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Feline coronavirus quantitative reverse-transcriptase polymerase chain reaction on effusion samples in cats with and without feline infectious peritonitis

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Abbreviated short title: Feline coronavirus polymerase chain reaction in the diagnosis of wet feline infectious peritonitis

Abstract

Objectives: To determine whether feline coronavirus (FCoV) RNA in effusion samples can be used as a diagnostic marker of feline infectious peritonitis (FIP), and in FCoV RNA positive samples, to examine amino acid codons in the FCoV spike protein at positions 1058 and 1060 where leucine and alanine, respectively, have been associated with systemic or virulent (FIP) FCoV infection.

Methods: Total RNA was extracted from effusion samples from 20 cats with confirmed FIP and 23 cats with other diseases. Feline coronavirus RNA was detected using a reverse transcriptase quantitative polymerase chain reaction assay (qRT-PCR) and positive samples underwent pyrosequencing of position 1058 and Sanger sequencing of position 1060 in the FCoV spike protein.

Results: Seventeen (85%) of effusion samples from 20 cats with FIP were positive for FCoV RNA, whereas none of the 23 cats with other diseases were positive. Pyrosequencing of the 17 FCoV positive samples showed that 11 (65%) of cats had leucine and 2 (12%) had methionine at position 1058. Of the two samples with methionine, one had alanine at position 1060.

Conclusions and relevance: A positive FCoV qRT-PCR result on effusions appears specific for FIP and may be a useful diagnostic marker for FIP in cats with effusions. The majority of FCoVs contained amino acid changes previously associated with systemic spread or virulence (FIP) of the virus.

Introduction

Feline coronavirus (FCoV) infection is common in domestic cat populations worldwide[1-3]. Most infections are enteric and self-limiting. In a small number of cases, FCoV infection can lead to the development of feline infectious peritonitis (FIP), a significant cause of mortality in young cats.

Definitive diagnosis of FIP relies on histopathological examination of affected tissues, ideally with detection of intracellular FCoV antigen by immunostaining[1, 4, 5]. Obtaining tissue samples is invasive and problematic for *ante mortem* diagnosis. In many FIP cases, abdominal, pleural and/or pericardial effusions develop[2], which can usually be easily obtained for diagnostic testing. Previous studies have reported the use of FCoV antigen staining in effusion samples in the diagnosis of FIP, with sensitivity and specificity of 57-100% and 71.5-100%, respectively[6-9]. Feline coronavirus RNA can be detected in samples using conventional or quantitative reverse transcriptase polymerase chain reaction assays (qRT-PCR). Studies on tissues using qRT-PCRs have found that cats with FIP have significantly higher FCoV loads in tissues than healthy or sick (non-FIP) FCoV infected cats[5, 10, 11]. It is possible that the same is true for effusion samples. Previous studies performing FCoV conventional PCR on effusion samples from cats with FIP have shown promising results, but were limited either by lack of definitive diagnosis of cases[12], or lack of control non-FIP cats[13].

The aim of this study was to perform FCoV qRT-PCR on effusions collected from cats with and without confirmed FIP to investigate whether the presence of FCoV RNA in effusions is helpful in diagnosing FIP. In addition, it has been reported that key amino acid substitutions (methionine to leucine at position 1058 and serine to alanine at position 1060) in the spike protein of FCoV may be associated with FCoV virulence[14] or systemic infection[11], therefore these substitutions were evaluated in FCoV positive effusions.

Methods

Fifty-nine samples of surplus abdominal, pleural and pericardial effusion, from 45 cats, submitted to the Diagnostic Laboratories of Langford Veterinary Services 2011-2012, were used. Samples had been collected into tubes containing either RNAlater (Sigma-Aldrich, UK), EDTA, or no preservative and stored at -20°C upon receipt. All cases classified as FIP were diagnosed by histopathology and subsequent immunohistological demonstration of FCoV antigen within macrophages in the lesions, whilst all cases classified as non-FIP were confirmed to have other diseases based on either histopathology and/or the presence of definitive diagnostic features of another disease (Table 1). Cases that could not be definitively classified were excluded from further analysis.

Total RNA was purified from 100µl of each effusion sample using a NucleoSpin® RNA II kit (Macherey-Nagel, Fisher, UK), eluted in 50µl RNase-free water and stored at -80°C.

Quantitative RT-PCR was carried out as described previously[11]. A previous study has evaluated this qRT-PCR assay, and reported a reaction efficiency of 95.9%[15]. The assay has a sensitivity of between 1 and 10 copies of FCoV per assay (data not shown). Positive and negative controls (FCoV cDNA and RNase-free water, respectively) were used in all PCR runs. In cats where more than one type of effusion was collected and/or into different preservatives, only the sample yielding the lowest threshold cycle (C_T) value was used in analysis.

Pyrosequencing was performed on the FCoV qRT-PCR positive samples to identify methionine to leucine substitutions at position 1058 (M1058L) in the spike protein. A second substitution at position 1060 (serine to alanine; S1060A), was investigated using Sanger sequencing on samples showing methionine at position 1058. Methods were as described previously[11]. Positive and negative controls (control oligonucleotide or FCoV cDNA and RNase-free water, respectively) were used in all pyrosequencing and PCR sequencing runs.

Sensitivity, specificity, and positive (PPV) and negative predictive values (NPV) of effusion qRT-PCR for the diagnosis of FIP were calculated (MedCalc Software bvba, Beligum).

Results

Of the 45 cats, 20 (44%) were classified as FIP, 23 (51%) as non-FIP and two (5%) were unclassified and thus excluded (Table 1). Of the 20 FIP cats, one effusion sample was obtained from 13 cats, two samples from six cats and three samples from one cat. Of the 23 non-FIP cats, one sample was obtained from 19 cats, two samples from three cats and three samples from one cat. Samples varied by collection site and/or preservative (Table 1). All collected samples were analysed by qRT-PCR, but as only one sample from each cat was used for analysis, a total of 43 samples were used.

Seventeen of 20 cats (85%) with FIP had FCoV positive effusions, with C_T values of 24.06-38.27 (median 31.05). None of the 23 non-FIP cats had FCoV positive effusions (Table 1). All negative and positive controls gave appropriate results. The effusion FCoV qRT-PCR assay had a sensitivity of 85%, a specificity of 100%, a PPV of 100% and a NPV of 88.5% for the diagnosis of FIP (Table 2). The 95% confidence intervals are also shown in Table 2.

Pyrosequencing showed that of the 17 FCoV positive effusion FIP cats, 11 (65%) had leucine, and two (12%) had methionine, at position 1058. Reliable sequence data could not be obtained for four (23%) cats (Table 1). Of the two cats with methionine at position 1058, only one had alanine at position 1060. Controls for all assays were appropriately positive and negative.

Discussion

We have investigated the presence of FCoV RNA in abdominal, pleural or pericardial effusion samples from cats with and without FIP. Our results show that in this group of samples, a positive FCoV qRT-PCR result was highly specific, with no non-FIP cats generating positive results. However, sensitivity was only 85%. These figures are similar to those recently reported

for cerebrospinal fluid FCoV qRT-PCR in cats with neurological and/or ocular FIP and non-FIP cats, where a specificity of 100% and sensitivity of 85.7% for FIP were reported[16].

The C_T values of positive qRT-PCR results were 24.1-38.3, representing a ~16,000 fold variation in the level of FCoV RNA present. Indeed, the C_T values of 7/17 FCoV positive cats were >34.0, representing relatively low levels of FCoV RNA. It is possible that the samples from the three FIP cases that generated negative FCoV qRT-PCR results had FCoV present, but at levels below the limit of detection of the PCR. Repeated analysis of samples containing levels of RNA close to the detection limit of the PCR assay can generate either positive or negative results, dependent on whether adequate template is present in the aliquot used in the PCR[15]. Additionally, levels of FCoV in cats with FIP vary in different tissues, likely mirroring the pathological changes present[5], and in some cases are too low to be detected by PCR[5, 11, 17], lending support to the premise that negative results in FIP cases may be due to the presence of very low levels of FCoV in these effusions. A recent study by Pedersen et al[5] reported that the cellular portion of ascitic FIP samples had 10-1000 times more viral RNA than the supernatant, with most FCoV within macrophages of the effusion. Thus, in the future, it would be interesting to perform FCoV qRT-PCR on effusion samples subjected to centrifugation, in an attempt to concentrate cellular material and any FCoV present, and potentially improve sensitivity.

The finding that FCoV was not detectable in any of the non-FIP cats contributed to the high specificity seen for the PCR. Feline coronavirus infection can be systemic in non-FIP cats[10, 11, 18-20], therefore some FCoV positive effusion samples might have been expected in our non-FIP group. Lack of such cases may be due to the nature of those included in the study. A large number of non-FIP cats had neoplasia and these cats tended to be older than the FIP cats, so may have been less likely to be infected with FCoV. The true FCoV status of the non-FIP cases could not be determined for this study. Furthermore, FCoV levels in systemic FCoV-infected

non-FIP cats are often low[10, 11], and may have been below the sensitivity of the FCoV qRT-PCR assay. A possible limitation of this study is the general recruitment of effusion samples submitted to a diagnostic laboratory, rather than targeting samples in which FIP was suspected as a major differential diagnosis. Non-targeted recruitment was performed to maximise case numbers, however, some cats in the non-FIP group presented with inflammatory disease, where FIP would have been considered a differential.

Our study found that the majority of effusions from FIP cats that generated FCoV sequence data for the amino acid positions 1058 and 1060 contained substitutions concordant with the systemic form of FCoV[11] and virulence[14]. Only one FIP cat generated sequence data previously associated with non-systemic (enteric) FCoV[11] or in healthy [14] cats, with methionine and serine at positions 1058 and 1060 respectively. The FCoV in this cat may have had alternative substitutions elsewhere in the genome responsible for systemic FCoV virulence.

In conclusion, this study suggests that a positive FCoV qRT-PCR result on effusions is highly indicative of FIP, and may therefore be a useful diagnostic tool in the investigation of suspected cases that present with an effusion. However, further evaluation of this test's sensitivity and specificity is required, using a larger sample size that includes FCoV-infected cats that do not have FIP.

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Conflict of Interest

The authors do not have any potential conflicts of interest to declare.

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232 **Table 1. Characteristics of effusion samples from the 45 cats recruited in the study.**

Cat number	FIP classification	Age (years)	Sex	Breed	Diagnosis	Source of effusion sample	Preservative	C_T value for FCoV qRT-PCR	Pyrosequencing result for position 1058
1	FIP	-	-	-	FIP	Abdominal	None	24.06	Leucine
2	FIP	0.6	M	DSH	FIP	Pleural	EDTA	24.38	Leucine
3	FIP	0.6	MN	Ragdoll	FIP	Abdominal	None	26.64	Leucine
4	FIP	0.4	-	DSH	FIP	Pleural	RNAlater	27.05	Leucine
5	FIP	-	-	DSH	FIP	Pleural	None	27.98	Methionine[1]
6	FIP	0.4	ME	Scottish Fold	FIP	Pleural	None	29.47	Leucine
7	FIP	-	M	DSH	FIP	Abdominal	None	30.10	Leucine
8	FIP	3	FN	-	FIP	Abdominal	None	30.66	Leucine
9	FIP	0.7	FE	Ragdoll	FIP	Abdominal	None	31.05	Methionine[2]
10	FIP	0.3	ME	Bengal cross	FIP	Abdominal	EDTA	33.94	No clear sequence

11	FIP	0.4	M	BSH	FIP	Pleural	None	35.02	No clear sequence
12	FIP	3	MN	DSH	FIP	Pericardial	EDTA	35.72	Leucine
13	FIP	1	F	BSH	FIP	Abdominal	EDTA	36.17	Leucine
14	FIP	0.7	-	Korat	FIP	Abdominal	RNAlater	36.96	Leucine
15	FIP	0.4	FE	Savannah	FIP	Abdominal	None	37.01	No clear sequence
16	FIP	0.3	M	Bengal	FIP	Abdominal	EDTA	37.81	Leucine
17	FIP	0.4	FE	DSH	FIP	Abdominal	RNAlater	38.27	No clear sequence
18	FIP	7	FN	DSH	FIP	Abdominal	None	No C_T	ND
19	FIP	0.9	MN	Bengal	FIP	Abdominal	None	No C_T	ND
20	FIP	7	FN	Birman	FIP	Abdominal	None	No C_T	ND
21	Non-FIP	13	FN	DSH	Thymoma with associated chylothorax	Pleural	None	No C_T	ND

22	Non-FIP	9	MN	DSH	Lymphohistiocytic thoracic neoplasm	Pleural	EDTA	No C_T	ND
23	Non-FIP	13	MN	DSH	Hyperthyroidism and hypertrophic cardiomyopathy associated congestive cardiac failure	Pleural	EDTA	No C_T	ND
24	Non-FIP	18	FN	DSH	Severe protein losing enteropathy	Abdominal	None	No C_T	ND
25	Non-FIP	0.3	M	Exotic	Idiopathic chylothorax	Pleural	EDTA	No C_T	ND
26	Non-FIP	8	FN	DSH	Intestinal carcinomatosis	Abdominal	EDTA	No C_T	ND
27	Non-FIP	10	FN	DSH	Cholangiocarcinoma with carcinomatosis	Abdominal	None	No C_T	ND
28	Non-FIP	1	FN	Maine Coon	Fibrous (non-inflammatory) lesions present throughout abdominal cavity – aetiology not known	Pleural	None	No C_T	ND
29	Non-FIP	10	FN	Somali	Feline triaditis (pancreatitis,	Abdominal	None	No C_T	ND

					cholangitis and inflammatory bowel disease)				
30	Non-FIP	15	MN	DSH	Large cell lymphoma of small intestine and liver	Abdominal	None	No C_T	ND
31	Non-FIP	8	FN	DSH	Thymoma	Pleural	EDTA	No C_T	ND
32	Non-FIP	11	FN	DSH	Possible mesothelioma, with mild neutrophilic inflammation	Pleural	EDTA	No C_T	ND
33	Non-FIP	4	FN	Persian	Intestinal lymphoma	Abdominal	EDTA	No C_T	ND
34	Non-FIP	10	FN	DLH	Abdominal carcinoma	Abdominal	EDTA	No C_T	ND
35	Non-FIP	1	FE	Russian Blue	Haemorrhagic effusion	Abdominal	None	No C_T	ND
36	Non-FIP	8	FN	DSH	Hepatic carcinoma	Abdominal	None	No C_T	ND
37	Non-FIP	8	MN	DSH	Chemodectoma	Pleural	None	No C_T	ND
38	Non-FIP	2	MN	Tonkinese	Abdominal carcinoma	Abdominal	EDTA	No C_T	ND
39	Non-FIP	13	MN	Birman	Restrictive cardiomyopathy	Pleural	EDTA	No C_T	ND

40	Non-FIP	3	F	BSH	Neutrophilic cholangitis	Abdominal	RNAlater	No C_T	ND
41	Non-FIP	7	MN	Devon Rex	Lymphoplasmacytic inflammation of the liver and kidney	Abdominal	None	No C_T	ND
42	Non-FIP	8	MN	DLH	Uroabdomen	Pleural	EDTA	No C_T	ND
43	Non-FIP	11	MN	Maine Coon	Diaphragmatic rupture	Pleural	None	No C_T	ND
44	Unclassified	1	FN	Maine Coon	Pyothorax but could not rule out FIP as an underlying cause	Abdominal	EDTA	No C_T	ND
45	Unclassified	12	MN	Russian Blue	Unable to determine definitive diagnosis	Abdominal	EDTA	No C_T	ND

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234 - = Unknown, C_T = Threshold cycle value, qRT-PCR = reverse transcriptase quantitative polymerase chain reaction, FCoV =

235 feline coronavirus

236 DSH = Domestic Shorthair, BSH = British Shorthair, DLH = Domestic Longhair, M = male, F = female, N = neutered, E =

237 entire

238 ND = Samples negative for FCoV RNA by qRT-PCR which were therefore not submitted for pyrosequencing

239 [1] Sequencing result for position 1060 = Alanine

240 [2] Sequencing result for position 1060 = Serine.

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Table 2. Sensitivity, specificity, and positive and negative predictive values of effusion reverse transcriptase quantitative polymerase chain reaction for the diagnosis of FIP

	Value	**95% Confidence intervals**
Sensitivity	85.0%	65.1 - 96.8%
Specificity	100.0%	85.2 – 100.0%
Positive predictive value	100.0%	80.5 – 100.0%
Negative predictive value	88.5%	69.9 - 97.6%
Prevalence of FIP	46.5%	31.5 – 62.2%

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