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

3
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22
23 **Keywords:** feline, diagnosis, coronavirus, mutation, effusion, feline infectious peritonitis

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

26 **Abstract**

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

32 **Methods:** Total RNA was extracted from effusion samples from 20 cats with confirmed FIP and
33 23 cats with other diseases. Feline coronavirus RNA was detected using a reverse transcriptase
34 quantitative polymerase chain reaction assay (qRT-PCR) and positive samples underwent
35 pyrosequencing of position 1058 and Sanger sequencing of position 1060 in the FCoV spike
36 protein.

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

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

45

46 **Introduction**

47 Feline coronavirus (FCoV) infection is common in domestic cat populations worldwide¹⁻³. Most
48 infections are enteric and self-limiting. In a small number of cases, FCoV infection can lead to
49 the development of feline infectious peritonitis (FIP), a significant cause of mortality in young
50 cats.

51 Definitive diagnosis of FIP relies on histopathological examination of affected tissues, ideally
52 with detection of intracellular FCoV antigen by immunostaining^{1, 4, 5}. Obtaining tissue samples is
53 invasive and problematic for *ante mortem* diagnosis. In many FIP cases, abdominal, pleural
54 and/or pericardial effusions develop², which can usually be easily obtained for diagnostic
55 testing. Previous studies have reported the use of FCoV antigen staining in effusion samples in
56 the diagnosis of FIP, with sensitivity and specificity of 57-100% and 71.5-100%, respectively⁶⁻⁹.

57 Feline coronavirus RNA can be detected in samples using conventional or quantitative reverse
58 transcriptase polymerase chain reaction assays (qRT-PCR). Studies on tissues using qRT-
59 PCRs have found that cats with FIP have significantly higher FCoV loads in tissues than healthy
60 or sick (non-FIP) FCoV infected cats^{5, 10, 11}. It is possible that the same is true for effusion
61 samples. Previous studies performing FCoV conventional PCR on effusion samples from cats
62 with FIP have shown promising results, but were limited either by lack of definitive diagnosis of
63 cases¹², or lack of control non-FIP cats¹³.

64 The aim of this study was to perform FCoV qRT-PCR on effusions collected from cats with and
65 without confirmed FIP to investigate whether the presence of FCoV RNA in effusions is helpful
66 in diagnosing FIP. In addition, it has been reported that key amino acid substitutions
67 (methionine to leucine at position 1058 and serine to alanine at position 1060) in the spike
68 protein of FCoV may be associated with FCoV virulence¹⁴ or systemic infection¹¹, therefore
69 these substitutions were evaluated in FCoV positive effusions.

70 **Methods**

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

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

82 Quantitative RT-PCR was carried out as described previously¹¹. A previous study has evaluated
83 this qRT-PCR assay, and reported a reaction efficiency of 95.9%¹⁵. The assay has a sensitivity
84 of between 1 and 10 copies of FCoV per assay (data not shown). Positive and negative controls
85 (FCoV cDNA and RNase-free water, respectively) were used in all PCR runs. In cats where
86 more than one type of effusion was collected and/or into different preservatives, only the sample
87 yielding the lowest threshold cycle (C_T) value was used in analysis.

88 Pyrosequencing was performed on the FCoV qRT-PCR positive samples to identify methionine
89 to leucine substitutions at position 1058 (M1058L) in the spike protein. A second substitution at
90 position 1060 (serine to alanine; S1060A), was investigated using Sanger sequencing on
91 samples showing methionine at position 1058. Methods were as described previously¹¹.

92 Positive and negative controls (control oligonucleotide or FCoV cDNA and RNase-free water,
93 respectively) were used in all pyrosequencing and PCR sequencing runs.

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

96 **Results**

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

104 Seventeen of 20 cats (85%) with FIP had FCoV positive effusions, with C_T values of 24.06-
105 38.27 (median 31.05). None of the 23 non-FIP cats had FCoV positive effusions (Table 1). All
106 negative and positive controls gave appropriate results. The effusion FCoV qRT-PCR assay
107 had a sensitivity of 85%, a specificity of 100%, a PPV of 100% and a NPV of 88.5% for the
108 diagnosis of FIP (Table 2). The 95% confidence intervals are also shown in Table 2.

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

113 **Discussion**

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

118 for cerebrospinal fluid FCoV qRT-PCR in cats with neurological and/or ocular FIP and non-FIP
119 cats, where a specificity of 100% and sensitivity of 85.7% for FIP were reported¹⁶.

120 The C_T values of positive qRT-PCR results were 24.1-38.3, representing a ~16,000 fold
121 variation in the level of FCoV RNA present. Indeed, the C_T values of 7/17 FCoV positive cats
122 were >34.0, representing relatively low levels of FCoV RNA. It is possible that the samples from
123 the three FIP cases that generated negative FCoV qRT-PCR results had FCoV present, but at
124 levels below the limit of detection of the PCR. Repeated analysis of samples containing levels
125 of RNA close to the detection limit of the PCR assay can generate either positive or negative
126 results, dependent on whether adequate template is present in the aliquot used in the PCR¹⁵.

127 Additionally, levels of FCoV in cats with FIP vary in different tissues, likely mirroring the
128 pathological changes present⁵, and in some cases are too low to be detected by PCR^{5, 11, 17},
129 lending support to the premise that negative results in FIP cases may be due to the presence of
130 very low levels of FCoV in these effusions. A recent study by Pedersen et al⁵ reported that the
131 cellular portion of ascitic FIP samples had 10-1000 times more viral RNA than the supernatant,
132 with most FCoV within macrophages of the effusion. Thus, in the future, it would be interesting
133 to perform FCoV qRT-PCR on effusion samples subjected to centrifugation, in an attempt to
134 concentrate cellular material and any FCoV present, and potentially improve sensitivity.

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

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

148 Our study found that the majority of effusions from FIP cats that generated FCoV sequence
149 data for the amino acid positions 1058 and 1060 contained substitutions concordant with the
150 systemic form of FCoV¹¹ and virulence¹⁴. Only one FIP cat generated sequence data previously
151 associated with non-systemic (enteric) FCoV¹¹ or in healthy ¹⁴ cats, with methionine and serine
152 at positions 1058 and 1060 respectively. The FCoV in this cat may have had alternative
153 substitutions elsewhere in the genome responsible for systemic FCoV virulence.

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

159

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168

169 **Conflict of Interest**

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

171

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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	RNA later	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	RNA later	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	Abdomi nal	RNA later	No C _T	ND
41	Non-FIP	7	MN	Devon Rex	Lymphoplasmacytic inflammation of the liver and kidney	Abdomi nal	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	Abdomi nal	EDTA	No C _T	ND
45	Unclassified	12	MN	Russian Blue	Unable to determine definitive diagnosis	Abdomi nal	EDTA	No C _T	ND

233

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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242
243 **Table 2. Sensitivity, specificity, and positive and negative predictive values of effusion**
244 **reverse transcriptase quantitative polymerase chain reaction for the diagnosis of FIP**
245

	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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