptor which is suspected to play an important role during virus entry. During this study it was tried to propagate field strain FCoV from clinical samples on this fDC-SIGN Huh7 cell line, in order to isolate type I field viruses. With these isolates, challenge studies could be performed to learn more about pathogenesis and efficacy of prototype vaccines. Samples from different lesions, organs and excretions from FIP-infected cats were collected, processed and inoculated on fDC-SIGN Huh7 cells. Virus strain UCDp was inoculated on fDC-SIGN Huh7 cells to serve as a positive control. Presence of virus was determined by using diagnostic methods like detecting cytopathogenic effect (CPE) and performance of immunofluorescence assay (IFA) and polymerase chain reaction (PCR). Each method revealed the same conclusion; the laboratory strain UCDp could be propagated on fDC-SIGN-Huh7 cells, while all other cells inoculated with sample were tested negative. Hence, it was concluded that the fDC-SIGN-Huh7 cell line is not suitable for the propagation of type I FCoV from clinical samples, at least not with the samples and conditions used in this study. This finding can be explained by several factors. It might be that UCDp has specific cell culture adapted virus characteristics and that field virus strains do not have the capability to infect this cell line. Another explanation could be that there was simply no or not enough infective virus in the samples in the first place, probably due to presence of defective particles and neutralizing antibodies. More knowledge is needed in order to obtain more reliable results and hopefully future studies will offer a way to grow type I FCoV field strains in a cell culture.

INTRODUCTION

Coronaviruses are viruses with an envelope and contain a positive, single-stranded RNA genome. With a genome of 27-32 kb, coronaviruses are believed to possess the largest RNA genome[1]. At the 5' end of the FCoV genome, ORF1a and ORF1b compromise approximately 20 kb of the entire genome and encode for 2 polypeptides that function as non-structural proteins for synthesis of viral RNA. The other genes of the genome encode for structural proteins; spike (S), nucleocapsid (N), membrane (M) and envelope (E), and 5 accessory proteins (3a-c, 7a, 7b)[2]. The S-protein, a 180-200 kDa glycoprotein arranged in peplomers, constitutes the characteristic spikes on the virus and contributes to the determination of the host and tissue tropism as well as of pathogenesis. The S-protein has a short C-terminal cytoplasmic tail and a long N-terminal ecto-domain. The ectodomain is divided into a domain (S1) responsible for binding to the cellular receptor and a domain (S2) responsible for membrane fusion[2–4]. The N protein, with a molecular weight of approximately 50kDa, forms with the viral RNA the helical nucleocapsid and is likely to be critical during viral replication.

During thousands of years, coronaviruses have adapted themselves in a way which makes them able to infect almost every species within mammals and birds. Infection with these viruses generally causes a transient enteritis and/or respiratory disease[5]. Coronaviruses are well known for their adaptability; they are able to recombine with closely related species to form new viruses as well as to change cell tropism and virulence within the same host, resulting in unpredictable new diseases[5,6].

Feline coronaviruses are the most common viruses found in the feces of cats and are endemic in the feline population; up to

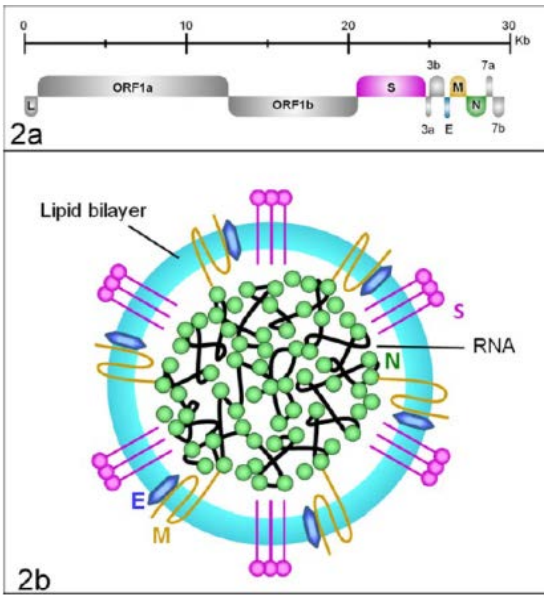

Figure 1: genome and structure of feline coronavirus. *Source: Kipar & Meli, Feline Infectious Peritonitis: Still an Enigma? Vet. Pathol.* ***51,*** *506 (2014).*

90% of the cats in multi-cat environments are seropositive for feline coronaviruses[5,7]. Feline coronaviruses occur as two pathotypes: the nonvirulent feline enteric coronaviruses (FECV) and the lethal feline infectious peritonitis viruses (FIPV)[6]. FECV replicates in intestinal epithelium cells and may cause a transient and mild diarrhea. Cats get horizontally infected through the faecal-oral route[1,5]. After infection, cats shed FECV in faeces within one week and continue for weeks, months, or even lifelong. Shedding may be transient, recurring or chronic for large periods of time and especially during the acute phase of infection, large quantities of virus are shed[1,8]. FCoV endemicity is maintained through these chronically infected, asymptomatic carriers[9]. FIPV, on the other hand, replicates in mononuclear cells, such as monocytes and macrophages, and causes a highly fatal systemic disease. The most common theory about the formation of FIP in cats is called the internal mutation theory, which proposes that FIPV evolves from FECV by mutation during intestinal replication in individually infected cats. Mutations in some non-structural genes and the S-gene are

associated with the formation of a virulent FIP strain. In particular, two amino-acid changes in the S-glycoprotein have been demonstrated to correlate with a FIP pathotype[6]. FIP occurs wherever FECV is found, and therefore FIP is ubiquitous among all cat populations in the world[8]. However, it is estimated that only 1-3% of the cats infected with a feline coronavirus may develop FIP[7]. FIP is after half a century of research still one of the most studied infectious diseases in cats. Because of its complex nature, FIP remains one of the leading infectious causes of death of cats all around the world for which there is no conclusive routine diagnostic test, no vaccine, no cure and no clear explanations about how virus-host interaction leads to clinical disease[5,8,10]. It is thought that especially an imbalance in humoral and cell-mediated immunity helps in avoiding viral clearance and subsequently contributes to development of disease. The production of antibodies against FIPV most likely has an adverse effect because antibodies enhance the uptake and replication of macrophages and thus the replication of FIP viruses[5]. Only the T cell-mediated response is thought to be efficient against disease progression. However, FIPV-induced T-cell depletion may rapidly lead to further progression of disease[9]. Next to the cat's immune system, the virus load may determine whether FIP will develop[1]. FIP is most prevalent among young cats, 70% of cases are less than 1 year old. Especially cats living in crowded environments, such as catteries and shelters, have a high risk on developing FIP. Moreover, stress is a big predisposition. Once clinical signs appear, chance of recovery is extremely low, and 95-100% of infected cats eventually die[1,10,11]. Activation of macrophages and monocytes is the direct cause of the pathologic phenomena; vasculitis, effusion of protein-rich fluids in the abdomen and/or thorax (ascites) and fibrinous and granulomatous inflammations[2]. Two forms of clinical signs are described, although both can occur in the same cat. The 'wet' or effusive form is characterised by ascites in the body cavities and vasculitis, while the 'dry' or non-effusive form is characterised by pyogranulomatous lesions in organs, also known as pyogranuloma. Pyogranuloma can be identified as small white plaques covering the affected tissues and consist of an accumulation of neutrophils, lymphocytes and macrophages that tend to aggregate around venules. Virus can be found in many organs due to the wide distribution of monocytes and macrophages. However, significant more virus particles can be found in haemolymphatic tissues, like the kidneys, lungs, liver, spleen, mesenteric lymph nodes, omentum and brain. An antibiotic unresponsive fever, lethargy, anorexia and weight loss are also common non-specific signs[1].

The diagnosis ante-mortem is difficult to make, especially when it is an infected cat with the dry form of FIP. No non-invasive confirmatory test for diagnosing FIP is available[1]. The background and history of a cat together with other predictive signs of FIP like abdominal expansion, jaundice, masses on the kidneys and/or mesenteric lymph nodes, uveitis and neurological signs might suggest an infection with FIPV[10]. Also haematology profiles may be suggestive for FIP, although most FIP cats do not have a specific haematology profile. Only the albumin/globulin ratio has a diagnostic value; a ratio above 0,8 makes FIP extremely unlikely. Antibody detection by indirect fluorescence assay provides diagnostic information, but should be interpreted with care because a high percentage of healthy cats are FCoV-positive. Seropositive results do not indicate FIP, while seronegative results do not exclude FIP. Presence of a characteristic type of ascites in peritoneal or, less frequently,

pleural cavity has a more important diagnostic value and analysis on ascites should be done to indicate FIP as the cause for disease[1]. The golden standard for diagnosing FIP is immunohistochemistry on effusions or lesions containing macrophages[8]. However, obtaining these biopsies is an invasive method.

Feline coronaviruses are divided into two serotypes: type I and II. The differences between these serotypes are based on differences in the genetic features of the S-glycoproteins, especially in the N-terminal receptor-binding domains[3,9]. Type I FCoVs have a distinctive feline S-glycoprotein and are the most prevalent in the field (80-90% of FIP infected cats). The primary receptor for type I coronaviruses is unknown. Type II FCoVs emerge by recombination of type I feline coronavirus and the canine coronavirus when these viruses meet in a common host[5]. For type II, the feline APN (aminopeptidase N) found in the intestinal brush border most likely is the primary receptor in vitro[2].

Feline coronavirus research has been inhibited by the inability to establish cell lines susceptible for propagation of type I FCoV's. In the past, only once a feline intestinal epithelial cell line has been established for propagation of type I feline enteric coronavirus, derived from feces[11]. Also a few serotype I FIPV strains have been adapted to grow in felis catus whole fetis (FCWF) cells, although most of these strains have lost their pathogenicity because of cell culturing adaptation[11]. Virus strains propagated in vitro may lack the 7b gene, which is present in non-culture adapted strains of FCoV, since this gene is not necessary for replication in cell cultures. Moreover, no study has succeeded so far in replicating type I FIPV strains in macrophage cell lines[8]. Type II FCoV is much less common but easy to replicate in vitro by employing fAPN as the cellular receptor[3,12]. Consequently, despite their lower prevalence, most studies on pathogenesis and vaccine development have focused on type II viruses[1,4]. FIPV WSU 79-1146, a type II serotype virus, is the best characterised FCoV strain and has become the 'golden standard' for both molecular biological analysis and in vivo studies. This virus strain has biased the FCoV field, because it may in fact be atypical because of its serotype, growth properties, and extreme pathogenicity[9]. Also the only vaccine available is a vaccine against a type II FCoV strain. Comparative studies between FIPV and FECV have only been done by comparing genomes of both naturally occurring strains. Hence, without better cell culture adapted virus strains grown on appropriate host cells, it remains unknown which genetic determinants are actually involved in the switch of FECV to FIPV and why FECV and FIPV show such a clinically and epidemiology different behaviour[11].

However recently, a cell line which enables the growth of type I feline enteric coronavirus to high titres was developed in the laboratory of the Virology Institute of Utrecht University[3]. This cell line is called fDC-SIGN-Huh7. Huh7 (humane hepatoma) is an immortal cell line of epithelial-like, tumorigenic cells[13]. Huh7 cells are susceptible to type I FCoV, but not to type II FCoV, meaning that these cells probably express a human receptor orthologue for type I. The cell line was transfected with a feline DC-SIGN, a nonspecific receptor which is suspected to play an important role during virus entry[14]. DC-SIGN means 'Dendritic Cell-specific Intercellular adhesion molecule-3-Grabbing Non-integrin' and is a calcium dependent lectin, meaning that it recognises viral glycoproteins containing high-mannose carbohydrate residues through calcium-dependant carbohydrate-recognition domains[14,15]. It has been shown to enhance infection of several enveloped viruses such as

human immunodeficiency virus, Ebola virus and hepatitis A virus as well as several coronaviruses[3]. DC-SIGN is widely expressed in monocytes and monocyte-derived cells and binding of the S-glycoprotein to DC-SIGN may induce a conformational change that stimulates S-glycoprotein fusion with target membranes and consequently facilitates the entry of virus[16]. It has been shown that type I FIPVs are capable of infecting macrophages and monocytes using DC-SIGN as an entry receptor[3,5,15]. During the study of Mou et al., three different cell lines were infected with a type I FECV strain called UCD. The three cell lines consisted of a feline embryonic fibroblast (FEA) cell line, a felis catus whole fetus (FCWF) cell line and the Huh7 cell line, all expressing fDC-SIGN. The two feline cell lines expressing DC-SIGN first proved insensitive to UCD, while the Huh7 cell line turned out to be immediately sensitive for FECV infection. Subsequent passaging of the virus in the cell line led to single mutations in the S-protein that already after one passage yielded a mutated virus that was able to infect the two feline cell lines and became even more infectious to the Huh7 cell line. Also, when binding to DC-SIGN was blocked, significant less infected cells could be detected. In summary, it was concluded that DC-SIGN contributes to the infection of FECV type I, and that a substitution in the FECV S-protein enhances entry into all cell lines. However, they proposed DC-SIGN unlikely to be the specific primary receptor for FECV, because enterocytes lack the expression of DC-SIGN and cells were also infected when binding to DC-SIGN was blocked. Especially the fDC-SIGN Huh7 cell line seemed to be susceptible for FECV type I infection. It was suggested that Huh7 cells express an orthologue receptor which can be used by serotype I FECV and that DC-SIGN increases the susceptibility of these cells. The study also elaborated if the mutation in the passaged virus confers macrophage tropism by inoculating the mutated virus on DC-SIGN positive macrophages. No infection could be seen in the macrophages, suggesting that besides this mutation, additional determinants are involved in obtaining macrophage tropism. Since most studies focused on type II viruses, it would be highly beneficial when type I viruses could be propagated routinely in a cell culture. The research described above has already shown that it is possible to efficiently replicate a type I FECV laboratory strain to high titres in Huh7-DC-SIGN cells. It would be interesting to take this finding a step further and to investigate the possibility to replicate type I FCoV field strains on this cell line. For this study we wanted to research whether it was possible to propagate clinical samples containing field strains FCoV type I on fDC-SIGN Huh7 cells. If this would succeed, this study could be of great value for future research, because it enables to perform investigations with type I isolates from the field. The impossibility to successfully prevent and control the disease has an enormous impact in a financial, emotional and ethical way and makes FIP the most feared infectious cause of death in cats. Isolating type I FIPV makes it possible, for example, to perform challenge studies in order to learn more about pathogenesis and efficacy of prototype vaccines, and subsequent a better understanding of how to prevent and control the disease.

To address this question, we collected samples from different lesions, organs and excretions from FIP-infected cats where feline coronaviruses are expected to be found. These samples were processed, inoculated on fDC-SIGN Huh7 cells and passaged. Thereafter, presence of virus in cell culture was determined by using diagnostic methods like detecting cytopathogenic effect (CPE),

immunofluorescence assay (IFA) and polymerase chain reaction (PCR).

MATERIAL AND METHOD

CLINICAL SAMPLES

Clinical samples for this study were obtained from tissues, blood, ascites and faeces of FIP-infected or FIP-suspected cats. A total of 52 samples were used during this study. Most samples originated from the biobank of the Virology Institute of the faculty of Veterinary Medicine in Utrecht. The biobank contains samples that were delivered for research between the period of 2012 to 2017. A total of 16 faeces samples, 3 tissue samples and 16 ascites samples were derived in this way. The other samples used in this research were obtained during the course of this study and originated from veterinary practices in and around Utrecht. These samples were mostly less than one day old and consisted of 6 tissue samples, 4 blood samples and 7 ascites samples collected from a total of 9 different cats. The tissue samples were obtained from two FIP cats that were delivered for autopsy to the pathology department.

Generally, cats were around one year old or younger and lived in multi-cat households with one or more FIP cats. Besides, many cats originated from catteries and shelters. One faeces sample was obtained from a cat that was experimentally infected with a FECV type I virus. FIP was diagnosed by elaborating the results of PCR, antibody testing by immunofluorescence (IFA), and haematology profiles and the occurrence of characteristic phenomena like fever, enlarged abdomen filled with sticky yellow liquor, lethargic appearance and anorexia. Of five cats, an autopsy report with an pathological confirmation of FIP was available. All feces and ascites sample were already indicated as positive in PCR during previous studies.

CELLS AND VIRUS

The DC-SIGN expressing Huh7 cell line, produced by HuiHui Mou[3], was available from a -150℃ freezer at passage 10. Cells were maintained in Dulbecco's modified Eagle's medium (DMEM) containing 10% foetal calf serum (FCS), penicillin/streptomycin (p/s), puromycin and GlutaMAX™. The cells were allowed to grow at 37°C in a 5% CO2 setting. Cells grew relatively slowly, so cells were split twice a week in a new flask ($25\mathrm{cm}^2$) to a ratio of 1:3 to 1:5.

Virus strain UCDp ($2 \cdot 10^7$ TCI50/ml on Huh7-fDC-SIGN), a cell adapted type 1 FECV strain (UCD), available from the study of Mou et al., 2017 was used as a positive control in this study. This virus was originally replicated on a recently established feline intestinal epithelial cell line.

PROCESSING OF SAMPLES BEFORE INOCULATION

TISSUE SAMPLES

Multiple lesions of organs were taken, especially the characteristic white spots, from the following organs: mesenteric lymph node, liver, pancreas, kidney, lung and omentum. After collecting the samples, tissue samples were weighted and phosphate-buffered saline with Mg/Ca (PBS) was added in dilution 1:10 weight/volume (w/v). Every organ was shredded separately, except the liver and mesenteric lymph node of one cat offered for autopsy, these were shredded together to one suspension. Subsequently, the suspension was centrifuged 3000 rpm at 10 minutes and the supernatant was isolated. Several portions of 0,5 ml of the supernatant were made to inoculate on the Huh7-DC-SIGN cells.

BLOOD SAMPLES

EDTA blood samples from highly suspected FIP cats from veterinary practices in and around Utrecht were sent to the Virology Division. Mononuclear cells were first isolated to be co-cultivated with fDC-SIGN-Huh7 cells. Therefore, the buffy coat layer containing white blood cells was separated from the rest of the blood. First, blood samples (3 ml) were centrifuged for 10 min at 2000 rpm to separate the plasma from the blood cells. Plasma was taken off and stored for IFA. The blood cells were diluted 1:1 with phosphate-buffered saline without Mg/Ca (PBS0). Four to six ml of Lymphoprep™, a density gradient medium (1,077 g/ml) for the isolation of mononuclear cells, was added to a 12 ml tube. Thereafter, the diluted blood cells were gently added to the Lymphoprep™. The blood cells together with Lymphoprep were centrifuged for 30 minutes at 1500 rpm. During centrifugation, granulocytes and erythrocytes flowed through the Lymphoprep layer, due to having a higher density than mononuclear cells. After centrifugation, the clear liquid (left-over plasma + PBS0) was discarded from the tube. The buffycoat layer with mononuclear cells was isolated by directly pipetting off the interface with cells. PBS0 was added to the isolated buffycoat layer to a volume of 12 ml and centrifuged again for 10 minutes at 2000 rpm to wash off all Lymphoprep. The pellet was resuspended with 14 ml PBS0 and centrifuged for 10 min at 1000 rpm to wash off the platelets. After this centrifugation, the pellet was mixed with 2 ml Roswell Park Memorial Institute medium (RPMI) containing 5% FCS, 0,1 µg/ml GlutaMAX™, 10 µg/ml Concanavalin A and penicillin/streptomycin (p/s), beneficial for survival and growth of mononuclear cells. The cells were cultured overnight at 37°C in a 5% CO2 setting in a well of a 6-well culture plate (Costar®).

FECES AND ASCITES SAMPLES

All feces samples and some ascites samples were taken from the biobank. After defrost, feces and ascites samples were suspended to 10% (w/v) in PBS. Subsequently, the suspensions were centrifuged for 10 min at 3000 rpm. The supernatants were pulled through a bacterial filter (0,2 µm) to avoid bacterial contamination during cell culturing.

The fresh ascites samples (max 1 day old) from FIP-suspected cats were sent to the faculty. These samples were centrifuged for 10 min at 1200 rpm and not pulled through a bacterial filter, because the mononuclear cells in the ascites were needed for co-cultivation. The supernatant was taken off and the cell pellet was resuspended with 2% FCS DMEM. Two milliliter of this mixture was added to a well of a 6-well culture plate, and cultured overnight in a 5% CO2 37°C setting in order to let the mononuclear cells differentiate and grow.

HUH7-FDC-SIGN INOCULATION

When flasks with Huh7-fDC-SIGN cells were full enough, the cells were passaged and seeded in a concentration of $2 \cdot 10^5$cells/ml into wells of a 6-well culture plate. After approximately 1-2 days, when cells were at 80-90% confluency, the cells were ready to be inoculated. Medium was removed and the cells were washed with PBS+DEAE (diethylethanolamine). Half a millilitre of the processed samples from the biobank was used for inoculation on the cultured Huh7-fDC-SIGN cells. After inoculation, the plates were incubated for approximately one hour in a 5% CO2 and 37°C setting. Thereafter, suspensions were taken off and instead of DMEM with 10% foetal calf serum (FCS), DMEM with 2% FCS was added to each well, giving the virus the capacity and time to replicate as much as possible and to prevent cells from overgrowing. As the samples probably

contained less virus than the laboratory strain (UCDp), the inoculated cells were passaged again into a new 6-well culture plate after 3-4 days. In this new plate, IFA slides were prepared by placing five 12 mm coverslips in each well. Then medium was removed from the inoculated wells and cells were washed with PBS0 and detached by trypsine. Cells were passaged to the new 6-well plate with coverslips in a ratio of 1:4. The removed medium was centrifuged and 0,5 ml of this supernatant was also added to each well, since medium may also contain infective virus particles. Finally, to each well an extra 2 ml DMEM (10% FCS) was added. This 6-well culture plate was again incubated for 3-4 days.

POSITIVE AND NEGATIVE CONTROL

Wells for positive and negative IFA controls were made by adding 12 mm coverslips in each well of a 6-well culture plate and seeding the wells with Huh7-DC-SIGN cells. Cells were infected with a MOI (multiplicity of infection) of 1, meaning that approximately 50% of cells will be infected with virus. It was calculated that 60 µl UCDp was needed for infection with MOI of 1. Consequently, a mixture of 60 µl UCDp + 440 µl 2% FCS DMEM was made to inoculate each well. For negative control, wells with Huh7-DC-SIGN cells were inoculated with 500 µl 2% FCS DMEM only. In contrast to the cells inoculated with samples, these cells were fixated after already 24 hours, because cells would otherwise detach due to high toxicity of the virus strain.

FRESH ASCITES AND BLOOD SAMPLES

The monocytic cells cultures from the fresh ascites samples were co-cultured with Huh7-fDC-SIGN cells after approximately 5 days. Co-cultivation occurred as described in the study of Gunn-Moore et al. 1998[17]. After one night culturing the monocytic cells were co-cultivated. Non-adherent cells were rinsed off with PBS and the adherent cell sheet was covered with 500 µl of fDC-SIGN-HUH7 cells in a concentration of $1 \cdot 10^5 \text{cells/ml}$ DMEM (10%FCS). These six-well culture plates were also incubated at 37℃ in a 5% CO2-setting for approximately three days. The subsequent procedures were the same as described above.

IMMUNOFLUORESCENCE ASSAY (IFA)

Before IFA could be performed, the cells attached to the coverslips had to be fixated first. For each well inoculated with sample, the medium was discarded and stored in -80℃ freezer for potential later use. Then, each well was washed with 2 ml PBS followed by adding one millilitre ethanol/acetic acid fluid (95%/5%) for quick fixation of cells. The plate was put in -20℃ for 10 minutes and washed three times with 1 ml PBSO. The plate was stored at 4℃ with 1 ml PBS0 in each well if IFA was not performed directly after fixation. The cells infected with UCDp functioned as a positive control and the uninfected cells as a negative control. All cells inoculated with ascites, blood and faeces samples were tested for presence of virus by IFA.

The IFA test was carried out as follows: two fixated coverslips of each well from the 6-well culture plates were transferred to a 24-well plate. Each well in the 24-well culture plate was incubated with 1 ml block buffer (PBSO containing 3% bovine serum albumin (BSA) and 0.1% Tween 20) for one hour at room temperature to block non-specific binding sites. Droplets of 25 µl from FIP positive serum (A36), containing specific FCoV antibodies, diluted 1:100 with PBS0+1%BSA+0.1%Tw20), were made and dropped on parafilm for each sample. Subsequently, coverslips with cells down were placed on the droplets, to let the antibodies bind. Also droplets of 25 µl of plasma from specific pathogen free (SPF) cats, negative for FCoV antibodies, diluted 1:50 with PBS0+1%

BSA+0.1%Tw20 were made and coverslips were again put on these droplets. Moreover, in all droplets 1:500 anti-double-strand RNA (α-dsRNA) was added, which binds to double stranded RNA. Since the coronavirus genome consists of double stranded RNA, α-dsRNA binding serves as an extra conformation for FCoV infection. After an hour of incubation at room temperature, the coverslips were transferred back to the 24 well plate and washed three times with 1ml PBS0 + 0.05% Tw20 to wash away the unbound first antibodies. Fluorescein isothiocyanate-conjugated (FITC) goat anti-cat IgG(MP Bio), diluted 1:150 in buffer (PBS0, 1% BSA and 0.1%Tween 20), was used as the secondary antibody against the first antibodies for fluorescence. Alexa594 Goat Anti Mouse IgG, diluted 1:200 in the same buffer, was used as the secondary antibody for fluorescence of α-dsRNA antibodies. Again, droplets of 25 µl of these diluted secondary antibodies were put on a parafilm and the coverslips were placed upside down on the drops. After incubation in the dark at room temperature, the coverslips were washed three times with 1ml PBS0 + 0.05% Tw20 and once with 500 µl PBS0 to wash off all unbound antibodies. To examine the glasses, 8 µl mounting medium was dripped onto microscope slide and the coverslips were placed upside down on the drops. Thereafter, the microscope slides were ready to be explored for fluorescence by immunofluorescence microscopy.

VIRAL RNA EXTRACTION

Viral RNA had to be extracted in order to perform polymerase chain reaction (PCR). No RNA was extracted from fecal and tissue samples, because PCR was already performed on these samples. Besides, no RNA was extracted from blood, since PCR on blood is not a reliable diagnostic method (see introduction). Only ascites and cell culture supernatants of the fDC-SIGN-Huh7 cells inoculated with samples were used to extract viral RNA. Because it was not sure whether or not viral RNA was present in the supernatants and because of the high costs of performing PCR with large numbers, three supernatants, each from a different sample, were pooled together before extraction of viral RNA was performed.

Following the manufacturer's protocols (Qiagen Inc., Valencia, CA), viral RNA was extracted from 138µl of the sample/supernatant, using the QIAamp® Viral RNA Mini Kit (cat no: 52906), a special viral RNA purification kit for plasma, serum, cell-free body fluids and cell-culture supernatants. First, two microliter of phocine distemper virus (PDV) was added as a control to check afterwards whether RNA isolation and the PCR itself were correctly performed. 140µl of sample/supernatant + PDV were added to AVL buffer containing carrier RNA, followed by short vortexing and 10 minutes incubation at room temperature, to ensure efficient lysis. Thereafter, it was mixed with 560µl ethanol, placed in a QIAamp Mini column (in a 2 ml tube) and centrifuged at 8000 rpm for 1 minute. The tube containing the filtrate was discarded. These proceedings were repeated with 500µl AW1 buffer and consecutively 500µl buffer AW2 instead of ethanol, except that the buffer AW2 mixture was centrifuged at full speed for 3 minutes to ensure that all buffer was discarded. Finally, the QIAamp Mini column was placed in a 1,5 ml tube and viral RNA was eluted from the column by adding 60µl AVE-buffer, followed by centrifugation at 8000 rpm for one minute. The residual filtrate contained the extracted viral RNA. The column was discarded and the isolated RNA was frozen in the PCR lab freezer (-20°C) until PCR was performed.

Real-time polymerase chain reaction

For this study, a fluorogenic probe-based one-tube FCoV RT-PCR assay was used to perform a quantitative PCR, according to the study of Gut et al., 1999[18]. The assay is based on the reverse transcription and amplification of a portion of the FCoV 7b gene in the 3' domain of the FCoV genome, a highly conserved gene among coronavirus I isolates. A fluorogenic probe (TaqMan), designed to hybridise to a sequence located between the PCR primers, was used. During the PCR polymerisation steps, this probe should be cleaved, resulting in generation of fluorescence. See below for the specific sequences and positions of the primers and probe.

Primer or probe	Sequence (5'→3')	Length of fragment (bp)
Forward primer FCoV1128f	GAT TTG ATT TGG CAA TGC TAG ATT T	102
Reverse primer FCoV1229r	AAC AAT CAC TAG ATC CAG ACG TTA GCT	
Probe FCoV1200p	TCC ATT GTT GGC TCG TCA TAG CGG A	

Table 1 FCoV primers and probe for the FCoV fluorogenic assay

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1001  GGCAACCCGA TGTCTAAAAC TGGTCTTTCC GAGGAATTAC GGGTCATCGC

1051  GCTGCCTACT CTTGTACAGA ATGGTAAGCA CGTGTAATAG GAGGTACAAG

                                          FCoV1128f ⟶
1101  CAACCCTATT GCATATTAGG AAGTTTAGAT TTGATTTGGC AATGCTAGAT

                                    ⟵ FCoV1200p
1151  TTAGTAATTT AGAGAAGTTT AAAGATCCGC TATGACGAGC CAACAATGGA

            ⟵ FCoV1229r
1201  AGAGCTAACG TCTGGATCTA GTGATTGTTT AAAATGTAAA ATTGTTTGAA

1251  AATTTTCCTT TTGATAGTGA TG
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Table 2 Position of primers and probe in the sequence of the FCoV 7b gene

Also for PDV, a different forward primer (PDVf), probe (PDVp) and reversed primer (PDVr) were added. PCR was performed with 2 µl of undiluted RNA sample and 18 µl PCR mix containing 7,4 µl Master Mix, 1,3 µl Activator, 4,3 µl water, 0,4 µl forward primer FCoV1128f (10 µM), 0,4 µl reverse primer FCoV1229R (0,4 µM), 1,5 µl probe FCoV1200p (4 µM), 1,2 µl PDV-f (10 µM), 1,2 µl PDV-r (10 µM) and 0,3 µl PDV-p (10 µM). According to the dilutions below, a FCoV RNA standard was made, that contained a fixed amount of FCoV RNA in each dilution.

After preparing the PCR mix and adding all samples to the plate, the plate was placed in the LightCycler. After a reverse transcription step of 3 minutes at 63°C and a denaturation step of 30 seconds at 95°C, the amplification step followed. Amplification consisted of 46 cycles, each cycle started at 95°C for 15 seconds, followed by 60°C for 30 seconds and then 72°C for 1 second. The final step, the cooling, occurred at 40°C for 10 seconds. During amplification, fluorescence intensity was measured by the ABI Prism 7700 Sequence Detector. In this study the 'real time' mode was run, which means that the fluorescence was monitored after each cycle. Data were transferred to a connected computer and saved.

Results

Cytopathic Effect (CPE)

After each inoculation on fDC-SIGN Huh7 cells, cells were cultured and checked for CPE every day. The cell culture-adapted virus strain UCDp generated identifiable cytopathic effect after already 24 hours, including formation of extensive syncytia and cell detachment (figure 2A). In the mock cells, naturally, no differences in cells could be detected (figure 2B). After three days of cell culturing, significant changes were rarely seen with any of the cells inoculated with clinical samples (figure 2C). Some inoculated cells looked different than the mock cells, but it was difficult to tell if this was due to virus or toxicity of other substances in the samples.

Furthermore, no distinction could be found between the different type of samples, all inoculated cells looked the same.

In the blood and fresh ascites samples, mononuclear cells were first cultured before co-cultivation. It was difficult to tell whether monocytes were present, since mainly large amounts of small cells, probably lymphocytes or red blood cells, were seen in the cultures. Moreover, the amount of white blood cells in each sample was highly variable and therefore it was challenging to compare the samples with each other. Many white blood cells were lost after co-cultivation with fDC-SIGN Huh7 cells, probably due to another use of medium and to the invasion of large number of different type of cells. Also in the co-cultivated fDC-SIGN-Huh7 cells no CPE could be found.

Immunofluorescence Assay

IFA was adjusted multiple times in different ways, e.g. other concentrations of first antibodies and conjugates, different kinds of antibodies, changes in incubation time, more intensive washing steps, before conducting the final protocol.

In the positive control that was infected with UCDp, clear fluorescence was found. The negative control was clearly negative (figure 3). In the cells inoculated with samples, it was challenging to determine the fluorescence. Some cells looked slightly positive, especially some cells inoculated with feces (figure 5). However, the fluorescence more likely seemed to be a consequence of non-specific binding of antibodies, regarding the small bright spots and absence of syncytia. Since the difference in fluorescence between the positive control and cells inoculated with samples was that big and no clear difference in staining between a positive and negative serum on inoculated cells was found, it was concluded that all cells inoculated with samples were negative for FCoV virus.

Also anti double strand RNA was used, to serve as an extra conformation for FCoV infection. None of the cells showed any fluorescence, except for the cells infected with UCDp (figure 4). Subsequently, just as stated before, only UCDp cells could be indicated as positive.

Polymerase Chain Reaction

In this study, PCR was performed on a total of 21 ascites samples. PCR was performed multiple times, following the manufacturer's protocol. However, not all times a reliable result was achieved, since the controls (PDV) were not always positive. Eventually, out of 21 ascites samples, only 11 samples tested positive.

PCR was also performed on the supernatants for determination of extracellular virus titres. None of the supernatants were tested positive, meaning that no feline coronavirus could be detected after inoculation of the fDC-SIGN-Huh7 cells with the clinical samples.

Discussion

The aim of this study was to investigate whether it was possible to isolate type I feline coronavirus from clinical samples. Samples from several body parts where FCoV infection occurs were collected, processed and inoculated on fDC-SIGN-Huh7 cells. In this study, one positive well with cells inoculated with sample would be enough to prove that it is possible to propagate a field feline coronavirus strain on fDC-SIGN-Huh7 cells. Three diagnostic methods were performed to detect virus and each method revealed the same conclusion; only the laboratory strain UCDp could be isolated from fDC-SIGN-Huh7 cells. This conclusion contributes to the study of Mou et al., which proposed fDC-SIGN-Huh7 a compatible cell line for replicating UCDp.

All other cells inoculated with sample were tested negative. Hence, we concluded that the fDC-SIGN-Huh7 cell line is not suitable for the propagation of type I FCoV from clinical samples, at least not with the samples and conditions we used. This finding could be explained by the inability of the field virus to infect this cell line. It may be that UCDp has specific cell culture adapted virus characteristics and therefore infects cells in a different way than virus strains from the field. UCDp is a FECV type I virus, made by propagation on enteric cell lines. In this study, mainly samples from FIP-cats were taken. FIP viruses have, as explained before, a different pathogenesis than FEC viruses and it might be that the fDC-SIGN Huh7 cell line is just not a suitable cell line for propagation of FIP viruses. However, we used feces samples, that should contain FECV and should be able to infect enteric cells just as UCDp does. The cells inoculated with these feces samples were tested negative for virus, although they seemed less negative on the IFA slides compared to the cells inoculated with the other clinical samples. However, because IFA with anti-double-strand RNA antibodies as well as the PCR resulted negative for these cells, it was concluded that the field strain FECV was not able to infect the fDC-SIGN-Huh7 cells.

It is known that FIPV in an infected cat's tissues and cells uses other receptors next to DC-SIGN. The primary receptor for type I FCoV is still unknown and this forms the biggest complication for type I virus propagation in a cell culture. It might be that fDC-SIGN Huh7 does not contain the right primary receptor for type I FCoV infection. RNA viruses have high error rates in their replication and therefore occur in several groups of related genotypes. Since with every RNA replication several point mutations occur, the variety in feline coronaviruses is high. Subsequently, pathogenesis changes, because host and tissue specificity as well as receptor usage and distribution are dependent on sequence variations of the S protein[2]. The high variation in S protein mutations may be a reason for the difficulty of propagation of these field viruses in cell cultures.

Also the cell line itself could be a contributing factor to obtaining negative results, because the cell line was passaged multiple times, starting at passage 10 to passage 36. Cells grew relatively slow and a couple of times cells were contaminated with bacteria, probably due to contamination with feces samples. This resulted in a long time before cells were ready to be inoculated. Because of the many passages, it might be possible that DC-SIGN was not expressed anymore, making the cells less susceptible for infection. It was tried once to test the expression of DC-SIGN by immunofluorescence staining using mouse monoclonal antibody against c-Myc antibody (Invitrogen) and Goat-anti-mouse Alexa 488 (Invitrogen) as a conjugate, just as in the study of Mou et al. In our study, no results could be obtained and therefore no conclusions could be made about the expression of fDC-SIGN. However, in the study of Mou et al., the same cell line was used and also older passages were shown to express DC-SIGN. Another contributing factor for the negative results may be that fDC-SIGN-Huh7 is only susceptible for infection of a type I FCoV. Therefore, type II FCoV that could potentially have been present in the clinical samples, might not have been able to infect this cell line. The samples were not screened for type II viruses. However, it is known that type II viruses are less prevalent and it is very likely that especially type I viruses were present in the samples.

Besides that the inability of replication of type 1 FCoV from clinical samples on fDC-

SIGN-Huh7 cells could originate from the cell line or virus, another explanation could be that there was simply no or not enough infective virus in the samples in the first place. Already half of the ascites samples tested negative in the PCR, although almost all samples were expected to be positive regarding the clinical profiles of these cats. All feces and tissue samples were tested positive in the PCR during previous studies. However, some of these samples were stored in the -80°C freezer for a long period of time, some already for 4 years. Also it was not sure whether the samples from the biobank were frozen and thawed before, which may have inactivated the virus particles. Besides, it was unknown how the fresh samples from veterinary practices were sampled and stored until they arrived at our laboratory.

In a study by Desmarets et al. 2013[11], it was shown that infectious titres of feces samples were between $10^{3,05}$ to $10^{5,77}$ times lower compared to the total titre virus, meaning that a lot of virus is needed in order to gain enough infectious virus to infect a cell culture. Moreover, it has been shown that viruses from FIP cats did not always cause infections upon inoculation of laboratory cats, meaning that despite the presence of a high viral load, a low amount of infectious virus can be found[11]. The lower infective virus titres may be attributed to the presence of defective particles. The most probable cause of a lower amount of infectious particles, however, are neutralizing antibodies[11]. Moreover, blood samples were generally delivered in small volumes, resulting in not that many white blood cells, and a reducing chance of obtaining enough, if infected, monocytes. In the fresh ascites samples it was difficult to find any mononuclear cells.

The lack of virus could be due to a wrongly executed processing of the samples, although we tried to follow the protocols as strictly as possible. For some samples, lots of procedures had to be followed before it could be inoculated on cells, which may increase the chance on human errors significantly. Although we kept everything on ice during the procedures, it may be possible that still some virus was inactivated.

For the blood samples, the protocol for co-cultivation as described in the study of Gunn-Moore et al. 1998[17] was used. However, this study stated that a minimal of four weeks of culturing was needed in order to be able to culture field strains from blood samples. Gunn-Moore et al. suggested that directly after co-cultivation, most of the input virus failed to replicate and that as the left-over adapted virus began to grow more strongly, it became detectable. Four weeks of culturing were needed to get a high titre of adapted virus. Because of the lack of time in this study, culturing occurred shorter than 4 weeks, which may have leaded to a lower infectious viral titre.

Since this study was never performed earlier, no determined protocols were available and proceedings were performed by trial and error. As stated before, IFA did not give sufficient results immediately. At first, we tried to do the IFA as described by the study of Mou et al., thus by using a mouse monoclonal antibody anti-FIPV-nucleocapsid protein followed by conjugating with goat-anti-mouse Alexa 488. However, by performing this method, none of the cells were stained positive. Thereafter, the protocol was adjusted multiple times, with different antibodies, conjugates, washing steps and longer incubation times, until a final protocol was made. Following this protocol, reliable results were obtained with the control samples, although it was difficult to compare the results with those of the study of Mou et al. For future research, it may be wise to gain results by using the same monoclonal

antibodies, in order to be able to make comparable conclusions.

Also with PCR, results were not directly clear. First, the samples as well as the control (PDV) were negative, suggesting a fault in the RNA extraction, preparation of the PCR mix or the PCR itself. However, the FCoV RNA standard curve corresponded to the fixed amounts of RNA in each dilution, suggesting that the LightCycler functioned fine. Eventually approximately half of the ascites samples that were tested in the PCR were positive, although we expected almost all ascites samples to be positive. Because the PDV controls were positive, it was concluded that RNA extraction and PCR mix preparation were performed correctly and the samples contained not enough/no virus in the first place. PCR was also performed on the cell culture supernatants at the same time as the ascites samples. Supernatants were collected from the inoculated fDC-SIGN-Huh7 cells after the second passage and pooled together in a group of three to perform PCR. By pooling three supernatants together, a threefold dilution was made. Diluting does not have a large impact on PCR results, and may only turn samples from positive in negative when they already contain a very low amount of virus particles. Therefore, it was concluded that there was no virus production after the second passage of inoculated fDC-SIGN cells, because none of the supernatants were found positive for type I FCoV in the PCR. However, also cell lysates from all cells inoculated with samples were taken and stored during this study. These cell lysates should be screened as well to confirm the absence of virus production.

In conclusion, despite the use of many samples, all originating from FIP-suspected or –infected cats, we were unable to propagate virus from any clinical sample on fDC-SIGN-Huh7 cells. Many factors could have contributed this finding, as previously described. Because not that many studies have been performed in this way, more knowledge is needed in order to obtain more reliable results. A recommendation for future studies could be that it first should be tried if FECV from fresh feces samples can be propagated on fDC-SIGN Huh7 cells. UCDp is a FECV type I strain and feces should contain FECV type I as well. To increase the chance of positive result, some factors need to be adjusted. We recommend to use feces samples less than one day old from cats in the acute phase of infection. Samples should be self-obtained by the researcher to avoid as much as possible errors in collecting and storing. Feces samples should be inoculated on cells immediately on the same day. Besides, all samples should be screened on presence of a high viral load a short time before inoculation. Hopefully, future studies will offer a way to isolate type I FCoV field strains, in order to be able to perform challenge studies with these isolates and subsequently learn more about prevention and control of this fatal and complex disease.

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APPENDIX

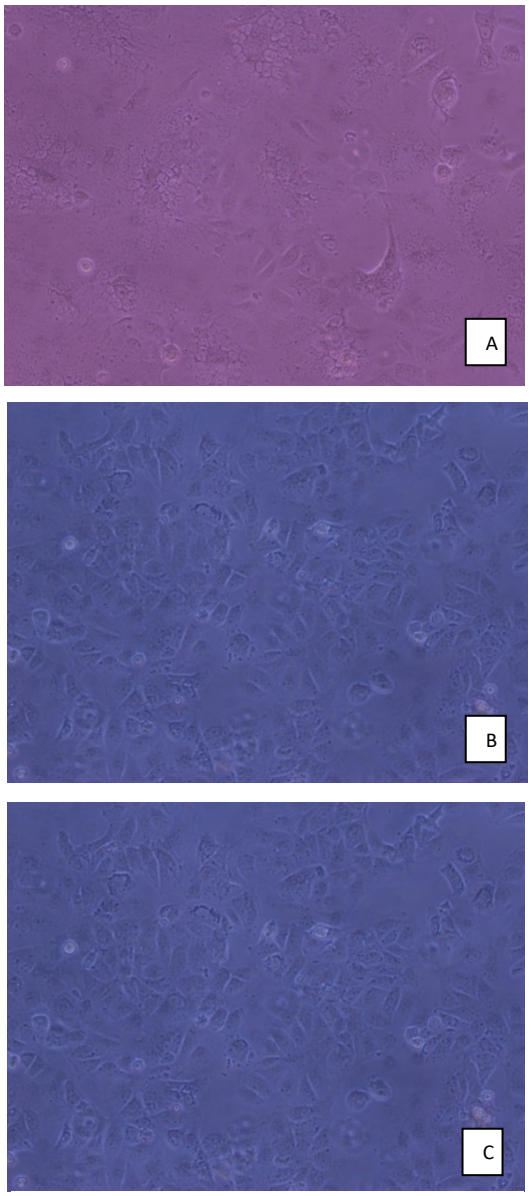

Figure 2: A: fDC-SIGN-Huh7 cells infected with UCDp Clear syncytia and cell detachment can be detected. B: mock fDC-SIGN-Huh7 cells. No sign of CPE is present. C: fDC-SIGN-Huh7 cells inoculated with sample. No sign of CPE is present.

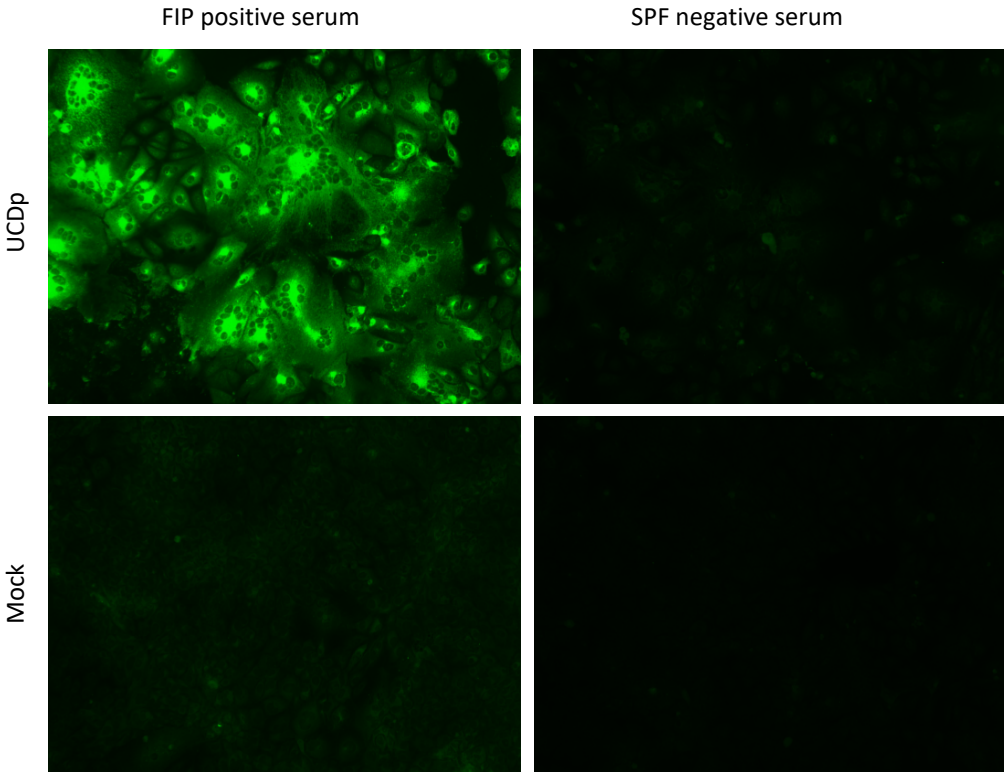

Figure 3: IFA results of positive and negative controls, 24h post infection. Infected cells were visualised by immunofluorescence staining against the primary antibodies (Fluorescein isothiocyanate-conjugated (FITC) goat anti-cat IgG(MP Bio)).

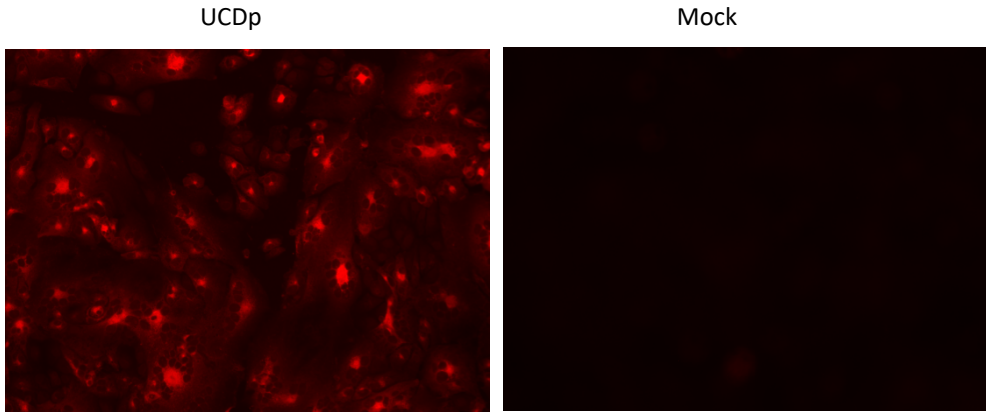

Figure 4: IFA results of positive and negative control 24h post infection. Infected cells were visualised by immunofluorescence staining against α-dsRNA (Alexa594 Goat Anti Mouse IgG). All other cells were comparable with the mock cells.

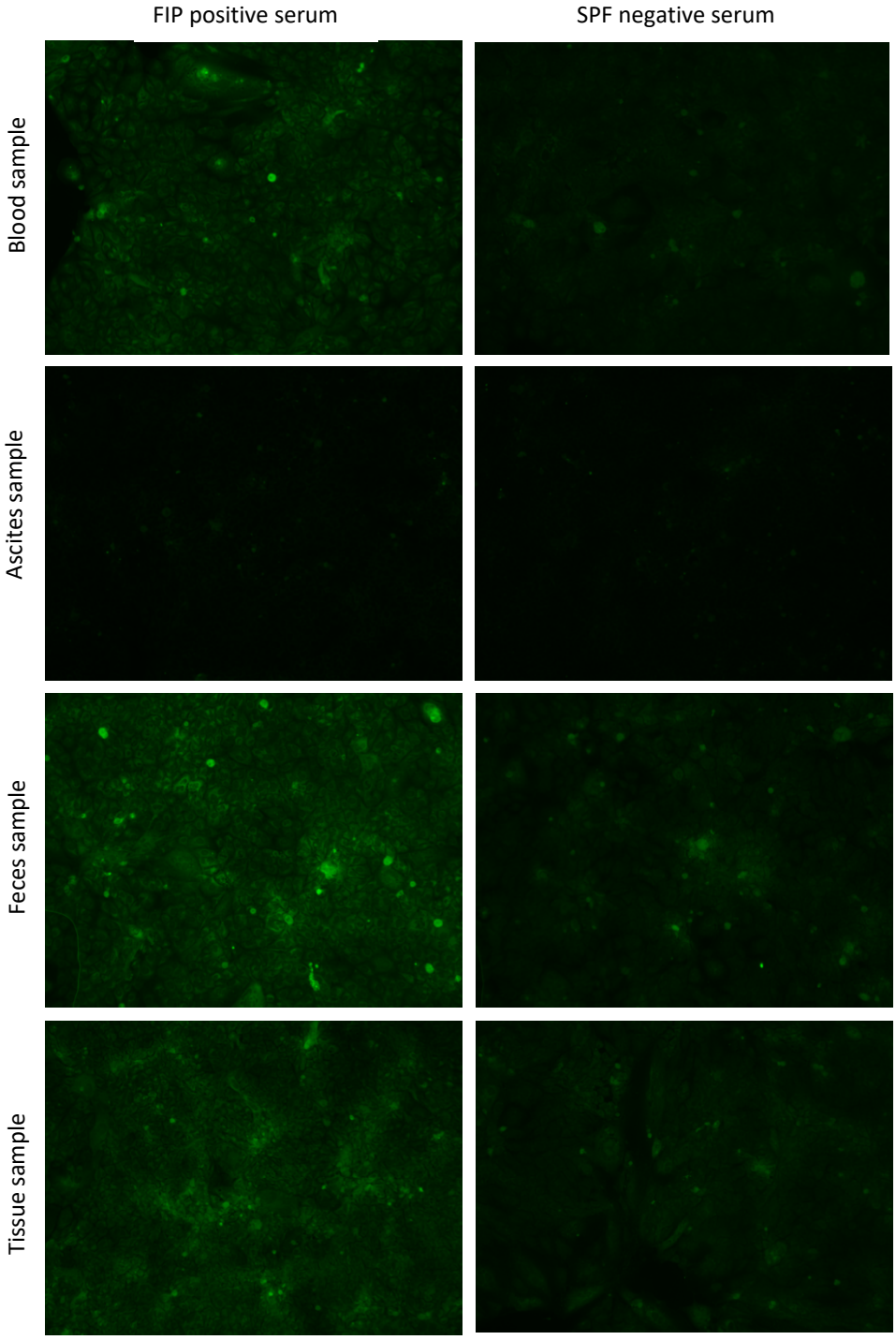

Figure 5: IFA results of cells inoculated with samples, 3-4 days post infection. Infected cells were visualised by immunofluorescence staining against the primary antibodies (Fluorescein isothiocyanate-conjugated (FITC) goat anti-cat IgG(MP Bio)). All cells were comparable with each other.

Sample (cat number)	Date of sampling	Description	Date of inoculation on fDC-SIGN-Huh7	Date of second passage	Date of fixation of cells	PCR result
1066-C	22-03-2015	Two cats living in household where another cat died because of FIP	18-10-2017	20-10-2017	23-10-2017	Positive CT-value 25,94
1066-I	22-03-2015		18-10-2017	20-10-2017	23-10-2017	Positive CT-value 29,8
1055-A	10-07-2014	FIP-suspected kitten living in a shelter	18-10-2017	20-10-2017	23-10-2017	Positive
1058-E	30-10-2014	FIP-suspected cat that tested positive for all 4 performed PCR's.	18-10-2017	20-10-2017	23-10-2017	Positive
3-A	22-03-2017	Experimentally FECV infected cat (same cat, different sampling dates)	25-10-2017 (1:10 diluted with 2% FCS DMEM instead of PBS)	27-10-2017	31-10-2017	Positive CT-value 22
3-B	10-2017		25-10-2017 (1:10 diluted with 2% FCS DMEM instead of PBS)	27-10-2017	31-10-2017	Positive CT-value 24
1064-E	13-03-2015	Three cats living in the same household as a FIP-infected cat	08-11-2017	13-11-2017	16-11-2017	Positive CT-value 28,22
1064-F	14-03-2015		08-11-2017	13-11-2017	16-11-2017	Positive CT-value 26,83
1064-J	11-03-2015		08-11-2017	13-11-2017	16-11-2017	Positive CT-value 26,07
1041-1	03-02-2014	Three kittens diagnosed with FIP	14-11-2017	17-11-2017	23-11-2017	Positive
1041-2	03-02-2014		14-11-2017	17-11-2017	23-11-2017	Positive
1041-5	17-03-2014		14-11-2017	17-11-2017	23-11-2017	Positive
1058-D	Unknown	Tested three times positive during PCR	14-11-2017	17-11-2017	23-11-2017	Positive
1064-D	11-03-2015	Cat living in the same household as a FIP-infected cat	14-11-2017	17-11-2017	23-11-2017	Positive CT-value 25,11
1057-G	30-10-2014	Two cats living in a shelter with FIP-suspected cats	21-11-2017	24-11-2017	28-11-2017	Positive
1057-R	30-10-2014		21-11-2017	24-11-2017	28-11-2017	Positive

TABLE 3 DESCRIPTION AND CELL CULTURING DETAILS OF FECES SAMPLES

Sample (cat number)	Date of sampling	Description	Date of culturing the mononuclear cells	Date of co-cultivation with fDC-SIGN-Huh7	Date of second passage	Date of fixation of cells
1097	27-10-2017	FIP-suspected kitten: sticky fluid in thorax and abdomen and low albumin/globulin ratio	27-10-2017 (white blood cells diluted with 2% FCS DMEM instead of 5% RPMI)	01-11-2018	06-11-2017	09-11-2017
1099	06-11-2017	Blood from mother of kitten 1096 (see table below), FIP-suspected: anorexia, fever, low albumin/globulin ratio and titre>2560	07-11-2017	08-11-2017 (1) 13-11-2017 (2)	15-11-2017 (1) 20-11-2017 (2)	20-11-2017(1) 23-11-2017(2)
1101	16-11-2017	One year-old FIP-suspected cat, high gammaglobulin concentration.	16-11-2017	20-11-2017	27-11-2017	01-12-2017
1105	27-11-2017	Six year-old FIP-suspected cat	27-11-2017	28-11-2017 (1) 30-11-2017 (2)	01-12-2017 (1) 04-12-2017(2)	?

TABLE 5 DESCRIPTION AND CELL CULTURING DETAILS OF BLOOD SAMPLES

Sample (cat number)	Date of sampling	Description	Date of inoculation on fDC-SIGN-Huh7	Date of second passage	Date of fixation of cells	PCR result
1040-1	20-01-2014	Liver (1040-1) and lung (1040-2) from 1-year old FIP-cat. FIP was pathologically confirmed.	10-11-2017	13-11-2017	16-11-2017	Positive
1040-2	20-01-2014		10-11-2017	13-11-2017	16-11-2017	Positive
1043	24-03-2014	Kidney with granuloma from 1-year old cat. FIP was pathologically confirmed.	10-11-2017	13-11-2017	16-11-2017	Positive
1099-1	17-11-2017	Mesenteric lymph node + liver (1099-1), pancreas (1099-2) and kidney (1099-3) from highly suspected FIP cat. Antibody titre (blood) was >2560	17-11-2017	20-11-2017	23-11-2017	Positive
1099-2	17-11-2017		17-11-2017	20-11-2017	23-11-2017	Positive
1099-3	17-11-2017		17-11-2017	20-11-2017	23-11-2017	Positive
1100-1	17-11-2017	Mesenteric lymph node (1100-1) and omentum (1100-2) from FIP-cat. FIP was pathologically confirmed.	17-11-2017	20-11-2017	23-11-2017	Positive
1100-2	17-11-2017		17-11-2017	20-11-2017	23-11-2017	Positive

TABLE 6 DESCRIPTION AND CELL CULTURING DETAILS OF TISSUE SAMPLES

TABLE 4 DESCRIPTION AND CELL CULTURING DETAILS OF ASCITES SAMPLES

Sample (cat number)	Date of sampling	Description	Date of inoculation	Date of second passage	Date of fixation of cells	PCR result
1093	28-08-2017	Kitten from cattery with fever	09-10-2017	12-10-2017	16-10-2017	Positive CP value 30,3
1073	2015	1 year-old icteric and apathetic cat	09-10-2017	12-10-2017	16-10-2017	Negative
1071	16-12-2015	Cat with fever and hydrothorax	09-10-2017	12-10-2017	16-10-2017	Positive CP value 28,4
1028	08-02-2013	1 year-old cat with extremely enlarged abdomen and weight-loss	09-10-2017	12-10-2017	16-10-2017	Positive CP value 30,41
1036	13-09-2013	Pale cat with fever and an antibody titre >2560.	11-10-2017	13-10-2017	17-10-2017	Positive
1074	Unknown	Cat from household with another cat that probably died because of FIP-infection.	11-10-2017	13-10-2017	17-10-2017	Positive CP value 30,07
1039B	06-01-2014	1 year-old cat with ascites in thorax, FIP pathologically confirmed.	11-10-2017	13-10-2017	17-10-2017	Positive
1051		FIP-suspected cat	11-10-2017	13-10-2017	17-10-2017	Negative
1095	13-10-2017	Cat from shelter with high fever, low albumin/globulin ratio and antibody titre of 640	13-10-2017	17-10-2017	23-10-2017	Positive CP value 28,08
1097	27-10-2017	Supernatant of ascites	27-10-2017	30-10-2017	02-11-2017	Negative
1096	28-08-2017	Cat highly suspected of FIP with antibody titre of 640	27-10-2017	30-10-2017	02-11-2017	Positive CP value 27,05
1097	27-10-2017	Cultured mononuclear cells	27-10-2017	01-11-2017	09-11-2017	Negative
1035	03-09-2013	Young cat with antibody titre of 320.	02-11-2017	06-11-2017	09-11-2017	Positive
1040	30-01-2014	1 year-old icteric and lethargic cat with pu/pd, anorexia and an antibody titre of 1280. FIP pathologically confirmed.	02-11-2017	06-11-2017	09-11-2017	Negative
1047	Unknown	9 month-old cat suspected of FIP	02-11-2017	06-11-2017	09-11-2017	Positive
1031	28-03-2013	Cat suspected of FIP	07-11-2017	10-11-2017	15-11-2017	Negative
1033	10-05-2012	FIP suspected cat with antibody titre of 320.	07-11-2017	10-11-2017	15-11-2017	Positive
1061	09-12-2014	FIP-suspected cat, another cat in the same household was euthanized for FIP.	07-11-2017	10-11-2017	15-11-2017	PCR not possible (fluid too sticky)
1067	20-04-2015	FIP-suspected cat	07-11-2017	10-11-2017	15-11-2017	Negative
1079	26-05-2016	FIP-suspected kitten with fever, cat in same household died because of FIP	07-11-2017	10-11-2017	15-11-2017	PCR not possible (too little volume)
1098	03-11-2017	FIP-suspected cat	07-11-2017	10-11-2017	15-11-2017	Negative
1103	21-11-2017	Kitten with enlarged abdomen and fever	21-11-2017	24-11-2017	28-11-2017	Negative