Discrepancies between feline coronavirus antibody and nucleic acid detection in effusions of cats with suspected feline infectious peritonitis

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Running title: FCoV antibodies and RNA in effusions from FIP-suspected cats.

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Abstract

Intra-vitam diagnosis of feline infectious peritonitis (FIP) is a challenge for veterinary diagnosticians, since there are no highly specific and sensitive assays currently available. With the aim to contribute to fill this diagnostic gap, a total of 61 effusions from cats with suspected effusive FIP were collected intra-vitam for detection of feline coronavirus (FCoV) antibodies and RNA by means of indirect immunofluorescence (IIF) assay and real-time RT-PCR (qRT-PCR), respectively. In 5 effusions there was no evidence for either FCoV RNA or antibodies, 51 and 52 specimens tested positive by IIF and qRT-PCR, respectively, although antibody titres ≥ 1:1600, which are considered highly suggestive of FIP, were detected only in 37 effusions. Three samples with high antibody levels tested negative by qRT-PCR, whereas 18 qRT-PCR positive effusions contained no or low-titre antibodies. qRT-PCR positive samples with low antibody titres mostly contained low FCoV RNA loads, although the highest antibody titres were detected in effusions with C_T values >30. In conclusion, combining the two methods, i.e., antibody and RNA detection would help improving the intra-vitam diagnosis of effusive FIP.

Key words: Feline infectious peritonitis; diagnosis; effusions; antibodies; viral RNA.

1. Introduction

Feline infectious peritonitis (FIP) is a lethal disease of cats caused by a hypervirulent variant of feline coronavirus (FCoV), an alphacoronavirus that usually causes self-limiting infections of the intestinal epithelium, leading to mild or no gastroenteric signs (Addie et al., 2009). Two different FCoV genotypes are currently known, FCoV type I (FCoV-I) and type II (FCoV-II), both involved in the occurrence of mild gastroenteritis or fatal FIP (Decaro and Buonavoglia, 2011). FIP is a perivascular pyogranulomatosis that may occur in two clinical forms, effusive and non-effusive FIP, which are characterized by prevalence of effusions in the body cavities and of pyogranulomatous lesion in organs, respectively. FIP diagnosis is challenging since the 'gold standard' is the post-mortem demonstration of FCoV antigens in tissues by immunohistochemistry. Therefore, alternative tools are commonly used for the intra-vitam diagnosis. Haematological and biochemical analyses can support a presumptive diagnosis of FIP, but they usually require further investigations, such as assessment of the FCoV antibody titers and molecular detection of FCoV RNA in the effusions (effusive form) or bioptic samples (non-effusive FIP) from ill cats. Unfortunately, both methods lack specificity and sensitivity, thus often leading to an inconclusive diagnosis (Addie et al., 2009). Recently, a comparison between the intra-vitam detection of FCoV antibodies and that of FCoV RNA in the effusions of cats with confirmed FIP has been carried out, showing a trend towards negative or low antibody levels in cats with high viral RNA titers (Meli et al., 2013). However, these findings have not been confirmed by other studies.

In the present paper, a total of 61 effusions from cats with confirmed FIP have been screened for FCoV antibodies and RNA, suggesting that intra-vitam diagnosis of effusive FIP needs to be assessed by means of combined antibody- and virus-detection methods.

2. Materials and methods

2.1. Sample collection

Effusions were collected intra-vitam from 61 cats whose FIP diagnosis was highly suspected since the clinical cases fulfilled all, or most, of the criteria for FIP diagnosis given in the European Advisory Board of Cat Disease recommendations (Addie et al., 2009,), as previously reported (Meli et al., 2013). All samples were sent to our lab for FIP confirmation by diagnostic labs that had carried out some preliminary analyses on the effusions, including Rivalta's test, total proteins, albumin/globulin ratio, total leukocyte counts and identity of cells (Table 1). Collected samples included 58 ascitic fluids and 3 pleuric effusions.

2.2. Detection of FCoV antibodies

For FCoV antibody detection and titration, an indirect immunofluorescent (IIF) assay was used (Campolo et al., 2005), with minor modifications. Briefly, FCoV-II strain 25/92 (Buonavoglia et al., 1995) was cultivated on Crandell feline kidney (CrFK) cells grown on coverslips. Infected cells were fixed in acetone 100% and twofold dilutions of the effusion (starting from dilution 1:100 to 1:51,200) were tested. Goat anti-cat IgG conjugated with fluorescein isothiocyanate was used as secondary antibody solution (Sigma Aldrich srl). The assay was proven to detect both FCoV-I and FCoV-II antibodies (Addie and Jarret, 1992; Campolo et al., 2005). Effusion with qRT-PCR positive and IIF-negative results were treated with ammonium thiocyanate to dissociate immune complexes, as previously described (Pullen et al., 1986; Macdonald et al., 1988).

2.3. Detection of FCoV RNA

For FCoV RNA detection, 140 µl of the effusions were used for RNA extraction by means of QIAamp® Viral RNA Mini Kit (Qiagen S.p.A., Milan, Italy), following the manufacturer's protocol and the RNA templates were stored at –70°C until their use. FCoV reverse-transcriptase quantitative PCR (FCoV qRT-PCR) was performed as previously described (Gut et al., 1999), with minor modifications. In brief, a one-step method was adopted using Platinum® Quantitative PCR SuperMix-UDG (Invitrogen srl, Milan, Italy) and the following 50-µl mixture: 25 µl of master mix, 300 nM of primers FcoV1128f (GATTTGATTTGGCAATGCTAGATTT) and FcoV1229r (AACAATCACTAGATCCAGACGTTAGCT), 200 nM of probe FCoV1200p (FAM- TCCATTGTTGGCTCGTCATAGCGGA-BHQ1) and 10 µl of template RNA. The employed oligonucleotides bind to the 3' untranslated region (Gut et al., 1999). The thermal profile consisted of incubation with UDG at 50°C for 2 min and activation of Platinum Taq DNA polymerase at 95° C for 2 min, followed by 45 cycles of denaturation at 95° C for 15 s, annealing at 48° C for 30 s and extension at 60° C for 30 s. Threshold cycle (C_T) number was used as the measure of viral load. The lower the C_T, the more virus present in the sample.

2.4 Statistical analysis

Spearman's rank correlation coefficient was calculated to evaluate the possible correlation between viral RNA loads and antibody titres by the use of the online tool Social Science Statistics (http://www.socscistatistics.com/tests/spearman).

3. Results

Fifty-one (48 ascitic and 3 pleuric fluids) of the 61 tested samples had FCoV antibody (Table 2 and Fig. 1), although only 37 positive effusions contained antibody levels ≥ 1:1,600, which are considered highly suggestive of FIP diagnosis (Hartmann et al., 2003). Additional 13 samples presented FCoV antibody titres between 1:200 and 1:800, which are quite high for an enteric infection but cannot be considered enough high for a systemic infection. Only one effusion had an antibody titre of 1:100 and two samples displayed an antibody titre of 1:51,200.

By means of qRT-PCR, FCoV RNA was detected in a total of 52 samples (49 ascitic and 3 pleuric fluids). C_T values were generally above 30 (mean C_T value of 32.87), accounting for low viral titres, with higher viral RNA loads (C_T values <30) being detected in only 11 effusions.

By comparing the results qRT-PCR with those of IIF assay using an antibody titre ≥ 1:1,600 as cut-off (Fig. 1B), 6 samples tested negative by both assays (no viral RNA and no FCoV antibodies), possibly accounting for diseases other than FIP, and 3 samples tested negative only by qRT-PCR, although they contained FCoV antibody titres between 1:3,200 and 1:12,800, which were highly suggestive of FIP. Eighteen effusions were found to contain FCoV RNA in the absence of specific antibodies (or at least in the presence of antibody titres less than 1:1,600); 5 of these qRT-PCR positive specimens had no FCoV antibodies (or at least antibody titres less than 1:100), while additional 13 effusions contained antibody titres ranging from 1:100 to 1:800, which are not considered as suggestive of FIP. Therefore, based only on antibody detection, a total of 18 cats whose effusions contained viral RNA were predicted not to be affected by FIP, while taking advantage on molecular detection of FCoV RNA, 3 animals with high antibody titres would have been considered FIP negative. Unfortunately, samples with FCoV RNA tested negative by IIF even after treatment with the chaotropic thiocyanate ion, which had been proven to dissociate immune complexes Pullen et al., 1986).

Most effusions displaying the highest viral loads (C_T values <30) contained antibody titres ≥ 1:1,600; only one sample with a low C_T value displayed an antibody titre (1:200) not suggestive of FIP (Table 2). Therefore, qRT-PCR positive samples with low antibody titres mostly contained low FCoV RNA loads, although the highest antibody titres were detected in effusions with C_T values >30.

Overall, no statistically significant correlation (R=0.1178; two-tailed *P-value*= 0.36576) was found between viral RNA loads and antibody titres.

4. Discussion

Intra-vitam FIP diagnosis still represents a challenge for veterinarians and diagnosticians, since there is no available tool to unambiguously diagnose the disease. FIP cannot be differentiated from an FCoV enteric infection based on serology because the antibodies are directed against the same pathogen and there are no relevant antigenic differences between the enteric and hypervirulent strains. It is recognised that FIP-ill cats have very high antibody titres in their serum and effusions due to the systemic spreading of the virus through the infected monocytes/macrophages (Addie et al., 2009). However, detection of high antibody titres alone is not a confirmatory test. In addition, the absence of specific antibodies or the presence of very low antibody titres has been recently demonstrated in the effusions of cats with confirmed FIP, likely due to antibody sequestration by the high number of viral particles in the same sample of some cats (Meli et al. 2013). Hartmann et al. (2003) demonstrated that about 10% of cats with FIP tested seronegative for FCoV. However, in that study a transmissible gastroenteritis virus strain was used as antigen for, which could affect the sensitivity of FCoV-antibody testing (Giori et al., 2011). Accordingly, FCoV antibody titres were found to dramatically drop in terminal cases of FIP (Pedersen, 1995). This phenomenon is not restricted to FIP, but it has been also demonstrated for other viral infections characterised by high-level virus replication (Quirós-Roldán et al., 2000; Guihot et al., 2014). Overall, detection of FCoV antibodies in the effusions is affected by poor specificity and sensitivity.

Molecular methods have been used for detection of FCoV RNA in the effusions of cats with suspected FIP (Gut et al., 1999; Hornyák e al., 2012; Soma et al., 2013; Doenges et al., 2017; Felten et al., 2017; Longstaff et al., 2017). However, these methods display similar issues related to the diagnostic performances (lack of sensitivity and specificity). In fact, they are not able to distinguish between enteric and virulent FCoVs, since no specific genetic markers have been identified for the latter strains. In addition, the enteric FCoVs have been proven to cause transient viremia and even have a low replication in the blood (Can-Sahna et al., 2007; Kipar et al., 2010; Fish et al., 2017), thus potentially being able to passively spread to the effusions associated to other diseases.

A recent paper (Meli et al., 2013) has investigated the agreement between FCoV antibody titres and RNA detection in the effusions of 13 cats with confirmed FIP, showing a correlation between high amounts of virus and lower signals in IIF assay, likely due to the fact that antibodies bound to viral antigens of the effusions are not able to bind to the antigens of the FCoV-infected cells used in serological tests. Here, we have analysed by the same methods the effusions of 61 cats with suspected FIP, thus including also potential samples from animals with non-FIP related diseases. Accordingly, using an IIF antibody titre of 1:1,600 as a cut-off, 5 samples tested negative by both IIF and qRT-PCR assays, possibly accounting for diseases other than FIP, while 21 effusions gave contrasting results (low-titre or no antibodies in the presence of FCoV RNA or viceversa). These 21 samples with conflicting results are likely to be true positive since an IIF-negative result could be related to antibody sequestration by high viral loads (Meli et al., 2013). In addition, Addie et al. (2014) demonstrated that up to 43% antibody-positive effusions from FIP cases were negative for FCoV RNA, likely as a consequence of PCR inhibition by interfering substances or RNA degradation during sample transportation and storage. However, in the absence of alternative diagnosis, even those 5 cats with neither FCoV antibodies nor RNA in their effusions could not be definitively considered as non-FIP animals (Addie et al., 2014). Unfortunately, clinical cases were mostly untraceable and confirmatory necropsy was not done in any case, so that the lack of confirmatory testing represents the main limitation of the present study.

In contrast with what observed by Meli et al. (2013), there was no statistically significant correlation between high viral loads and low-titre or negative antibody results. In fact, most effusions with low or no FCoV antibody titres displayed low amounts of virus, although samples with very high levels of FCoV RNA contained slightly lower antibody titres (generally <1:3,200) in comparison with effusions with the lowest amounts of virus, which reached IIF antibody titres of 1:26,600-1:51, 200 (Table 2).

The present study confirms that, when performed singularly, neither the detection of FCoV nucleic acid nor that of specific antibodies in the effusions of cats with suspected FIP is able to warrant an affordable diagnosis of the disease. Therefore, in order to increase the diagnostic performances, we suggest combining the two methods (antibody and RNA detection) for an intra-vitam diagnosis of effusive FIP. Using this diagnostic approach, only 6 out of 61 cats whose effusions were analysed would be considered FIP negative, even if also in these cases FIP could not be completely ruled out (Meli et al., 2013). Thus, the combined serological and molecular protocol should improve the ability of laboratories to diagnose effusive FIP, especially if the test results are supported by clinical and haematological findings. However, intra-vitam diagnosis of non-effusive FIP still remains highly inconclusive, even if recent studies tried to address this issue (Doenges et al., 2016). Therefore, future studies are needed to develop and validate tools for the intra-vitam diagnosis of non-effusive FIP, which still represents a challenge for veterinary diagnosticians.

Conflict of interest statement

There is no conflict of interest of any authors in relation to the submission.

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Figure legend.

Fig. 1. Comparison between indirect immunofluorescence (IIF) assay and real-time RT-PCR (qRT-PCR) carried out on 61 effusions from cats with suspected feline infectious peritonitis (FIP). Numbers indicate the samples positive (+) or negative (-) for FCoV antibodies or RNA. Results according to both techniques are shown in bold. For IIF assay, the cut-off was set to 1:100 (A) or 1:1600 (B), the latter being considered highly suggestive of FIP.

Table 1. Effusion features used as criteria for FIP diagnosis.

Feature	Value
Rivalta's test	Positive
Total proteins	>35 g/l
Albumin/globulin	<0.8
Total leukocytes	$<2 \times 10^9$/l
Identity of cells	Neutrophils+macrophages

Table 2. FCoV antibody titres and RNA loads in the effusions of 61 cats with suspected FIP.

Cat no.	Sample type	FCoV RNA loads[a]	FCoV antibody titres[b]
1	Ascitic fluid	40.38	25,600
2	Ascitic fluid	>45	800
3	Ascitic fluid	>45	<100
4	Ascitic fluid	32.31	1600
5	Ascitic fluid	>45	<100
6	Ascitic fluid	>45	<100
7	Ascitic fluid	36.21	12,800
8	Ascitic fluid	34.82	1,600
9	Ascitic fluid	37.96	6,400
10	Ascitic fluid	35.79	1600
11	Ascitic fluid	35.14	1600
12	Ascitic fluid	>45	<100
13	Ascitic fluid	37.24	<100
14	Ascitic fluid	>45	<100

15	Ascitic fluid	34.04	12,800
16	Ascitic fluid	>45	6,400
17	Ascitic fluid	>45	12,800
18	Ascitic fluid	33.25	800
19	Ascitic fluid	37.46	<100
20	Ascitic fluid	30.63	400
21	Ascitic fluid	36.97	25,600
22	Ascitic fluid	>45	3,200
23	Ascitic fluid	26.34	1,600
24	Ascitic fluid	36.20	6,400
25	Ascitic fluid	40.42	1,600
26	Ascitic fluid	33.18	800
27	Ascitic fluid	32.66	800
28	Ascitic fluid	35.2	12,800
29	Ascitic fluid	31.02	400
30	Ascitic fluid	24.80	3,200
31	Ascitic fluid	21.11	3,200
32	Ascitic fluid	29.73	12,800
33	Pleuric effusion	33.34	12,800
34	Ascitic fluid	34.60	51,200
35	Ascitic fluid	37.04	12,800
36	Ascitic fluid	35.99	3,200
37	Ascitic fluid	38.48	800
38	Ascitic fluid	32,87	800
39	Ascitic fluid	24.18	6,400
40	Ascitic fluid	25.95	3,200
41	Ascitic fluid	26.94	1,600
42	Ascitic fluid	30.27	51,200
43	Pleuric effusion	29.09	200
44	Ascitic fluid	27.69	1,600
45	Ascitic fluid	34.10	400
46	Ascitic fluid	28.48	6,400
47	Ascitic fluid	34.35	800
48	Ascitic fluid	40.56	<100
49	Ascitic fluid	38.57	<100
50	Ascitic fluid	32.05	400
51	Ascitic fluid	35.17	1,600
52	Ascitic fluid	29.04	12,800
53	Ascitic fluid	37.56	6,400
54	Pleuric effusion	35.97	200
55	Ascitic fluid	35.35	<100
56	Ascitic fluid	39.23	25,600
57	Ascitic fluid	32.32	6,400
58	Ascitic fluid	36.25	6,400
59	Ascitic fluid	37.80	12,800
60	Ascitic fluid	41.14	1,600
61	Ascitic fluid	32.57	100

[a] FCoV RNA loads are expressed as C_T values. Values >45 are considered negative results.

[b] FCoV antibody titres are expressed as the reciprocal of the highest sample dilution able to generate fluorescence in FCoV-infected cells.