Comparison of the performance of laboratory tests in the diagnosis of feline infectious peritonitis

Angelica Stranieri, Alessia Giordano, Saverio Paltrinieri,[1] Chiara Giudice, Valentina Cannito, Stefania Lauzi

Department of Veterinary Medicine, University of Milan, Milan, Italy; Central Laboratory, Veterinary Teaching Hospital, University of Milan, Lodi, Italy.

[1]Corresponding author: Saverio Paltrinieri, Department of Veterinary Medicine, University of Milan, Via Celoria, 10 - 20133 Milan, Italy. saverio.paltrinieri@unimi.it

Running head: Performances of tests for FIP

Abstract. We compared the performance of clinicopathologic and molecular tests for the antemortem diagnosis of feline infectious peritonitis (FIP). From 16 FIP and 14 non-FIP cats, we evaluated retrospectively the sensitivity, specificity, and likelihood ratios (LRs) of serum protein electrophoresis, α_1-acid glycoprotein (AGP) on peripheral blood, screening reverse-transcription nested PCR (RT-nPCR) on the 3'–untranslated region (3'-UTR), and spike (S) gene sequencing on peripheral blood, body cavity effusions, and tissue, as well as body cavity cytology and delta total nucleated cell count (ΔTNC). Any of these tests on blood, and especially the molecular tests, may support or confirm a clinical diagnosis of FIP. A negative result does not exclude the disease except for AGP. Cytology, 3'-UTR PCR, and ΔTNC may confirm a clinical diagnosis on effusions; cytology or 3'-UTR PCR may exclude FIP. Conversely, S gene sequencing is not recommended based on the LRs. On tissues, S gene sequencing is preferable when histology is highly consistent with FIP, and 3'-UTR PCR when FIP is unlikely. Combining one test with high LR+ with one with low LR– (e.g., molecular tests and AGP on blood, ΔTNC and cytology in effusions) may improve the diagnostic power of the most used laboratory tests.

Key words: Clinicopathologic tests; feline coronavirus; feline infectious peritonitis; likelihood ratios; molecular tests; spike gene.

Feline infectious peritonitis (FIP) is a fatal disease of felids caused by a mutated feline coronavirus (FCoV) and its interaction with the immune response of the host.[14] Several candidate genes have been investigated, but how their mutation contributes to the onset of FIP is not completely understood.[16]

Two amino acid substitutions (mutation M1058L and S1060A) of the spike protein have been found in FCoVs from tissues of cats with FIP.[1] These mutations were later associated with the systemic spread of the virus rather than with FIP, confirming that, to date, there are no tests that can differentiate enteric from pathogenic strains.[17,18] Antemortem diagnosis relies on patient history and on the combination of different laboratory tests including serum protein electrophoresis (SPE), α_1-acid glycoprotein (AGP) measurement, and analysis of body cavity effusions including evaluation of the delta total nucleated cell count (ΔTNC) and the immunocytochemical staining of FCoVs in macrophages.[5,7,15,17] Few studies have investigated the diagnostic potential of mutations in the spike (S) gene.[3,12] Therefore, we compared the likelihood ratios (LRs) of clinicopathologic and molecular tests for the diagnosis of FIP. LRs are not influenced by the prevalence of disease and may be used to measure the increase or decrease in post-test probability of FIP.

Results recorded on samples submitted to our diagnostic laboratory from 2013 to 2015 were retrospectively analyzed and selected if samples fit the following criteria: 1) clinical suspicion of FIP, based on history and clinical signs including fever, lethargy, anorexia, weight loss, neurologic and/or ocular signs, and effusions; 2) availability of a final diagnosis as specified below; and 3) results of at least one clinicopathologic test (SPE and AGP on blood, cytology and ΔTNC on effusions) or reverse-transcription nested PCR (RT-nPCR) on the 3'–untranslated region (3'-UTR), and S gene sequencing on blood, effusions, and tissues. Because samples were analyzed for routine diagnostic purposes and collected under informed

consent of the owners, according to the guidelines of our Institution, a formal approval from the Ethical Committee was not required (EC decision 29 Oct 2012, protocol 02-2016).

Cats were assigned to the FIP group if histopathologic findings revealed typical lesions along with positive immunohistochemistry (IHC) in at least one tissue.[11] Cats were assigned to the non-FIP group when histology revealed diseases other than FIP along with negative IHC in all of the collected tissues, when follow-up demonstrated a complete recovery 18 mo from the first diagnosis, or when laboratory and imaging tests allowed diagnosis of a different disease. Euthanized cats were autopsied within 6 h. One specimen from all organs affected by gross anatomic lesions was collected, fixed in 10% buffered formalin, and embedded in paraffin. Another specimen from at least one affected organ (usually mesenteric lymph node) was immediately frozen at –80°C to perform both RT-nPCR for 3'-UTR and S gene sequencing.

Histology was performed on 5-μm sections stained with hematoxylin and eosin. IHC was performed using a mouse monoclonal antibody anti-FCoV (FIPV3-70 clone, Serotec, Bio-Rad, Segrate, Italy) using protocols described in other studies.[9] In FIP cats, IHC was performed on specimens with typical histologic lesions; in non-FIP cats, all of the collected tissues were tested with IHC as part of the routine autopsy in order to exclude the presence of FCoVs.

Total proteins were measured spectrophotometrically using the biuret method (Cobas Mira, Roche, Basel, Switzerland), then SPE was performed on agarose gel using an automated analyzer (Hydrasis, Sebia Italia, Bagno a Ripoli, Italy) and a specific kit (Hydragel 15 β1-β2, Sebia Italia) as described previously.[4]

Serum AGP concentration was measured using a radial immunodiffusion kit (SRID, Tridelta Development, Bray, Ireland) as described previously.[15]

Fresh body cavity effusions were analyzed with a commercial analyzer (Sysmex XT-2000iV, Sysmex Europe, Norderstedt, Denmark) to record the ΔTNC as reported previously.[5] Cytology was performed on smears stained with a rapid stain (Hemacolor, Merck, Darmstadt, Germany).

Whole blood and effusions collected in EDTA were centrifuged (3,500 × *g*, 5 min) immediately upon receipt at the laboratory, within 12 h of collection. Pellets were suspended in 200 µL of phosphate-buffered saline and immediately stored at –20°C for RNA extraction.

RNA was obtained (NucleoSpin RNA kit, Macherey-Nagel, Bethlehem, PA) according to the manufacturer's instruction, and used for RT-nPCR analysis. FCoV presence was investigated in the pellets extracted from whole blood, effusions, or from tissues collected during autopsy (mesenteric lymph nodes in 25 cats, spleen in 3 cats, small intestine in 1 cat, lung in 1 cat) using RT-nPCR targeting a 177-bp product of the highly conserved 3'-UTR.[8] FCoV RNA was used as positive control and RNase-free water as negative control. PCR products were visualized under an ultraviolet transilluminator on 2% agarose gel stained with ethidium bromide. RNA was also tested using a RT-nPCR assay targeting a 142-bp product of the S gene.[1] Positive samples were sequenced (Big Dye Terminator v.3.1 cycle sequencing kit, AB3730 DNA analyzer, Applied Biosystems, Foster City, CA), and forward and reverse primers were used for the second reaction.

Sequence data were assembled and manually corrected (BioEdit software v.7.0, https://goo.gl/eDyNHn). Consensus sequences were aligned with FCoV strains bearing, or not, the mutations M1058L or S1060A, retrieved from GenBank (Clustal X, BioEdit software).

Samples were classified as consistent with or not consistent with FIP according to study criteria (Table 1). Dubious features were considered "non-consistent with FIP" and included the lack of granular background in cytologic samples, ΔTNC values of 1.7–3.4 ×

10^9/L,[5] increased serum α_2-globulins but normal γ-globulins or vice-versa,[20] or AGP values between 0.56 (reference value of the laboratory) and 1.5 g/L.[15] When molecular tests were performed on >1 tissue specimen, cats were classified as positive when at least one of the tested organs provided a positive result, and negative when all of the specimens resulted negative (Table 2).

For each test, true-positive and false-positive results (results consistent with FIP in cats with and without FIP, respectively) and true-negative and false-negative results (results not consistent with FIP in cats without and with FIP, respectively) were recorded. Sensitivity, specificity, as well as positive and negative LRs (LR+ and LR–, respectively) were then calculated.[2]

Thirty cats (age: 4 mo to 13 y; median: 12 mo) suspected to have FIP were included in our study. The FIP group included 16 cats (age: 4–12 mo; median: 9 mo). The non-FIP group included 14 cats (age: 8 mo to 13 y; median: 5 y). In 3 cats in the non-FIP group, FIP was ruled out based on normalization of clinical and laboratory findings during an 18 mo follow-up (cats 20–22, with persistent fever, inappetence, and lethargy); in 3 cats, a disease other than FIP was diagnosed through cytology, imaging, and flow cytometry (cat 24 with hepatic carcinoma, and cats 28 and 29, both with lymphoma); in 8 cats, postmortem findings were consistent with a disease other than FIP, and IHC was negative (cats 17–19, 23, 25–27, and 30, with renal failure, pleuropericardial fibrosis, pleomorphic sarcoma, lymphocytic cholangitis, intestinal carcinoma, myelofibrosis, polycystic kidney disease, and lymphoma, respectively).

All hematologic tests had high or absolute specificity and a high LR+, whereas sensitivity was low, except for AGP (Table 3). The very low sensitivity of SPE was caused by dubious or negative patterns, in accord with one study,[20] but in disagreement with another report.[19] Even if the high specificity was possibly the result of the relatively low number of

inflammatory conditions in the non-FIP group, the LR ratios indicate that SPE cannot definitely rule out FIP, but it may be used as a confirmatory test.

AGP measurement had the highest sensitivity and LR+, even if lower than in a previous report,[6] and the lowest LR–. Nevertheless, the false-negative cases had values consistent with inflammation and may support the diagnosis of FIP in conjunction with other consistent laboratory results. Interestingly, AGP showed also the lowest specificity but the LR– was low enough to recommend the use of this test to rule out FIP. On the other hand, based on the absolute specificity and on the relatively high LR–, the molecular tests cannot be used to rule out FIP, but they may support the diagnosis of FIP in the case of positive results.

On effusions, all of the tests had high-to-absolute sensitivity and specificity and, consequently, high-to-absolute LR+ and low-to-excellent LR–, except for S gene sequencing, which had the worst performance in terms of sensitivity, LR+, and LR–. Cytology and the RT-nPCR 3'-UTR were the best tests on effusions, despite the presence of false-positive results. False-positive cytologic results may be explained by the fact that nonspecific inflammatory cytologic patterns are found in many inflammatory conditions,[13] and that the virus can be found using immunofluorescence in the effusion of cats with diseases other than FIP.[17] Therefore, in accord with previous reports, cytology and RT-nPCR 3'-UTR cannot be used to confirm FIP,[6,11] but, based on their high LR+, these remain the tests of choice for effusions. The ΔTNC was specific but not as sensitive as expected, possibly given the low cell concentration of the samples with features that provided false-negative results, as noted in a previous study.[5] Hence, ΔTNC may be used in addition to the other tests to support the diagnosis of FIP.

Spike gene sequencing had low sensitivity. Moreover, one false-positive result was recorded, as described by others[18] who found the S gene mutations described previously[1] in tissues of cats without FIP, but in contrast to another report of absolute specificity of S gene

sequencing on effusions.[12] Therefore, the risk of false-positive results, even if rare, make this test not optimal in the diagnosis of FIP. On tissues, the RT-nPCR 3'-UTR had the best, but not absolute, sensitivity and a low LR–, but also low specificity and low LR+. Conversely, S gene sequencing had high specificity and LR+ but lower sensitivity and slightly higher LR–. The negative results of this latter technique were the result not only of the absence of the mutated nucleotides, but also of negative results of the spike PCR (data not shown) and the resulting absence of sequencing templates. The low sensitivity of RT-nPCR 3'-UTR confirms a previous report regarding the spread of the virus in cats not affected by FIP and resulting false-positive results.[10] On the other hand, the specificity of S gene sequencing was high, as on the other specimens. Thus, the risk of false-positive results with the RT-nPCR 3'-UTR is alarmingly high whereas the use of S gene sequencing can be useful as a confirmatory test, but its use for the exclusion of FIP should be avoided based on the results of our study. Summary information about the suggested clinical utility of each test to either confirm or exclude FIP is reported in Table 4.

The limitations of our study are the low caseload, application of strict inclusion criteria that, however, increased the reliability of the results, and the low rate of inflammatory conditions in the non-FIP group that may have overestimated the specificity of tests suggestive of inflammation. However, we demonstrated that combining one test with high LR+ with one with low LR– (e.g., molecular tests and AGP on blood, ΔTNC and cytology on effusions) may improve diagnostic power.

Acknowledgments

We thank Dr. Valentina Gualdi, Parco Tecnologico Padano, Lodi, for her support in the sequencing of S gene PCR products. Preliminary results were presented as an oral communication at the 25th Eur College Vet Intern Med congress, Lisbon, September 2015.

Declaration of conflicting interests

The authors declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article

Funding

This work was partially funded by University of Milan funds (Fondo Sviluppo UNIMI).

References

1. Chang HW, et al. Spike protein fusion peptide and feline coronavirus virulence. Emerg Infect Dis 2012;18:1089–1095.

2. Christenson RH. Evidence-based laboratory medicine—a guide for critical evaluation of in vitro laboratory testing. Ann Clin Biochem 2007;44:111–130.

3. Felten S, et al. Detection of feline coronavirus spike gene mutations as a tool to diagnose feline infectious peritonitis. J Feline Med Surg 2017;19:321–335.

4. Giordano A, Paltrinieri S. Interpretation of capillary zone electrophoresis compared with cellulose acetate and agarose gel electrophoresis: reference intervals and diagnostic efficiency in dogs and cats. Vet Clin Pathol 2010;39:464–473.

5. Giordano A, et al. High diagnostic accuracy of the Sysmex XT-2000iV delta total nucleated cells on effusions for feline infectious peritonitis. Vet Clin Pathol 2015;44:295–302.

6. Giori L, et al. Performances of different diagnostic tests for feline infectious peritonitis in challenging clinical cases. J Small Anim Pract 2011;52:152–157.

7. Hartmann K., et al. Comparison of different tests to diagnose feline infectious peritonitis. J Vet Intern Med 2003;17:781–790.

8. Herrewegh AAPM, et al. Detection of feline coronavirus RNA in feces, tissues, and body fluids of naturally infected cats by reverse transcriptase PCR. J Clin Microbiol 1995;33:684–689.

9. Hsu SM, et al. Use of avidin-biotin-peroxidase complex (ABC) in immunoperoxidase techniques: a comparison between ABC and unlabeled antibody (PAP) procedures. J Histochem Cytochem 1981;29:577–580.

10. Kipar A, et al. Natural FCoV infection: cats with FIP exhibit significantly higher viral loads than healthy infected cats. J Feline Med Surg 2006;8:69–72.

11. Kipar A, Meli ML. Feline infectious peritonitis: still an enigma? Vet Pathol 2014;51:505–526.

12. Longstaff L, et al. Feline coronavirus quantitative reverse transcriptase polymerase chain reaction on effusion samples in cats with and without feline infectious peritonitis. J Feline Med Surg 2017;19:240–245.

13. Paltrinieri S, et al. In vivo diagnosis of feline infectious peritonitis by comparison of protein content, cytology, and direct immunofluorescence test on peritoneal and pleural effusions. J Vet Diagn Invest 1999;11:358–361.

14. Paltrinieri S, et al. Laboratory profiles in cats with different pathological and immunohistochemical findings due to feline infectious peritonitis (FIP). J Feline Med Surg 2001;3:149–159.

15. Paltrinieri S, et al. Critical assessment of the diagnostic value of feline α1-acid glycoprotein for feline infectious peritonitis using the likelihood ratios approach. J Vet Diagn Invest 2007;19:266–272.

16. Pedersen NC. An update on feline infectious peritonitis: virology and immunopathogenesis. Vet J 2014;201:123–132.

17. Pedersen NC. An update on feline infectious peritonitis: diagnostics and therapeutics. Vet J 2014;201:133–141.

18. Porter E, et al. Amino acid changes in the spike protein of feline coronavirus correlate with systemic spread of virus from the intestine and not with feline infectious peritonitis. Vet Res 2014;45:49–60.

19. Riemer F, et al. Clinical and laboratory features of cats with feline infectious peritonitis—a retrospective study of 231 confirmed cases (2000–2010). J Feline Med Surg 2016;18:348–356.

20. Stranieri A, et al. Frequency of electrophoretic changes consistent with feline infectious peritonitis in two different time periods (2004–2009 vs 2013–2014). J Feline Med Surg 2017;19:880–887.

Table 1. Study criteria of various laboratory tests to confirm or exclude feline infectious peritonitis (FIP) in 30 cats.[5,19-21,27]

Specimen/Test	Features and cutoffs consistent with FIP
Effusion	
Cytology	Presence of a nonspecific inflammatory process and of a proteinaceous background
ΔTNC	$>3.4 \times 10^9$/L
RT-nPCR 3'-UTR	Positive result
S gene sequencing	Presence of M1058L or S1060A mutations
Blood	
SPE	Increased α_2- and γ-globulin with a polyclonal peak
AGP	>1.5 g/L
RT-nPCR 3'-UTR	Positive result
S gene sequencing	Presence of M1058L or S1060A mutations
Tissues	
RT-nPCR 3'-UTR	Positive result
S gene sequencing	Presence of M1058L or S1060A mutations

AGP = α_1-acid glycoprotein; RT-nPCR 3'-UTR = reverse-transcription nested PCR on the 3'–untranslated region; S gene = spike gene; SPE = serum protein electrophoresis; ΔTNC = ratio between total nucleated cells counted on 2 channels of the Sysmex XT-2000iV.

244 **Table 2.** Results of laboratory tests for feline infectious peritonitis (FIP) in 30 cats.

	Blood				Effusion				Tissue	
Group/ID	SPE	AGP	RT-nPCR 3'-UTR	S gene seq	Cytology	ΔTNC	RT-nPCR 3'-UTR	S gene seq	RT-nPCR 3'-UTR	S gene seq
FIP										
1	–	–	NP	NP	NP	NP	NP	NP	NP	NP
2*	+	+	+	–	+	–	+	–	+	–
3	–	+	+	+	NP	NP	NP	NP	+	+
4*	–	+	+	+	+	+	+	+	+	+
5	–	–	–	–	NP	NP	NP	NP	+	+
6*	+	+	+	+	+	+	+	+	+	+
7*	–	+	+	–	+	+	+	–	NP	NP
8*	–	+	–	NP	+	+	+	+	+	+
9	+	+	NP	NP	NP	NP	NP	NP	NP	NP
10*	–	+	+	–	NP	+	+	–	+	+
11*	–	+	NP	NP	+	+	+	–	+	–
12*	+	+	NP	NP	+	+	+	–	NP	NP
13*	+	+	NP	NP	NP	+	+	+	+	+
14*	NP	NP	NP	NP	NP	–	+	–	+	–
15	NP	NP	NP	NP	NP	NP	NP	NP	–	NP
16	+	+	NP	NP	NP	NP	NP	NP	NP	NP
Total positive results	6/14	12/14	6/8	3/7	7/7	8/10	10/10	4/10	10/11	7/10
Non-FIP										
17	NP	–	NP	NP	NP	NP	NP	NP	+	–
18*	–	+	NP	NP	–	–	–	–	–	–
19*	–	–	–	–	–	–	–	–	+	–
20	–	–	–	–	NP	NP	NP	NP	NP	NP
21	–	–	–	NP	NP	NP	NP	NP	NP	NP
22	–	–	NP	NP	NP	NP	NP	NP	NP	NP
23	+	+	–	–	NP	NP	NP	NP	+	+

24*	NP	NP	NP	NP	–	–	–	–	NP	NP
25*	–	–	NP	NP	+	–	+	+	–	–
26	NP	NP	–	–	NP	NP	NP	NP	–	–
27	NP	NP	NP	NP	NP	NP	NP	NP	+	–
28*	–	–	–	–	–	–	–	–	NP	NP
29*	–	–	–	–	–	–	–	–	NP	NP
30	–	–	–	–	NP	NP	NP	NP	–	–
Total positive results	1/10	2/11	0/8	0/7	1/6	0/6	1/6	1/6	4/8	1/8

245 – = negative; + = positive; AGP= α_1-acid glycoprotein; NP = not performed; RT-nPCR 3'-UTR = reverse-transcription nested PCR on the 3'–

246 untranslated region; S gene = spike gene; SPE = serum protein electrophoresis; ΔTNC = ratio between total nucleated cells counted on 2

247 channels of the Sysmex XT-2000iV.

248 * = Presence of effusion.

249

Table 3. Sensitivity, specificity, and positive and negative likelihood ratios of laboratory tests and sample types for feline infectious peritonitis in 30 cats.

Specimen/Test	Se (%)	Sp (%)	LR+	LR–
Blood				
SPE	43	90	4.29	0.63
AGP	86	82	4.71	0.17
3'-UTR PCR	75	100	NC	0.25
S gene sequencing	43	100	NC	0.57
Effusions				
Cytology	100	83	6.00	0.00
ΔTNC	80	100	NC	0.20
3'-UTR PCR	100	83	6.00	0.00
S gene sequencing	40	83	2.40	0.72
Tissues				
3'-UTR PCR	91	50	1.82	0.18
S gene sequencing	70	88	5.60	0.34

AGP = α_1-acid glycoprotein; LR+ = positive likelihood rate; LR– = negative likelihood ratio; NC = not calculable based on 100% specificity; RT-nPCR 3'-UTR = reverse-transcription nested PCR on the 3'–untranslated region; S gene = spike gene; Se = sensitivity; Sp = specificity; SPE = serum protein electrophoresis; ΔTNC = ratio between total nucleated cells counted on 2 channels of the Sysmex XT-2000iV.

Table 4. Recommended laboratory tests to confirm or exclude feline infectious peritonitis based on results in 30 cats.

	Confirmatory test	Exclusion test
Blood	SPE, RT-nPCR 3'-UTR, S gene sequencing	AGP
Effusions	ΔTNC measurement, S gene sequencing	Cytology, RT-nPCR 3'-UTR
Tissues	S gene sequencing	RT-nPCR 3'-UTR

AGP = α_1-acid glycoprotein; RT-nPCR 3'-UTR = reverse-transcription nested PCR on the 3'–untranslated region; S gene = spike gene; SPE = serum protein electrophoresis; ΔTNC = ratio between total nucleated cells counted on 2 channels of the Sysmex XT-2000iV.