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Detection of feline coronavirus mutations in paraffin-embedded tissues in cats with feline infectious peritonitis and controls

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Abstract

Objectives The amino acid substitutions M1058L and S1060A in the spike protein of feline coronavirus (FCoV) have been postulated to be responsible for the development of the pathogenic feline infectious peritonitis virus (FIPV), which causes feline infectious peritonitis (FIP). The aim of the following study was to investigate the presence of mutated virus in tissue samples of cats with and without FIP.

Methods The study population consisted of 64 cats, 34 of which were diagnosed with FIP and 30 control cats. All cases underwent autopsy, histopathology and immunohistochemistry (IHC) for FCoV. Furthermore, a genotype-discriminating quantitative reverse transcriptase PCR (RT-qPCR) was performed on shavings of paraffin-embedded tissues to discriminate between cats with FIP and controls, and the sensitivity and specificity of this discriminating RT-qPCR were calculated using 95% confidence intervals (CIs).

Results Specificity of genotype-discriminating RT-qPCR was 100.0% (95% CI 88.4–100.0), and sensitivity was 70.6% (95% CI 52.5–84.9). In cats with FIP, 24/34 tested positive for FIPV. In samples of three control cats and in seven cats with FIP, FCoV was found, but genotyping was not possible owing to low FCoV RNA concentrations. Out of the positive samples, 23 showed the amino acid substitution M1058L in the spike protein and none the substitution S1060A. One sample in a cat with FIP revealed a mixed population of non-mutated FCoV and FIPV (mixed genotype). For one sample genotyping was not possible despite high viral load, and two samples were negative for FCoV.

Conclusions and relevance As none of the control animals showed FCoV amino acid substitutions previously demonstrated in cats with FIP, it can be presumed that the substitution M1058L correlates with the presence of FIP. FCoV was detected in low concentration in tissues of control animals, confirming the ability of FCoV to spread systemically. The fact that no negative controls were included in the IHC protocol could potentially lead to an underestimation of the sensitivity of the RT-qPCR.

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Introduction

Feline infectious peritonitis (FIP) predominantly affects young cats, with those <1 year of age being especially prone to developing this fatal disease.[1] FIP is caused by a certain genotype of feline coronavirus (FCoV). FCoV exists in two genotypes: feline enteric coronavirus (FECV), which is benign but very common, whereas the sporadically appearing FIP virus (FIPV) triggers a lethal immune response in affected cats.[2]

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FIPV develops out of FECV within infected cats.[3,4] Two mutations in nucleotide 23531 and nucleotide 23537 in the spike (*S*) gene, resulting in the substitution of a methionine to leucine codon at position 1058 (M1058L) and a substitution of a serine to alanine codon at position 1060 (S1060A) of the spike protein, respectively, have been considered responsible for the genotype switch and for an enhanced macrophage tropism.[5,6] However, other mutations have also been hypothesised to play a role, such as changes in the 3c, 7a, 7b gene,[7,8] or, more recently, in the furin cleavage site in the region between receptor-binding (S1) and fusion (S2) domains of the spike gene, enhancing virus uptake by macrophages.[3]

Production of cytokines via infected macrophages and activation of neutrophils result in histological lesions typical of FIP,[9] consisting of granulomas with focal and perivascular lymphoplasmocytic infiltrates and pyogranulomatous–necrotising vasculitis.[10] Identification of such histopathological lesions with immunohistochemical (IHC) detection of FCoV antigen in tissue macrophages is considered the gold standard of FIP diagnosis.[11] Thus, organ biopsies for histopathology and IHC are necessary for a definitive diagnosis, which have to be obtained through invasive procedures.

Recently conducted studies on the presence of FCoV with a mutation in the region of the *S* gene have delivered contradictory results. A group in the Netherlands obtained full genome sequence data from FCoV strains in the faeces of healthy cats and tissues or ascites of cats with FIP, and found that the two gene mutations in nucleotide 23531 and nucleotide 23537 distinguished FIPV from FECV in >95% of all cases.[5]

Another study conducted in England included 45 tissue samples from 27 cats with FIP and 41 tissue samples obtained from 16 control animals (excluding faecal samples). Out of all of the tissue samples from cats with FIP, 43/45 samples were positive for FCoV using quantitative reverse transcriptase PCR (RT-qPCR), with 39 samples showing the leucine codon (M1058L), which was identified using pyrosequencing. Of the 41 tissue samples from control animals, nine were positive for FCoV, with eight samples presenting the leucine codon at position 1058. As the leucine codon (M1058L) in the FCoV spike protein had previously only been found in cats with FIP, this questions the hypothesis that the M1058L substitution only occurs in cats with FIP and is responsible for the genotype switch. Thus, the authors of the latter study concluded that the M1058L substitution would only be an indicator for the ability of FCoV to spread systemically from the gastrointestinal tract and not for confirming FIP.

It still remains under debate whether the M1058L and S1060A substitutions in the spike protein are responsible for the genotype switch or not. Therefore, it was the aim of the present study to assess the presence of mutated virus in tissues of a large and well-defined population of cats with and without FIP.

Materials and methods

Animals

The study included 64 cats, 34 of which were diagnosed with FIP and 30 were classified as control animals. All cats were presented to the Clinic of Small Animal Medicine or were submitted directly to the Institute of Veterinary Pathology for autopsy between 2012 and 2015. Only cats in which a full-body examination, as well as IHC staining for FCoV antigen in tissue macrophages was performed, were included. Cats were classified as having FIP if they demonstrated macroscopic, as well as histological, changes consistent with FIP and a positive IHC staining of tissue samples (Figure 1, Table 1). The main criteria for inclusion of control cats were the possibility to perform a full-body necropsy and presence of at least one clinical problem indicative of FIP, such as ascites, thoracic effusion, pericardial effusion, dyspnoea, icterus, fever, glomerulonephritis, neurological signs or uveitis (Table 2). Only cats in which negative IHC staining for FCoV antigen in organ tissue macrophages was obtained were included in the control group. The animals were not age-matched. Every cat that was presented to the Clinic of Small Animal Medicine and that fulfilled the inclusion criteria was included into the study. The study was approved by the ethical committee of the Centre for Clinical Veterinary Medicine of LMU (reference number 52-27-07-2015).

IHC

In all cats, IHC was performed to diagnose or exclude FIP as described previously.[12] As primary antibody,

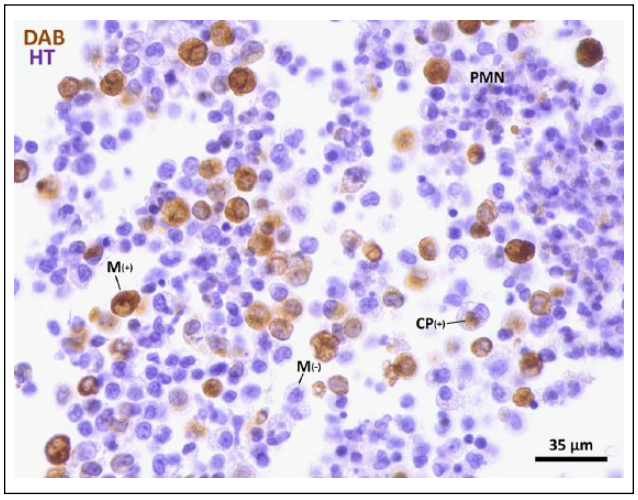

Figure 1 Immunohistochemical staining for feline coronavirus antigen using chromogen 3,3′-diaminobenzidine (DAB; brown) and haematoxylin (HT; violet) as counterstain. Note immunopositive (M(+)) vs immunonegative (M(–)) macrophages and those engulfing immunopositive cells via cytophagocytosis (CP(+)). Apart from macrophages, the infiltrate contains some lymphocytes and aggregates of polymorphonuclear neutrophils (PMN)

anti-FCoV mouse monoclonal IgG2A (FIPV3-70; Linaris) was used. Samples were then left to incubate for at least 17 h overnight at 4°C and treated on the second day with the secondary antibody (biotinylated goat anti-mouse; Dako). An avidin-biotinylated horseradish peroxidase (HRP)-complex (ABC, Vectastain ABC Kit; Vector Laboratories) was applied to bind to the biotin of the secondary antibody, presenting the biotin-bound HRP for the final step. 3,3′-Diaminobenzidine tetrahydrochloride (DAB; pH 7.0; Kem-En-Tec Diagnostics) was combined with 3% hydrogen peroxide to catalyse the oxidation of DAB to a visibly brown chromogen. To rule out staining errors, positive tissue controls were part of every protocol, but no negative controls were included.

RT-qPCR to discriminate the FCoV genotype

Tissue samples of all cats were obtained post-mortem. Tissue samples were elected retrospectively, including samples of cats with FIP from previous studies,[12,13] whereas processing and investigation was undertaken prospectively using manufactured paraffin blocks. Samples of various tissues had been collected of each animal during necropsy and subsequently fixed in paraffin blocks (Tables 3 and 4). The samples for RT-qPCR were then shaved off each block with a thickness of 16–32 µm per sample. As one block and therefore one shaved sample could contain several different samples of tissues, a differentiation of the tissues was not possible. Five samples from each animal were then pooled for RT-qPCR analysis, yielding only one result per animal.

Genotype-discriminating RT-qPCR was performed blinded with regard to the underlying diagnoses. Total nucleic acid was extracted from paraffin-embedded tissue samples by a QIAamp DNA Blood BioRobot MDx Kit on an automated Qiagen platform according to the manufacturer's instructions with slight modifications (IDEXX Laboratories). In order to determine the presence of FCoV and to discriminate the genotype, three real-time PCR assays were performed in parallel as single-plex reactions (IDEXX Laboratories). The first real-time PCR was based on the *7b* gene to detect FCoV RNA and quantify viral burden;[14] the other two real-time PCRs were targeting the M1058L and S1060A single nucleotide polymorphisms (SNPs) described before to correlate with the occurrence of the lethal FIPV genotype.[5] These two PCR assays allow typing of an FCoV strain based on the presence (FIPV) or absence (FECV) of one of the two SNPs within the fusion peptide of the spike gene. Briefly, highly specific hydrolysis probes were designed to either detect the mutation at position 3174 or 3180 (corresponding to M1058L and S1060A) or wild-type sequences by using an allelic discrimination real-time PCR approach. Fluorescence intensities were used to calculate ratios of the probes detecting the mutation or the wild-type sequences. FIPV was assigned if the mutation probe exceeded a two-fold higher fluorescence than the wild-type probe.

Genotype-discriminating real-time PCR was run with six quality controls, including: (1) PCR-positive controls (quantitatively; using synthetic DNA covering the real-time PCR target region; Integrated DNA Technologies); (2) PCR-negative controls (PCR-grade nuclease-free water); (3) negative extraction controls (extraction positions filled with nucleic acid-free water only); (4) RNA pre-analytical quality control targeting feline ssr rRNA (18S rRNA) gene complex; (5) a swab-based environmental contamination monitoring control; and (6) spike-in internal positive control (using lambda phage DNA). These controls assessed the functionality of the PCR test protocols (1), for the absence of contamination in the reagents (2) and laboratory (5), absence of cross-contamination during the extraction process (3), quality and integrity of the RNA as a measure of sample quality (4), and absence of PCR inhibitory substances as a carry-over from the sample matrix (6). The analytical, as well as the clinical, specificity of the RT-qPCR was confirmed by re-sequencing amplification products with dedicated sequencing primers located 5′ and 3′ to those used in the routine RT-PCR protocol. For Cp values regarding the endogenous quality control (18S rRNA gene) as well as the detection of FCoV RNA (7b gene), see the supplementary material.

Corresponding to the outcome of the discriminating RT-qPCR, six different results were obtained (Table 5).

1 Genotype FIPV: the mutated genotype was detected at much higher levels than the wild-type in the sample of paraffin-embedded tissues.
2 Genotype FECV: FCoV without the mutation was found in the sample.
3 Mixed genotype: mutated as well as non-mutated FCoV RNA was detected.
4 Below limit of determination (BLD): FCoV RNA was detected at low viral burden, with the mutation assays not yielding a result.
5 Indeterminate (IND): FCoV RNA was found in high concentration, with the mutation assays not yielding a result.
6 Negative: no FCoV RNA was detected.

Statistical evaluation

Samples were evaluated for the presence of mutated and non-mutated virus. In order to define parameter uncertainty, a confidence interval (CI) of 95% was calculated. Sensitivity, specificity, positive predictive value (PPV) and negative predictive value (NPV) were determined. The overall accuracy was calculated as the sum of true positive and true negative test results divided by the total number of test results. Sample results classified as BLD or IND were defined as negative in statistical analysis, owing to the inability to determine the underlying genotype. Samples containing a mixed genotype were regarded as positive.

Table 1 Presence of effusion, results of the genotype-discriminating RT-qPCR, amount and thickness of shavings per block in 34 cats with feline infectious peritonitis (FIP)

Cat	Presence of effusion due to FIP	Major additional clinical problems	Amount and thickness of shavings per block (µm)	Result of genotype-discriminating RT-qPCR
1	Ascites	Anorexia, lethargy	5 × 16	BLD
2	Ascites and fluid in scrotal cavity	Anaemia, icterus	5 × 16	BLD
3	Ascites	Fever, icterus	5 × 16	FIPV, M1058L
4	Ascites	Anaemia, diarrhoea, icterus	5 × 32	FIPV, M1058L
5	Pericardial effusion	Anaemia	5 × 16	FIPV, M1058L
6	Ascites, thoracic effusion	Dyspnoea	5 × 16	BLD
7	No effusion	Fever, hyperglobulinaemia	5 × 16	FIPV, M1058L
8	Ascites	Anaemia, hyperglobulinaemia	5 × 16	FIPV, M1058L
9	Ascites	Dyspnoea, fever, icterus	5 × 16	BLD
10	Ascites	Hyperglobulinaemia, icterus	5 × 16	FIPV, M1058L
11	Ascites	Anaemia, icterus	5 × 16	FIPV, M1058L
12	Ascites	Anorexia, fever, icterus	5 × 16	FIPV, M1058L
13	No effusion	Anaemia, fever, hyperglobulinaemia	5 × 16	FIPV, M1058L
14	Ascites	Anaemia, fever, hyperglobulinaemia, uveitis	5 × 16	FIPV, M1058L
15	Ascites, pericardial effusion	Anaemia, hyperglobulinaemia, icterus	5 × 16	FIPV, M1058L
16	Ascites	Fever, icterus, cachexia	5 × 16	BLD
17	Ascites, thoracic effusion	Anaemia, uveitis	5 × 16	FIPV, M1058L
18	Ascites	Anaemia, hyperglobulinaemia, icterus	5 × 16	FIPV, M1058L
19	Ascites	Fever, lethargy	5 × 16	Negative
20	No effusion	Anaemia, dyspnea, hyperglobulinaemia, icterus	5 × 16	Negative
21	Ascites	Anaemia, icterus	5 × 32	FIPV, M1058L
22	Pleural, pericardial effusion	Fever, uveitis	5 × 32	FIPV, M1058L
23	Pleural, pericardial effusion	Anaemia, icterus	5 × 32	FIPV, M1058L
24	Thoracic effusion	Anaemia, dyspnoea, fever	5 × 32	FIPV, M1058L
25	Ascites	Anaemia, fever, icterus	5 × 32	IND
26	Ascites	Anaemia, icterus, hyperglobulinaemia, neurological signs	5 × 32	FIPV, M1058L
27	Ascites	Anaemia, dyspnoea, fever, hyperglobulinaemia	5 × 32	FIPV, M1058L
28	Ascites	Weight loss, icterus	5 × 32	FIPV, M1058L
29	Ascites	Icterus, lethargy, weight loss	5 × 32	BLD
30	Ascites	Anaemia, icterus	5 × 32	Mixed genotype
31	Ascites	Anorexia, fever	5 × 32	FIPV, M1058L
32	Ascites	Diarrhoea, icterus, lethargy	5 × 32	BLD
33	Ascites	Anaemia, fever	5 × 32	FIPV, M1058L
34	Pleural and pericardial effusion	Dyspnoea, neurological signs	5 × 32	FIPV, M1058L

BLD = feline coronavirus (FCoV) present but below limit of detection; FIPV = feline infectious peritonitis virus; IND = FCoV present, but indeterminate strain variations

Table 2 Diagnosis, clinical presentation, results of the genotype-discriminating RT-qPCR and amount and thickness of shavings per block in 30 control cats

Cat	Diagnosis at necropsy	Major clinical problems	Amount and thickness of shavings per block (µm)	Result of genotype-discriminating RT-qPCR
1	Globoid cell leukodystrophy	Pericardial effusion, tetraplegia, head tremor	5 × 32	Negative
2	Lymphocytic meningoencephalitis	Icterus	5 × 32	BLD
3	Intracranial neoplasia	Ataxia, paraparesis	5 × 32	BLD
4	Intestinal lymphoma, meningoencephalitis	Seizures	5 × 32	Negative
5	Hypertensive intracranial bleeding	Neurological deficits	5 × 32	Negative
6	Squamous cell carcinoma	Seizures	5 × 32	Negative
7	Heart failure, chronic interstitial nephritis	Seizures	5 × 32	Negative
8	Renal lymphoma, hippocampal sclerosis	Seizures	5 × 32	Negative
9	Pulmonary adenocarcinoma	Thoracic effusion	5 × 32	Negative
10	Bronchial carcinoma	Thoracic effusion	5 × 32	Negative
11	Pancreatitis, hepatic lipidosis, enteritis	Icterus, ascites	5 × 32	Negative
12	Toxoplasmosis, hypertensive encephalopathy, chronic nephritis	Thoracic effusion	5 × 32	Negative
13	Salmonellosis	Icterus	5 × 32	BLD
14	Chronic glomerulonephritis, conjunctivitis	Glomerulonephritis	5 × 32	Negative
15	Enteritis, cholangiohepatitis	Ascites, icterus	5 × 32	Negative
16	Persisting foramen ovale	Ascites, thoracic effusion	5 × 32	Negative
17	Mediastinal angiosarcoma	Thoracic effusion	5 × 32	Negative
18	Lymphoma (liver, pancreas)	Ascites	5 × 32	Negative
19	T-cell lymphoma	Thoracic effusion	5 × 32	Negative
20	Carcinoma	Thoracic effusion	5 × 32	Negative
21	Lymphoplasmacellular cholangiohepatitis	Icterus, fever	5 × 32	Negative
22	Cholangitis, degenerative poliomyelopathy	Seizures, icterus	5 × 32	Negative
23	Pulmonary adenocarcinoma	Thoracic effusion	5 × 32	Negative
24	Laryngitis, otitis media	Dyspnoea, uveitis	5 × 32	Negative
25	Acute renal failure	Dyspnoea, thoracic effusion	5 × 32	Negative
26	Chronic nephritis	Renal failures, ascites	5 × 32	Negative
27	Meningoencephalitis	Seizures, opisthotonus	5 × 32	Negative
28	Pulmonary carcinoma	Anorexia, dyspnea	5 × 32	Negative
29	Chronic nephropathy	Anorexia, fever	5 × 32	Negative
30	Osteosarcoma, biliary cirrhosis	Ascites	5 × 32	Negative

BLD = feline coronavirus present but below limit of detection

MS Excel , as well as an online program that calculates a non-asymptotic binomial CI (https://stattools.crab.org/Calculators/binomialConfidence.htm), were used for statistical analysis.

Results

In 32/34 samples of paraffin-embedded tissue of cats with FIP, FCoV RNA was detected, and in 24 of these, the FIPV genotype could be determined. Twenty-three samples showed the amino acid substitution M1058L (Table 5). One sample contained a mixed genotype (mixed population of FECV and FIPV). In seven of the FCoV-positive samples from cats with FIP, genotyping was not possible because of a low virus load (BLD). One sample revealed a high FCoV load (in a cat with FIP), but genotyping was not possible (IND), owing to the occurrence of an

Table 3 Details of the tissues used for genotype-discriminating RT-qPCR for 34 cats with feline infectious peritonitis

Cat	Result of genotype-discriminating RT-qPCR	Tissues sampled
1	BLD	Intestine, kidney, liver
2	BLD	Kidney, liver, spleen
3	FIPV, M1058L	Kidney, lung, muscle, spleen
4	FIPV, M1058L	Kidney, liver, lung, spleen
5	FIPV, M1058L	Intestine, kidney, liver
6	BLD	Intestine, liver, lung, lymph node, spleen
7	FIPV, M1058L	Kidney
8	FIPV, M1058L	Intestine, liver, lung, muscle, pancreas
9	BLD	Kidney, liver, lung
10	FIPV, M1058L	Intestine, liver, lung, spleen
11	FIPV, M1058L	Intestine, liver, lung, spleen
12	FIPV, M1058L	Intestine, kidney, liver, lung, muscle, spleen
13	FIPV, M1058L	Kidney, liver, pancreas, spleen, testis
14	FIPV, M1058L	Intestine, liver, lung, spleen, thyroid
15	FIPV, M1058L	Kidney, spleen
16	BLD	Intestine, kidney, liver, lymph node, spleen
17	FIPV, M1058L	Kidney, spleen
18	FIPV, M1058L	Intestine, liver, lung, spleen
19	Negative	Kidney, liver, lung, lymph node, muscle, spleen, pancreas
20	Negative	Intestine, kidney, lung, lymph node, pancreas, spleen
21	FIPV, M1058L	Heart muscle, intestine, liver
22	FIPV, M1058L	Kidney, liver
23	FIPV, M1058L	Kidney, spleen
24	FIPV, M1058L	Adrenal gland, kidney, liver, lung, spleen
25	IND	Intestine, kidney, lung, muscle, spleen
26	FIPV, M1058L	Adrenal gland, intestine, lymph node, spleen, thyroid, uterus
27	FIPV, M1058L	Intestine, kidney, liver, lymph node, spleen
28	FIPV, M1058L	Kidney, liver, lung, muscle, spleen, thyroid
29	BLD	Kidney, lung, muscle, spleen
30	Mixed genotype	Intestine, kidney, liver, lymph node, spleen
31	FIPV, M1058L	Intestine, kidney, liver, lung, spleen
32	BLD	Intestine, kidney, liver, lung, lymph node, pancreas, spleen
33	FIPV, M1058L	Intestine, kidney, liver, lung, pancreas, spleen
34	FIPV, M1058L	Intestine, kidney, liver, lung, pancreas, spleen

BLD = feline coronavirus (FCoV) present but below limit of detection; FIPV = feline infectious peritonitis virus; IND = FCoV present but indeterminate strain variations

unknown FCoV strain (failed amplification) or infection with a serotype 2 FCoV strain. These eight samples were classified as negative in statistical analysis. Two samples from cats with FIP yielded negative results.

In the control group, 27/30 samples were negative for FCoV RNA. The samples of three control cats yielded positive results in RT-qPCR for FCoV (Tables 5 and 6). However, virus load was too low to allow genotyping (BLD). Sensitivity, specificity, NPV, PPV and overall accuracy of discriminating RT-qPCR to differentiate between cats with FIP and controls are listed in Table 6.

Discussion

The aim of this study was to demonstrate the presence of non-mutated or mutated FCoV in paraffin-embedded tissues using RT-qPCR, addressing the question of whether the M1085L and S1060A substitutions in the spike protein are responsible for the switch to the FIP disease phenotype or indicate systemic spread of FCoV. Previous studies have looked into the use of PCR in the detection of FCoV in various sample materials of cats with FIP, reporting conflicting results.[13,15–20] So far, no other study has investigated paraffin-embedded tissues using the recently commercially available genotype-discriminating RT-qPCR.

FIP is always fatal once diagnosis is confirmed. Thus, specificity of a diagnostic test plays a more important role than sensitivity, as cats falsely diagnosed with FIP might be unnecessarily euthanased. In the present study, specificity of the discriminating RT-qPCR in

Table 4 Details of the tissues used for genotype-discriminating RT-qPCR for 30 control cats

Cat	Result of genotype-discriminating RT-qPCR	Tissues sampled
1	Negative	Intestine, kidney, lung, lymph node, muscle, spleen
2	BLD	Intestine, kidney, liver, lung, lymph node, muscle, spleen
3	BLD	Adrenal gland, intestine, kidney, lung, muscle, spleen, thyroid
4	Negative	Adrenal gland, heart muscle, intestine, kidney, liver, lung, spleen
5	Negative	Intestine, liver, muscle
6	Negative	Intestine, kidney, liver, lung, lymph node, spleen
7	Negative	Intestine, kidney, liver, lung, muscle, pancreas
8	Negative	Intestine, kidney, liver, muscle, pancreas
9	Negative	Intestine, kidney, liver, muscle
10	Negative	Kidney, liver, lung
11	Negative	Intestine, kidney, liver, lung, thyroid
12	Negative	Liver, lung, spleen, thyroid, parathyroid
13	BLD	Intestine, lung, pancreas, spleen
14	Negative	Intestine, liver, lung, spleen
15	Negative	Kidney, liver, lung, muscle, pancreas, spleen
16	Negative	Heart muscle, intestine, kidney, liver, lung, spleen
17	Negative	Heart muscle, liver, lung
18	Negative	Intestine, kidney, liver, lung, muscle, spleen, thyroid
19	Negative	Intestine, kidney, liver, lung, spleen
20	Negative	Adrenal gland, intestine, kidney, liver, lung, thyroid
21	Negative	Adrenal gland, intestine, kidney, liver, lymph node, spleen
22	Negative	Intestine, kidney, liver, lung, muscle, pancreas, spleen, stomach, thyroid
23	Negative	Kidney, lung, lymph node
24	Negative	Intestine, liver, pancreas, spleen, thyroid
25	Negative	Intestine, kidney, liver, lung, spleen, thyroid
26	Negative	Heart muscle, intestine, kidney, lung
27	Negative	Intestine, kidney, liver, lung, spleen
28	Negative	Intestine, kidney, liver, lung, spleen
29	Negative	Intestine, kidney, liver, lung, pancreas, spleen
30	Negative	Intestine, kidney, liver, lung, spleen

BLD = feline coronavirus present but below limit of detection

paraffin-embedded tissues and thus of detecting one of the two spike gene mutations was 100% (95% CI 88.4–100.0). Low FCoV RNA load was detected in tissues of 3/30 cats from the control group. One of the three cats had a parafalxial meningioma, one was diagnosed with a lymphocytic meningoencephalitis and one had salmonellosis.

As virus load was too low to allow differentiation, either mutated or non-mutated virus could have been present in these three cats. An infection with non-mutated FCoV could occur due to the ability of non-virulent FCoV to spread systemically, as it was shown that FCoV can persist in the organs of healthy cats for up to 80 days post-infection.[19]

The presence of mutated virus and therefore an early stage of FIP cannot, however, be excluded. This might have been the case in the cat with lymphocytic meningoencephalitis, as FIP can affect the central nervous system (CNS) and lead to, among other things, meningitis, with lymphocytes and macrophages predominating.[20,21] However, necropsy, histopathology and IHC of tissues (including the brains) of both cats with CNS involvement were negative and it therefore seems very unlikely that this was an early stage FIP. It cannot be completely ruled out, though, as there is also the possibility of false-negative staining in IHC due to the occurrence of uneven viral distribution in the tissue samples. Negative IHC staining in a cat in which all other clinical and histopathological findings are suggestive of FIP would not entirely exclude the possibility of FIP. Also, the possibility of mutated virus being present in cats without FIP cannot be completely ruled out, as the presence of the M1058L amino acid substitution has been considered responsible rather for systemic spread of FCoV than for causing FIP.[6]

Contrary to the UK study, in which control cats also showed the M1058L substitution in the spike protein,[6] this could not be confirmed in the present study. As

Table 5 Results of genotype-discriminating RT-qPCR in paraffin-embedded tissue samples of 64 cats

Group	FIPV* M1058L	FIPV* S1060A	FECV†	Mixed genotype*	BLD†	IND†	Negative†	Total
FIP	23	0	0	1	7	1	2	34
Controls	0	0	0	0	3	0	27	30
Total	23	0	0	1	10	1	29	64

*Defined as positive for statistical analysis
†Defined as negative for statistical analysis
FIPV = feline infectious peritonitis virus; FECV = feline enteric coronavirus; BLD = feline coronavirus (FCoV) present, but below limit of detection; IND = FCoV present, but indeterminate strain variations; FIP = feline infectious peritonitis

Table 6 Sensitivity, specificity, negative predictive value (NPV), positive predictive value (PPV) and overall accuracy of genotype-discriminating RT-qPCR in 64 cats, including the 95% confidence interval (CI) and prevalence of feline infectious peritonitis (FIP)

	Genotype-discriminating RT-qPCR (n = 64)	95% CI
Sensitivity (%)	70.6	52.5–84.9
Specificity (%)	100.0	88.4–100.0
NPV (%)	75.0	58.8–87.3
PPV (%)	100.0	85.8–100.0
Overall accuracy (%)	84.4	73.1–92.2
Prevalence of FIP (%)	53.1	

samples in the former study were collected post mortem, control cats in that study could have been lacking clinical signs but already harbouring mutated FCoV. This is not very likely, as histopathology, as well as IHC, for mutated virus were negative, but cannot be completely ruled out. FCoV with the respective mutations was not detected in any of the 30 control cats in the present study, thereby supporting the hypothesis that these substitutions truly determine the development of FIP.

Sensitivity of the detection of FIPV in paraffin-embedded tissues was 70.6% (95% CI 52.5–84.9%). In the few existing studies in which PCR was performed on organ tissues, sensitivity was similar and ranged from 87.5–87.9%.[18,22] However, in one of these studies, the diagnosis of FIP was only based on history, clinical signs and/or pathological changes that were suggestive of FIP, but not all cats underwent histological examinations and in none of the cases was IHC performed to confirm FIP. Furthermore, the PCR used in these studies was not able to definitely distinguish non-mutated FECV from mutated FIPV, which leaves the possibility of animals systemically infected with benign FCoV being falsely detected as positive. In the present study, sensitivity calculated for the presence of 'any FCoV' was 94.1% (95% CI 80.3–99.3) and therefore higher than in previous studies.

Of the 34 cats with FIP, 24 had positive results in the discriminating RT-qPCR. Of these, 23 cats showed the substitution M1058L in the spike protein. One sample revealed a mixed population of FECV and FIPV, indicating a possible transition state with mutated virus already being present. Also, a superinfection with FECV, which has been described previously,[23] cannot be ruled out. The predominance of the substitution M1058L in the spike protein is very similar to the original study conducted in the Netherlands, in which the amino acid substitution M1058L was present in a majority of tissue samples in cats with FIP.[5]

One sample of a cat with FIP showed an indeterminate result. In this case, genotyping was not possible, despite a high FCoV load in the tissue sample. This might be owing to the occurrence of an unknown FCoV strain (failed amplification) or infection with a serotype 2 FCoV strain, as the two mutations are serotype I-specific. Studies conducted in Austria and Japan revealed that 7% and 30% of cats with FIP were infected with serotype 2 FCoV, respectively.[24,25]

Two cats with FIP were completely negative for FCoV in the discriminating RT-qPCR, although IHC was positive. This indicates inhomogeneous distribution of virus within the tissue and makes the irregular occurrence of virus in the samples a major limitation of this study. Seven samples of cats with FIP showed the presence of FCoV RNA, although in such a low concentration that genotyping could not be performed. The low amount of virus could be caused by tissues being over-fixated in formaldehyde, making it extremely difficult to extract long, intact fragments of nucleic acid. As samples used in this study were fixed for less than 72 h and subsequently

embedded in paraffin, over-fixation is extremely unlikely. Uneven distribution of virus, as discussed above, could also lead to a low amount of virus being detected. Also, specific antibodies were formerly detected in lesions of cats with FIP,[10] possibly binding antigen and therefore reducing the possibility of virus being detected by RT-qPCR. Last, but not least, false-positive staining in the IHC assay cannot be fully excluded in these cats. This possibility cannot be completely ruled out, as no negative controls were included in the IHC protocol, which is a limitation of the study. However, false-positive staining is very unlikely, as both of the negative cats showed typical clinical signs of FIP with effusion, as well as corresponding histopathological changes.

In none of the samples in this study was the substitution S1060A detected. In only one of the samples was FECV detected, and this was in a cat with mixed infection. This makes an assessment of the value of the RT-qPCR regarding the discrimination between the different genotypes of FCoV difficult and a major limitation of this study. It would have strengthened the study if FECV had been detected in more control cats. Further studies should be performed in which a second control group should be included. Furthermore, it was not possible to extract the RNA separately from each tissue and no quantification of virus in tissue samples was performed, although it would have been very interesting to compare different tissues within each cat and to compare the results between the cats. A follow-up study using samples collected prospectively and comparing different tissues including quantification of copy numbers should be performed in the future. No negative controls were included in the IHC protocol, which is another limitation of the study that could potentially have lead to an underestimation of the sensitivity of the RT-qPCR.

Conclusions

This study aimed to provide clarity concerning the question of whether or not the M1058L or S1060A substitutions in the spike protein are responsible for the genotype switch reported to cause FIP. In this study, detection of the substitution M1058L correlated 100% with the presence of confirmed FIP, whereas the S1060A substitution was not found in this cohort. It can therefore be concluded that the M1058L substitution in the spike protein appears strongly associated with the genotype switch from FECV to FIPV.

Other, as-yet-unknown strain variations also seem to play a role in the development of FIP, demonstrating the complexity of this fatal disease.

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Supplementary material Cp values retrieved from the endogenous quality control (18S rRNA gene) as well as Cp values for the detection of FCoV RNA (7b gene).

Conflict of interest Christian Leutenegger is the Director of Molecular Diagnostics at IDEXX Laboratories. Hans-Jörg Balzer and Nikola Pantchev are employed at IDEXX Laboratories, Ludwigsburg. This laboratory offers the FCoV and FIPV RT-qPCR on a commercial basis and performed the testing in this study. IDEXX played no role in the study design, in the collection and interpretation of data, or in the decision to submit the manuscript for publication.

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