

A comparative study of techniques used for the diagnosis of effusive feline infectious peritonitis

Een comparatieve studie van technieken die gebruikt worden voor de diagnose van effusieve feliene infectieuze peritonitis

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ABSTRACT

Feline infectious peritonitis (FIP) is a fatal disease caused by feline infectious peritonitis virus (FIPV). At present, neither a licensed treatment nor an accurate ante-mortem diagnosis are available. In the present study, three available tests were evaluated for their diagnostic power on effusion samples. High feline coronavirus antibody titers, measured with an immunoperoxidase monolayer assay (IPMA), were correlated with FIP but its low specificity precluded a reliable diagnosis. The in-house 5' reverse transcriptase-quantitative polymerase chain reaction (RT-qPCR) provided a much better specificity and high sensitivity. Given the low sensitivity of immunofluorescence staining (IF) of effusive cells, the RT-qPCR alone or in combination with IPMA represents a good alternative for IF. In the majority of the effusion samples from FIP positive animals, Sanger sequencing of the open reading frame encoding the spike protein (ORF S) revealed not only mutations that were previously associated with FIP (M1058L, S1060A, I1106T and D1108Y/E/G) but also two new, closely related mutations (T1112S/N).

SAMENVATTING

Feliene infectieuze peritonitis (FIP) is een fatale ziekte die veroorzaakt wordt door het feliene infectieuze peritonitisvirus (FIPV). Momenteel zijn een geregistreerde behandeling en een accurate antemortem-diagnose niet beschikbaar. In de huidige studie werden drie beschikbare diagnostische technieken geëvalueerd op hun diagnostische waarde bij effusiestalen. Hoge feliene coronavirus-antistoftiters, gemeten met een immunoperoxidase monolayer assay (IPMA), correleerden goed met FIP, maar de lage specificiteit sloot een betrouwbare diagnose uit. De "in-house 5' reverse transcriptase-quantitative polymerase chain reaction" (RT-qPCR) gaf een betere specificiteit in combinatie met een hoge sensitiviteit. Gezien de lage sensitiviteit die geassocieerd wordt met het gebruik van immunofluorescentiekleuringen (IF) op cellen uit effusies, schuiven deze resultaten de RT-qPCR, al dan niet gecombineerd met IPMA, naar voren als een zeer goed alternatief voor IF. Door Sanger-sequencing van het openleesraam dat codeert voor de spike-proteïne (ORF S) werden niet alleen mutaties aangetroffen die reeds geassocieerd werden met het FIP-fenotype (M1058L, S1060A, I1106T en D1108Y/E/G), maar ook twee nieuwe, verwante mutaties (T1112S/N).

INTRODUCTION

Coronavirus infections in cats are associated with subclinical infection, enteritis (ranging from mild to severe) as well as highly lethal infectious peritonitis (FIP) (Kipar et al., 1998; Kipar and Meli, 2014). Feline coronavirus (FCoV) is endemic in cat populations worldwide (Addie et al., 2009). In about 12% of those cats, a highly virulent mutant, known as feline infectious peritonitis virus (FIPV) arises, causing death in the majority of cats (Addie et al., 2009). The two biotypes differ from each other in terms of cell tropism. After orofecal transmission, the enteric biotype or feline enteric coronavirus (FECV) infects mainly enterocytes and to a limited extent monocytes and macrophages, allowing systemic spread of the virus (Porter et al., 2014). As a consequence, clinical signs are usually self-limiting (Rottier et al., 2005; Drechsler, 2011). FIPV, on the other hand, has a greatly increased tropism for monocytes and macrophages, leading to an increased systemic spread from the intestines via the bloodstream to internal target sites (primarily small to medium sized vessels), causing vasculitis, fibrinous granulomatous inflammatory lesions and subsequent exudation of proteinaceous fluids into the peritoneal and pleural cavities (Rottier et al., 2005; Kipar and Meli, 2014). Besides the pathogenic differences, FCoV can be divided in two serotypes based on their antigenic properties. Serotype I and II differ in their spike protein (S protein). The latter originates from recombination of FCoV with canine coronavirus (CCoV) (Herrewegh et al., 1998; Decaro and Buonavoglia, 2008; Kipar and Meli, 2014). It should be noted that both biotypes occur with both serotypes (Rottier, 1999).

The most accepted hypothesis to date states that FIPV arises from FECV after (an) in-vivo mutation(s) in the FECV-infected cat, presumably due to a selection process for mutant viruses with a remarkably increased tropism for the monocyte/macrophage cell type (Vennema et al., 1998; Dewerchin et al., 2005; Rottier et al., 2005; Pedersen, 2014b). However, a minority of cats with FIP has been shown to shed mutant virus, and horizontal transmission in these cases is not excluded (Pedersen et al., 2009; Pedersen et al., 2012; Wang et al., 2013; Porter et al., 2014). So far, associations have been made between FIP and the genes encoding the spike (S) and accessory 3c protein (Vennema et al., 1998; Chang et al., 2012; Licitra et al., 2013; Bank-Wolf et al., 2014; Lewis et al., 2015).

Until recently, an accurate ante-mortem diagnosis could only be made by immunohistochemistry or immunocytochemistry on biopsies or cells from effusions. These techniques are based on the detection of intracellular FCoV antigens in monocytes/macrophages and offer a specificity (Sp) close to 100%. The sensitivity (Se) however, is rather limited (Tasker, 2018). Serological tests for FCoV must be interpreted with care because cats with conditions other than FIP

may be coincidentally seropositive (Pedersen, 2014a). Only IIF/IPMA antibody titers of >1600 measured in serum might have an adequate positive predictive value (PPV) of 94%, providing additional evidence for the presence of FIP (Hartmann et al., 2003). When effusion was used instead of serum for the detection (presence or absence) of FCoV antibodies, an even higher diagnostic value was noticed in the study by Hartmann et al. (2003). Remarkably, when using effusion, no correlation was found between the antibody titer and the FIP status (Hartmann et al., 2003). Moreover, given the high Se of serology, low antibody titers are often used in practice to exclude FIP. Recently, this has been questioned by Meli et al. (2013) who have reported an inverse correlation between antibody titers and RNA load. Polymerase chain reaction (PCR)-based techniques can be used for the detection and quantification of FCoV RNA. Because the majority of FECV infected cats go through an initial viremia, during which viral RNA can be detected in blood and tissue samples, care must be taken with the interpretation of these results (Gunn-Moore et al., 1998; Kipar et al., 2010). Nonetheless, Sp and Se have been found to be significantly increased when using effusion samples instead of serum (Egberink et al., 1995; Herrewegh et al., 1995). The most promising results were obtained in two recent studies by Barker et al. (2017) and Longstaff et al. (2017) after applying a 3' reverse transcriptase-quantitative polymerase chain reaction (RT-qPCR) on effusion. The authors reported a Se of 78.4-85% and Sp of 97.9-100% for the diagnosis of FIP (Barker et al., 2017; Longstaff et al., 2017). At least theoretically, the Sp of this technique could be further improved by using an RT-qPCR with primers targeting the 5' end of the genome, giving a more adequate estimate of the true genomic viral RNA load (Desmarets, 2015; Tasker, 2018). Ultimately, a definite diagnosis of FIP requires the identification of specific genetic determinants that discriminates FIPV from FECV. To date, only one commercial test (RT-qPCR) is available detecting two specific mutations M1058L and S1060A (FIP Virus RealPCR™ Test, IDEXX, USA) in open reading frame S (ORF S), but it lacks sensitivity (Chang et al., 2012).

In the present study, both serological and molecular techniques were compared using immunofluorescence staining (IF) (ante-mortem gold standard) on effusion samples of feline patients.

MATERIAL AND METHODS

Sample collection

Twenty-four abdominal (n= 16) or pleural (n= 8) effusion samples originating from 22 cats, of which eight were suspected of FIP, were collected in veterinary practices and the Faculty of Veterinary Medicine of Ghent University. All accessible clinical data such

as the patient's history, physical examination, laboratory and medical imaging results or post-mortem data were collected. A minimum volume of 12 ml abdominal or pleural effusion was collected in tubes containing heparin and stored at 4 °C for no longer than 24 hours after collection. The samples were used either immediately for IF or frozen at -70 °C for RT-qPCR (Desmarests et al., 2013) and immunoperoxidase monolayer assay (IPMA) as described below. Samples displaying a positive result with the RT-qPCR underwent amplification of ORF S by a second RT-PCR and Sanger sequencing (Chang et al., 2012; Barker et al., 2017).

Indirect immunofluorescence staining

The intracellular presence of FCoV was assessed by IF. Cells were pelleted and fixed with paraformaldehyde 4 %. After incubation at room temperature (RT) for ten minutes and washing in phosphate buffered saline (PBS), the cells were permeabilized with Triton X-100 (Sigma-Aldrich, USA) for three minutes at RT. After washing with PBS, the remaining cell pellet was treated with 10 % normal goat serum (NGS) to block non-specific staining. Following incubation at 37 °C for twenty minutes and centrifugation (16000 x g, ten minutes), 1/30 diluted 10A12 mouse monoclonal primary antibodies (characterized and produced by the laboratory of the department of Virology (Faculty of Veterinary Medicine, Ghent University) were added to specifically target the FCoV nucleocapsid. In order to assess the specificity, irrelevant 13D12 mouse monoclonal antibodies, which react against pseudorabies virus gD (Nauwynck and Pensaert, 1995), were used in parallel to serve as an isotype control. The samples were incubated at 37 °C for sixty minutes and centrifuged. The cells were resuspended in 1/1000 diluted goat-anti-mouse IgG1 FITC antibodies (Goat anti-Mouse IgG1 Cross-Adsorbed Secondary Antibody, Molecular Probes, USA) and incubated at 37 °C for fifty minutes. After staining the cell nuclei with Hoechst 33342 (Invitrogen, USA) at 37 °C for ten minutes, the cells were resuspended in PBS, mounted on a microscopic slide in a drop of glycerin/DABCO (Janssen Chimica, Belgium), and analyzed by fluorescence microscopy.

Immunoperoxidase monolayer assay

Antibody titers were measured by means of an IPMA. Multiwell plates with a monolayer of ST- (swine testicle) cells were infected with a first passage of porcine respiratory coronavirus (PRCV) 1488, grown in ST-cells. Before use, the plates were fixed with paraformaldehyde 4 % and subsequently washed in a PBS/Tween 80 0.5 % solution for five minutes. Next, a solution of methanol and H₂O₂ was added to eliminate any background staining. Thereafter, the plates were washed twice for five minutes with PBS/Tween 80 0.5 % solution. Positive (from a

FIP-field case) and negative control (same volume of pure minimum essential medium, Glutamax, Gibco BRL, Belgium) samples were included. Serial dilutions were made from all samples, after which 50 µl of each dilution were transferred to the corresponding well of the plate. Next, the plates were incubated at 37 °C for sixty minutes to allow binding of the primary antibodies. After washing, the plates were incubated at 37 °C for sixty minutes with horseradish peroxidase (HRP)-labeled rat-anti-cat IgG antibodies (Nordic-MUBio BV, the Netherlands). Subsequently, AEC-substrate (amino-ethylcarbazole/Na-acetate/H₂O₂) was added to each well. After removing the supernatant and blocking with Na-acetate, the titers were determined by conventional light microscopy.

ORF 1b 5' RT-qPCR

The genomic viral RNA load was assessed by an in house 5' RT-qPCR (Desmarests et al., 2013). After centrifugation (350 x g, ten minutes) and removal of the supernatant, the cell pellet was resuspended in Dulbecco's Modified Eagle Medium (DMEM). For viral RNA extraction, the QIAamp Cador Pathogen Mini Kit (Qiagen, Belgium) was used according to the manufacturer's instructions, after which the extract was stored at -70 °C. The RT-qPCR was performed as described by Desmarests et al. (2013) with a forward primer ORF1bFW (5'-3': TGGACCATGAGCAAGTCTGTT) and reverse primer ORF1bRV (5'-3': CAGATCCATCATTGTGTACTTTGTAAGA) to specifically allow amplification of a 137 bp fragment ORF 1b (Desmarests et al., 2013). A volume of 10 µl mastermix containing SYBR green/ROX (Precision OneStep RT-qPCR Mastermix, PrimerDesign, United Kingdom) was mixed with 0.4 µl forward primer, 0.8 µl reverse primer, 5.8 µl RNase/DNase free water and 3 µl RNA to form the final PCR mix. A quantitative RT-PCR analysis was carried out by a Step One Plus real-time PCR system (Applied Biosystems, USA). In brief, a reverse transcription reaction (55 °C, ten minutes) was followed by enzyme activation (95 °C, eight minutes), 40 PCR-cycles (95 °C, ten seconds; 58 °C, sixty seconds) and melting curve analysis (95 °C, fifteen seconds; 60 °C, one minute; 95 °C). The number of RNA copies/ml in each sample was calculated by the Step One Plus real-time PCR system using the comparative CT ($\Delta\Delta CT$) method.

RT-PCR and Sanger sequencing

A one-step RT-PCR (OneStep RT-PCR Kit, Qiagen, Belgium) was carried out according to the manufacturer's instructions on FCoV positive RNA extracts, as determined by the ORF 1b 5' RT-qPCR. Two primer sets were used, of which one targets a specific 607 bp fragment of ORF S of serotype I FCoV (Chang et al., 2012) (forward primer 5'-3':TCCCGCAGAAAC-CATACCTA; reverse primer 5'-3':TCCCGCAG-AAACCATACCTA) and another targets a 1820 bp

fragment in ORF S of serotype II FCoV (Barker et al., 2017). To produce the final PCR mix, 2 µl dNTP mix, 10 µl buffer, 27 µl RNase/DNase free water, 2 µl enzyme mix, 3 µl (0.6 µM) forward primer, 3 µl (0.6 µM) reverse primer and 3 µl template RNA were mixed subsequently. The mixture was loaded on a preheated (50 °C) PCR plate (Thermocycler T100, Bio-Rad, USA). In brief, a reverse transcription step (thirty minutes, 50 °C) was followed by enzyme activation (fifteen minutes, 95 °C), 30 PCR cycles (94 °C, one minute; 50 °C, one minute; 72 °C, one minute) and terminal elongation (72 °C, one minute), after which the mix was cooled down to 4 °C. Finally, Sanger sequencing (GATC Biotech, Germany) was performed for all samples displaying the 607 (Chang et al., 2012) or 1820 bp fragment (Barker et al., 2017).

Statistical analysis

The test characteristics sensitivity (Se), specificity (Sp), positive predictive value (PPV) and negative predictive value (NPV) with corresponding 95 %-confidence intervals were calculated using Win Episcopo 2.0 software (University of Zaragoza, Spain). The

sample mean (xg) and standard deviations (SD) were calculated in IBM SPSS 24.0. The Pearson correlation coefficient for the RNA load and antibody titer was calculated using IBM SPSS 24.0.

RESULTS

Twenty-four samples of abdominal (n= 16) and pleural (n= 8) effusion, originating from twenty-two cats, were classified being either FIP positive (n= 8) or negative (n= 16) by IF on effusion cells. Twenty-four samples were assessed by IPMA and RT-qPCR to respectively determine the antibody titer and RNA load. In 9 RNA positive samples, RT-PCR and sequencing of ORF S were successful. Briefly, 41.6 % (10/24) of the samples were negative for all tests, 33.3 % (8/24) were positive for all tests, 12.5 % (3/24) were positive for IPMA and RT-qPCR, but negative on IF. In 77.8 % of the samples, one or more mutations leading to an amino acid (AA) shift in ORF S were detected. A schematic overview of all the available clinical data and test results is shown in Table 1.

Indirect IF staining of effusion cells was consid-

Table 1. Clinical data such as patient history, FIP diagnosis (based on FIP flowchart as described by Desmarets, 2015) (Figure 2), presence of alternative pathologies and type of effusion, are shown left. Results from IF, IPMA, RT-qPCR and RT-PCR with sequencing are shown on the right. To allow better interpretation of these results, cats are grouped according to FIP (IF) diagnosis. Cat 4 and 5 presented with both abdominal and pleural effusion.

Cat Nr.	Breed	Age	FIP diagnosis	Other pathology	Type effusion	IF	Antibody titer (IPMA)	RNA-copies (Log10)/ml (RT-qPCR)	Ct value	Mutations (Sanger sequencing)
1	BSH	1 y	FIP certain	n/a	A	Positive	10240	4.76	26.05	None
3	DSH	7 m	FIP certain	n/a	A	Positive	51200	2.22	31.36	n/a
10	BSH	9 m	FIP certain	n/a	A	Positive	51200	4.96	25.35	M1058L + I1106T + T1112S
11	DSH	7 m	FIP certain	n/a	P	Positive	20480	5.63	23.04	T1112S
15	BSH	3 y	FIP certain	n/a	P	Positive	12800	5.99	21.84	n/a
16	BSH	5 m	FIP certain	n/a	A	Positive	6400	5.92	22.06	M1058L + D1108G + T1112S
20	BSH	6 m	FIP certain	n/a	A	Positive	640	4.84	25.01	M1058L + D1108Y + T1112S
21	BSH	7 y	FIP certain	n/a	A	Positive	1280	6.21	21.12	M1058L
8	DSH	3 y	FIP possible	n/a	A	Negative	12800	5.52	23.43	S1060A + D1108E + T1112S
9	BSH	8 m	FIP possible	n/a	A	Negative	6400	5.95	21.99	M1058L + T1112N
12	DSH	2 y	FIP possible	n/a	A	Negative	25600	0	Negative	n/a
18	DSH	11 y	FIP possible	Pulmonary adenocarcinoma	P	Negative	320	0	Negative	n/a
19	DSH	2 y	FIP possible	n/a	A	Negative	160	4.91	25.56	M1058L + I1106T + T1112S
22	DSH	3 y	FIP possible	n/a	A	Negative	0	0	Negative	n/a
2	DSH	9 y	FIP very unlikely	Suspicion lymphoma	A	Negative	0	0	Negative	n/a
4	DSH	7 y	FIP very unlikely	Right congestive heart failure + Chronic kidney failure	A	Negative	0	0	Negative	n/a
5	RD	14y	FIP very unlikely	Hyperthyroidism and chronic triaditis	A	Negative	0	0	Negative	n/a
6	DSH	16 y	FIP very unlikely	n/a	P	Negative	0	0	Negative	n/a
7	DSH	12 y	FIP very unlikely	Left congestive heart failure	A	Negative	0	0	Negative	n/a
13	DSH	9 y	FIP very unlikely	n/a	P	Negative	0	0	Negative	n/a
14	BSH	2 y	FIP very unlikely	Hypertrophic cardiomyopathy	P	Negative	6400	0	Negative	n/a
17	DSH	12 y	FIP very unlikely	Suspicion lymphoma	P	Negative	0	0	Negative	n/a

A= abdominal, BSH= British Shorthair, DSH= Domestic Shorthair, P= pleural, RD= Ragdoll, SPX= Sphynx.

ered to be the gold standard for ante-mortem diagnosis of FIP in the present study. FCoV antigen was detected by IF in eight out of 22 cats as exemplified in Figure 1. Both positive and negative results were compared to their index of suspicion as determined by FIP-flowchart (Desmarets, 2015) or the presence of other diseases (imaging, biopsies or autopsy) (Figure 2).

Antibody titers for FIP positive samples varied between 640 and 51200, while titers for FIP negative samples varied between 0 and 25600. In effusions where FCoV antibodies were detected, FIP was only confirmed by IF in 57.1 % (8/14) of the cases. However, where antibodies were absent, IF was negative in all cases. On average, higher titers were detected in animals with FIP (FIP positive: sample mean (\bar{x}) = 19280.0, standard deviation (SD)= 20702.9; FIP negative: \bar{x} = 3230.0, SD= 6996.8).

FCoV genomic RNA was detected in 45.8 % (11/24) of the samples. If RNA was present, 72.7 % (8/11) of the samples were FIP (IF) positive. If viral RNA was not detected, all samples were FIP (IF) negative and in 69.2 % (9/13) of them, an alternative disease was diagnosed. FIP positive samples demonstrated RNA loads between $10^{2.22}$ and $10^{6.21}$ genome copies/ml. FIP negative samples were FCoV RNA negative in 81.3 % (13/16) of the cases. If RNA was present, antibodies were detected. On the contrary, in absence of antibodies, no RNA was present in the sample. There was no linear correlation between the antibody titer and RNA load (Pearson correlation $r = -0.015$, $p = 0.94$). Finally, when no RNA or antibodies were detected, there was no expression of viral antigen in the cell population being considered, as assessed by IF staining. When both RNA and antibodies were present in the sample, FIP could be confirmed in 72.7 % (8/11) by IF staining of effusion cells.

When viral RNA was detected, RT-PCR was carried out with primers targeting ORF S of FCoV serotype I or II. A positive result was recorded in nine samples when a first set of primers (serotype I) (Chang et al., 2012) was used. Two samples showed no response, necessitating the use of a second set of primers (serotype II) (Barker et al., 2017). None of the two samples showed any replication during this second RT-PCR, although the positive control sample (FIPV

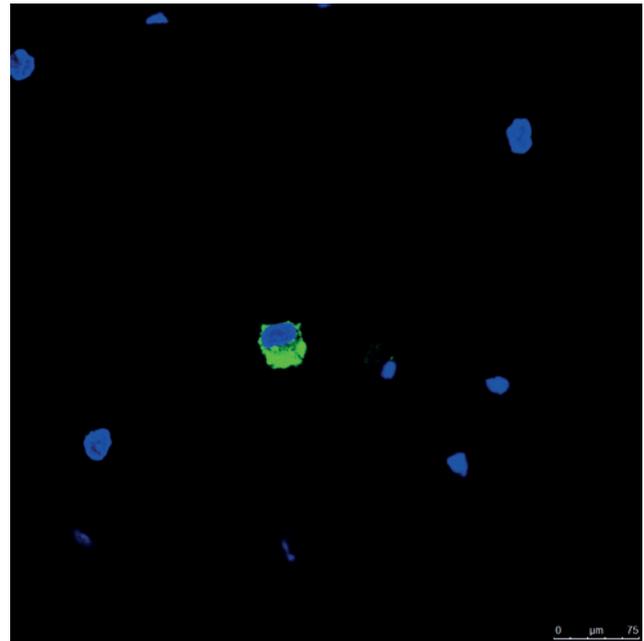


Figure 1. IF staining for FCoV antigen in macrophages. Blue (Hoechst) and green (goat-anti-mouse IgG1 FITC) fluorescence staining are compatible with the cell nucleus and the presence of intracellular FCoV antigen, respectively.

serotype II 79-1146) ensured correct implementation.

In eight out of nine samples, sequencing revealed one or more mutations in ORF S (Tables 1 and 2). Mutation M1058L at the level of the fusion peptide (FP) was the most commonly observed, being present in 66.7 % (6/9) of the sequenced samples. The methionine (M) - leucine (L) shift was caused by a A - T or A - C point mutation. In 66.7 % (4/6) of these samples, FIP was confirmed by IF staining. Mutation S1060A, representing a serine (S) - alanine (A) shift at AA residue 1060 within the S protein, was only detected in one FIP (IF) negative sample. In that case, a T - G shift was observed. In 22.2 % (2/9) of the samples, mutation I1106T was present at the level of the heptad repeat (HR) 1 region. The isoleucine (I) - threonine (T) AA shift at position 1106 was caused by a T - C point mutation. FIP was confirmed by IF in one out of two cases. In an additional 33.3 % (3/9) of the samples, a second mutation D1108Y/E/G was

Table 2. Sequencing results of ORF S.

Region	Original codon	Original AA	Sequenced codon	Corresponding AA	Mutation code
Fusion peptide	ATG	Methionine	TTG CTG	Leucine	M1058L
Fusion peptide	TCT	Serine	GCC	Alanine	S1060A
Heptad repeat 1	ATC	Isoleucine	ACA	Threonine	I1106T
Heptad repeat 1	GAT	Aspartic acid	TAC	Tyrosine	D1108Y
			GAA	Glutamic acid	D1108E
			GGG	Glycine	D1108G
Heptad repeat 1	ACT	Threonine	AGT	Serine	T1112S
			AAT	Asparagine	T1112N

Table 3. Test characteristics for FCoV antibody titers with increasing cut-off values, the presence of RNA or serial testing for the presence of antibodies (1) and FCoV RNA (2).

	Titer >0 ⁽¹⁾	Titer >1600	Titer >3200	Titer >6400	FCoV RNA ⁽²⁾	Serial (1) + (2)
Sensitivity	100 %	75.0 %	75.0 %	62.5 %	100 %	100 %
Specificity	62.5 %	75.0 %	75.0 %	87.5 %	81.2 %	93.0 %
PPV	57.1 %	60.0 %	60.0 %	71.4 %	72.7 %	87.6 %
NPV	100 %	85.7 %	85.7 %	82.3 %	100 %	100 %
True prevalence	33.3 %	33.3 %	33.3 %	33.3 %	33.3 %	33.3 %
Apparent prevalence	58.3 %	41.7 %	41.7 %	29.2 %	45.8 %	38.0 %
Sample size	24	24	24	24	24	24

dered to be the reference test in this study, no results for Se or Sp could be obtained. Nevertheless, the IF results were compared with the index of suspicion and the presence of an alternative diagnosis (depending on the available data). For two samples, there were indications to believe that IF led to a false negative result. The first sample was obtained from Cat 8, a three-year-old European Shorthair, which presented with anorexia, lethargy and abdominal distension for three weeks. After abdominocentesis, a yellow translucent exudate (total protein >75 g/L) was obtained with a moderate neutrophilia. The sample had a high antibody titer of 12800 in conjunction with a high RNA load ($10^{5.52}$ copies/ml). The second sample was collected from Cat 9, an eight-months-old British Shorthair who developed anorexia, diarrhea and pleural effusion shortly after adoption. No additional ante- or post-mortem examinations were performed. Again, a high antibody titer (6400) and RNA load ($10^{5.95}$ copies/ml) were found. As samples were collected and processed within 24 hours, it could not be excluded that differences within this storage interval attributed to a decrease of the Se of the IF staining in some of the samples, as observed in a previous study by Litster et al. (2013). The presence of a false negative IF testing in Cat 9 could not be excluded in absence of post-mortem analysis. As subjectively more background staining was observed in thoracic than in abdominal effusion, the use of the latter is recommended by the authors, if available.

Next, the use of antibody titers in effusion samples for diagnosing FIP was evaluated. Whereas in previous studies, enzyme-linked immunosorbent assay (ELISA), immunofluorescence assay (IFA) or rapid immunomigration (RIM) on serum were primarily used, in the current study, IPMA was used to determine titers on effusion (Pedersen, 2009; Addie et al., 2015). On average, higher antibody titers were detected in cats with FIP than in cats without (FIP positive: \bar{x} = 19280.0, SD= 20702.9; FIP-negative: \bar{x} = 3230.0, SD= 6996.8). However, the presence of antibodies only correlated with FIP in 57.1 % (8/14) of the cases. The Sp (62.5 %) and PPV (57.1 %) for IPMA with cut-off value titers >0 was lower than reported by

Hartmann et al. (2003), who observed a much higher Sp (85 %) and PPV (90 %). Furthermore, they found no correlation between the magnitude of the antibody titer and the presence of FIP (Hartmann et al., 2003). In the present study, only a mild increase in Sp (75.0 %) and PPV (60.0 %) was found if a higher cut-off value of >1600 or 3200 was selected. When only samples with antibody titers of over 6400 were analyzed, Sp and PPV increased to 87.5 % and 71.4 %, respectively; however, Se and NPV decreased further to 62.5 % and 82.3 %. Antibody titers of more than 6400 were only present in just over half of the samples of cats with FIP. In the present study, NPV was 100%, indicating that a lack of FCoV antibodies makes a diagnosis of FIP very unlikely. PPV for FCoV serology alone was 57%; however, the authors want to point out that a lack of data in the literature on FCoV seroprevalence in Belgium and other European countries further complicated the interpretation of these results, thus emphasizing the need for seroprevalence studies in these regions.

PCR-based techniques are gaining importance for diagnosing many infectious diseases, and FIP is no exception. Again, since clear genetic markers for FIPV are still unknown, results should be interpreted with care. Interpretation also highly depends on the sample type: PCR of blood samples is not recommended due to absence of viral RNA in blood samples in most FIP cases (Tasker, 2018). In the current study, an in-house developed 5' RT-qPCR was employed with primers targeting ORF 1b, as a more accurate estimation of the RNA load had been observed in the original study using fecal material (Desmarests et al., 2013). However, it could not be stated that the outcome resulted from using effusion instead of feces. For this reason, a specific amount of virus was added to an FCoV negative feces sample, an FCoV negative ascites sample and medium, showing no significant difference in RNA load (data not shown). The calculated Se of 100 % proved to be much higher than the 78.4 to 85.0 % observed in earlier studies using a 3' RT-qPCR. But more importantly, the calculated Sp of 72.7 % was lower than the previously reported 97.9 % to 100 % (Barker et al., 2017; Longstaff et al., 2017). None-

theless, the authors believe that using the cell pellet instead of the untreated effusion as done in previous studies, might have increased the Se. This is based on the fact that FCoV serotype I is cell-associated, resulting in intracellular FCoV RNA concentrations that can be up to a thousand times higher than the supernatant (Pedersen et al., 2015). It could be confirmed that when the RNA load was measured on the cell pellet, the supernatant or the untreated effusion, the highest viral RNA load was found when using the cell pellet (data not shown). The primers used in this assay are less likely to be the cause of the apparently lower Sp, as they are expected to increase the Sp in comparison with classic 3' RT-qPCRs by specifically amplifying genomic RNA instead of subgenomic RNA, thus providing a better estimation of the true viral particle load (Desmarets, 2015; Tasker, 2018). The question arises as to what extent false negative results on IF might have contributed to the lower Sp of the RT-qPCR. If present, possibly those cases represent cats with a very acute onset of disease, whereby the granulomatous periphlebitis lesions are still developing, leading to an insufficiently low cell count for IF. To test this hypothesis, sequential sampling is needed. Nevertheless, there was no association between the duration of the physical complaints of the cats and the RNA load in the present study. In previous studies by Barker et al. (2017) and Longstaff et al. (2017), post-mortem inspection was used followed by immunohistochemistry and exclusion of samples, for which no definite diagnosis was achieved, thus minimalizing the presence of false negative results. It is possible that the Sp of the current 5' RT-qPCR is higher when tested under the same circumstances. If this is true, RT-qPCR can prove to be a valuable alternative for IF in effusive FIP.

In a study by Meli et al. (2013), the RNA load and antibody titer of a series of serum and effusion samples from cats with FIP using different FCoV antibody tests were simultaneously investigated. The authors reported low antibody titers in the presence of high RNA loads, primarily when using in-house rapid immunomigration tests on effusion samples. One suggested explanation for this phenomenon is that virus-bound antibodies in virus-rich samples are unavailable for the detection with the antibody test. However, as ELISA and IFA test are less affected by this phenomenon and as individual variability exists between different RIM tests, the importance of the individual test sensitivity and quality has again been demonstrated (Meli et al., 2013). In a more recent and extensive study by Lorusso et al. (2017), simultaneous determination of the RNA load and antibody titer revealed no significant correlation between high RNA loads and low antibody titers. In contrast, the authors stated that mainly low RNA loads had been found in samples with a low antibody titer. The influence of antibody sequestration on diagnosing FIP is therefore questioned (Lorusso et al., 2017). In analogy with Lo-

russo et al. (2017), in the present study, no correlation between the RNA load and the antibody titer in effusion samples (Pearson correlation $r = -0.015$, $p = 0.94$) was found. However, it cannot be completely excluded that antibody sequestration was still present in the samples studied, albeit to a very limited extent. It would provide a logical explanation for the samples from Cats 20 and 21 presenting high RNA loads of $10^{4.84}$ and $10^{6.21}$ copies/ml, respectively and relatively low antibody titers of 640 and 1280. Furthermore, experiments performed by Lorusso et al. (2017) suggested the potential of combining molecular and serological techniques for the diagnosis of effusive FIP. The current study yielded results that support this hypothesis. It was predicted that if both tests were used (only RT-qPCR if IPMA titers >0), this would result in a maximum Sp and PPV of 93.0 % and 87.6 %, while both Se and NPV would remain at 100 %.

In 2015, biotyping became commercially available by RT-qPCR (FIP Virus RealPCR™ Test, IDEXX, USA) based on two FP mutations (M1058L and S1060A) described by Chang et al. (2012). Nonetheless, the same mutations have been demonstrated in 88.2 to 89 % of tissue samples from clinically healthy cats, suggesting that these mutations are no genetic markers for FIP phenotype, but rather correlate with the systemic spread of the virus (Porter et al., 2014; Barker et al., 2017). A possible explanation why both mutations in the study conducted by Chang et al. (2012) were only found in FIP-associated FCoV is that the study did not contain tissue samples from FIP-negative cats and were only compared with feces samples originating from clinically healthy animals (Porter et al., 2014). Given the high Se (78.4-85.0 %) and Sp (97.9-100 %), which were associated with the use of RT-qPCR for the diagnosis of FIP on effusion or CSF in earlier studies, the question arises to what extent the additional detection of M1058L and S1060A contributes to the accuracy of the technique (Porter et al., 2014; Doenges et al., 2015; Barker et al., 2017; Longstaff et al., 2017). In a study by Barker et al. (2017), it has been shown that an additional sequencing step to detect M1058L and S1060A reduces Se from 78.4 % to 60.0 %, while Sp (97.9 %) remains unchanged. A potential advantage RT-qPCR may offer over IF when applied on effusion, might be lost by additional detection of M1058L (and S1060L) (Tasker, 2018). In this study, consecutive RT-PCR and Sanger sequencing was performed successfully in nine out of eleven RNA-positive samples. No amplification was present for two samples (3 and 15). RT-PCR was repeated in these two samples using a second set of primers specifically targeting serotype II (Barker et al., 2017). Since again, no amplification of the correct fragment was observed, the RNA load was suspected to be too low to replicate.

Similar to previous studies, sequencing yielded a high mutation frequency across ORF S (Chang et al., 2012; Licitra et al., 2013; Bank-Wolf et al., 2014;

Lewis et al., 2015). Only in a few cases, mutation provoked an AA substitution, represented by M1058L, S1060A, I1106T, D1108Y/E/G, T1112S and T1112N. The most common mutation in this study was again M1058L (FP), as it was found in 66.7 % (4/6) of the FIP-positive samples. The results were in line with previous studies, in which a prevalence of 64 %, 65 % and 83 % was observed using effusion samples of FIP positive cats (Barker et al., 2017; Felten et al., 2017; Longstaff et al., 2017). A second mutation S1060A (FP), has been reported in 3-6 % of the effusion samples from FIP-affected cats (Barker et al., 2017; Felten et al., 2017; Longstaff et al., 2017). In the current study, S1060A was only found in the sample of Cat 8, for which a negative IF-result was obtained. The finding of these mutations in three IF-negative cats could possibly provide additional evidence for the hypothesis that these mutations described by Chang et al. (2012) merely account for the systemic distribution of the virus (Porter et al., 2014; Barker et al., 2017). It would require additional and presumably unknown mutations to occur in the viral genome, after which FIP develops through uncontrolled replication and a disturbed host response (Porter et al., 2014; Barker et al., 2017). Due to a lack of post-mortem testing, it was impossible to draw conclusions from the presence of these mutations in FIP-negative animals. Nevertheless, the absence of both M1058L and S1060A or only S1060A in 33.3 % and 100 % of the FIP-associated FCoV, respectively, allowed to assume that the above-mentioned hypothesis is very likely. Furthermore, two other mutations were observed in the HR1 region. However, unlike the original studies that reported the corresponding mutations I1106T and D1108Y/E/G in 100 % and 55.5 % of the FIPVs, in the present study, the mutations could only be observed in 16.7 % (1/6) and 33.3 % (2/6) of the FIPVs (Lewis et al., 2015; Bank-Wolf et al., 2014). According to Lewis et al. (2015), both AA changes associated with I1106T and D1108Y/E/G occur at the level of a fifteen AA-long chain characteristic for the heptad repeat structure of the HR1 region of *alphacoronaviridae* [NAIT(I/T)S(D/Y/E/G)GFNTMAS], by which the author suggested that a functional relationship between both mutations cannot be excluded (Bank-Wolf et al., 2014; Lewis et al., 2015). Remarkably, two other mutations (T1112S and T1112N) were found in this 15 AA chain, which appeared to be present in four isolates that tested positive for FIP on IF [NAIT-TISDGFN (T / S or T / N) MAS] in the present study. However, similar to both mutations in the FP, a mutated HR1 was also found in the samples from Cat 8 (D1108Y/E/G, T1112S), 9 (T1112N) and 19 (I1106T, T1112S), which were classified as being FIP-negative by IF. Thus, the absence of post-mortem investigation to firmly exclude FIP in these samples was a drawback in this study. Additionally, as no specific regional FECV sequencing data existed for comparison, it cannot be excluded that these mutations are common in Belgian FECV strains. The question therefore aris-

es to what extent mutations in the HR1 region offer clinical significance. After all, it is not inconceivable that in case of FCoV, the region concerned has already undergone functional loss, leading to uncontrolled accumulation of mutations. Therefore, the use of reverse genetics (in vitro and in vivo) appears to be necessary for future research. It should be noted that mutations in other genes have been associated with FIP (Pedersen, 2014b), for example specific mutations at the S1/S2 cleavage site of ORF S (Licitra et al., 2013). Their presence was not evaluated in the current study.

CONCLUSION

It can be concluded that in cats, higher FCoV antibody titers measured in effusion tend to correlate with the presence of FIP determined by IF. Nevertheless, even titers of more than 6400 as present in just above half of the cases, could not provide sufficient accuracy for the diagnosis of FIP. On the other hand, cats with no antibodies or only very low antibody titers were very unlikely to have FIP. The presence of FCoV RNA in effusion measured by an in-house developed 5' RT-qPCR provided a much better Sp with a very high Se than an in-house IPMA. These results suggest that RT-qPCR may serve as an alternative for IF, certainly if the sample volume (effusion) is insufficient for IF testing. Nonetheless, future studies are indispensable. Moreover, the authors believe that the combination of IPMA and RT-qPCR should be considered in clinical practice to diagnose FIP. The addition of T1112S/N to a group of nearby mutations more often seen in the HR1 of FIPV may point in the direction of a functional relationship, although more research is strictly necessary to demonstrate a possible function within the pathogenesis of FCoV.

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Uit het verleden

ZEEWATERKUUR VOOR PAARD GEBETEN DOOR RAZENDE HOND (1509-1510)

Zeekuren kwamen naar ons weten pas vanaf de 19^{de} eeuw in de mode, maar in het begin van de zestiende eeuw verwachtte men daar blijkbaar ook al iets van, en wel iets heel eigenaardigs. De brouwer van het Onze Lieve Vrouwe Hospitaal in Oudenaarde werd toen naar het Noordzeestrand gestuurd met een door een razende hond gebeten paard om het dier te laten genezen (of voorbehoedend te vrijwaren?) door het in het zoute water te laten lopen.

Ghijs, N., Martens, N., Castelain, R., Van Bockstaele, G. (2004). *Het Onze Lieve Vrouwehospitaal te Oudenaarde en de Zusters Bernardinnen*. Gent, Provincie Oost-Vlaanderen, p. 72

Luc Devriese