Preliminary investigation on feline coronavirus presence in the reproductive tract of the tomcat as a potential route of viral transmission

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Abstract

Objectives Feline infectious peritonitis (FIP) is an immune-mediated disease initiated by feline coronavirus (FCoV) infection. To date, the only proven route of transmission is the fecal–oral route, but a possible localization of FCoV in the reproductive tract of tomcats is of concern, due to the involvement of the male reproductive tract during FIP and to the presence of reproduction disorders in FCoV-endemic feline catteries. The aim of the study was to investigate the presence and localization of FCoV in semen and/or in the reproductive tract of tomcats, and its possible association with seroconversion and viremic phase. ***Methods*** Blood, serum, semen samples and/or testicles were obtained from 46 tomcats. Serology was performed on 38 serum samples, nRT-PCR and RT-qPCR were performed on 39 blood samples and on 17 semen samples, and histology, immunohistochemistry and nRT-PCR were performed on 39 testicles. ***Results*** Twenty-four out of 38 serum samples were positive on serology. Semen samples were negative at RT-PCR and RT-qPCR for FCoV, while all blood sample were negative at both molecular methods, except for one sample positive at RT-qPCR with a very low viral load. All testicles were negative at immunohistochemistry, while 6 were positive at nRT-PCR for FCoV. Serology and blood PCR results suggest that the virus was present in the environment, stimulating transient seroconversion. FCoV seems not to localize in the semen of tomcats, making the venereal route as a way of transmission unlikely. Although viral RNA was found in some testicles, it could not be correlated with the

viremic phase. ***Conclusions and relevance*** At the light of these preliminary results, artificial insemination appears safer than natural mating since it eliminates the direct contact between animals, thus diminishing the probabilities of fecal-oral FCoV transmission.

Introduction

Feline infectious peritonitis (FIP) is an immune-mediated disease of young cats. The causative agent is the feline coronavirus (FCoV), generated by a mutation of the widespread enteric pathotype, that gains the ability to replicate in macrophages, and spreads through infected monocytes.[1] The course of the infection depends in part upon the type and strength of the immune response of the host,[2-4] but environmental factors such as the level of stress and overcrowding also play a role.[5] FCoV infection is very common in cats; around 40% of the domestic cat population has been infected with FCoV, and this figure may increase up to 90% in multi-cat households.[6,7] Natural FCoV infections are transient in ~70% of cats, but persistent infections can occur in ~13% of cats,[8] while around 5–10% of cats are believed to be resistant to FCoV infection. In most cases, FCoV infection is asymptomatic, or results in only mild gastrointestinal clinical signs; however, in a small percentage of cases, FCoV infection results in FIP.[5] Asymptomatic FCoV infection was previously believed to be confined to the intestinal tract, but it is now known that healthy FCoV-infected cats can have systemic infection, albeit with lower viral loads than cats with FIP.[9] These recurrent phases of intestinal colonization and fecal shedding of the virus may lead to a transient localization in several organs and are followed by seroconversion and by negativization at the intestinal level.[10,11] During the viremic phase, it is possible that the virus localizes also

77 in the reproductive tract, and that it is shed with semen, contributing to the spread of the
78 FCoV by coupling or by artificial insemination (AI) in breeding cats.
79 AI has nowadays become reasonably successful in the domestic cat, sufficiently so to
80 contribute to genetic management of catteries.[12] Therefore, there is concern about the
81 possibility of sexual transmission of viruses also through AI. It has been demonstrated
82 that feline immunodeficiency virus (FIV) is shed with semen, and that it can be
83 transmitted horizontally by AI with fresh semen.[13] Feline leukemia virus (FeLV)
84 infection alters hormone production in the hypothalamic-pituitary-gonadal system,
85 decreasing testosterone, luteinising hormone (LH) and follicle stimulating hormone
86 (FSH) levels, but its exact localization in the reproductive system is still unknown.[14]
87 The involvement of the male reproductive tract during FCoV infection has previously
88 been described as scrotal swelling following abdominal effusion, orchitis, or
89 priapism.[15-18] In all these cases, cats with FCoV in the male reproductive tract were
90 affected by FIP. Nevertheless, the hypothesis of a possible association between FCoV
91 infection and reproductive disorders is supported also by the presence of hypofertility,
92 abortions and/or natimortality in FCoV-endemic catteries.[1] To the best of our
93 knowledge, the localization of the FCoV in the reproductive tract of healthy cats or its
94 presence in tomcat semen has never been demonstrated, but it could represent an
95 important step in the process of understanding the mechanisms of FCoV transmission,
96 as to date the only proven route of transmission is the fecal–oral route.[4] Therefore, the

aim of the study was to investigate the presence and localization of FCoV in semen and/or in the reproductive tract of healthy tomcats, and its possible association with seroconversion or with the viremic phase.

Material and methods

Sample collection

Blood, serum, semen and/or testicles were obtained from 46 cats aging from 6 months to 4 years. Seven cats were tomcats from breeding catteries whose semen samples were collected for AI purposes. All the remaining cats were client-owned, except two stray cats. One of these latter underwent orchiectomy after being placed in a shelter, the other was found severely injured and euthanized.

Blood samples were available if routine hematology and/or biochemistry were performed prior to semen collection and/or surgery. After routine diagnostic procedures performed at the site of collection, blood or serum samples, when available, were immediately frozen and periodically sent to the Laboratory of the Veterinary Teaching Hospital of the University of Milan in cold chain. Semen samples were collected as described below, either for AI purposes or before orchiectomy, with the owner's informed consent.

Testicle samples were obtained after orchiectomy from all the cats except two, whose testicles were collected during necropsy performed for diagnostic purposes.

Immediately after collection, half testicle was frozen in plain tubes while the other half was collected into 10% neutral-buffered formalin for histological and immunohistochemical examination. For the two cats on which necropsy was performed, tissue samples grossly affected by lesions were also collected into 10% neutral-buffered formalin for histology and immunohistochemistry to reach a definitive diagnosis.

The study protocol was approved by the IACUC of the University of Milan (approval number 109/2016).

Semen collection

Semen samples were collected at the Veterinary Reference Centre (Turin, Italy) via urethral catheterization using an injectable anesthesia protocol with 0.2 mg/kg methadone (Semfortan, Dechra) and 5 μg/kg dexmedetomidine (Dexdomitor; Pfizer Italia) premedication, followed by induction with 2 mg/kg propofol (Propovet, Esteve Veterinaria) to effect.[19] Immediately after collection, semen samples were frozen and sent to our laboratory maintaining the cold chain for molecular biology processing.

Serology

Anti-FCoV antibodies titres were assessed using an indirect immunofluorescence test performed on 10 multi-well slides produced at the University of Zurich according to Osterhaus et al,[20] by coating each well with $4{\cdot}5\times10^3$ PD-5 cells, half of which were infected with swine transmissible gastroenteritis virus (serologically cross-reacting with FCoVs). Twofold dilutions (1:25 to 1:400) of each serum sample were prepared and 20

137 μL of each dilution was applied to the wells. After incubation for 30 minutes at 37°C in
138 a moist chamber, slides were washed with phosphate-buffered saline (PBS), dried and
139 15 μL of fluorescein isothiocyanate-conjugated rabbit-anticat immunoglobulin (Nordic
140 Immunological Laboratories, Tilburg, The Netherlands) was added to each well. After
141 incubation for 30 minutes at 37°C in a moist chamber, slides were washed, dried and
142 observed on a fluorescence microscope. Dilutions were judged as positive when
143 showing a clear fluorescent signal in about half of the cells. Samples that were still
144 positive at a 1:400 dilution were further diluted on a twofold basis until negativization.
145 *RNA extraction, nRT-PCR and RT-qPCR*
146 RNA was obtained from blood and testicle samples using a NucleoSpin RNA kit
147 (Macherey-Nagel, Bethlehem, PA). Fifty μL of blood were suspended in 300 μL of
148 RA1 lysis buffer, while 20 mg of testicles were thinly shredded on sterile plates using
149 sterile scalpels, followed by vigorous vortexing in RA1 lysis buffer until completely
150 dissolved. All the further steps were performed according to the manufacturer's
151 instruction.
152 RNA was obtained from semen using TRIzol reagent (Invitrogen Corporation,
153 Carlsbad, CA, USA) according to Das et al.[21] Samples (starting mean volume: 50 μL; 8-
154 100 μL) were centrifuged (5 min at 7000 x g) and the supernatant was discarded. The
155 resulting pellets were washed two times using 100 μL of phosphate buffer saline (PBS)
156 for 5 minutes at 7000 x g. To each sample, a volume of TRIzol (Thermo Fischer

157 Scientific, Waltham, USA) equal to 10 times the starting volume of semen was added.
158 After incubation for 5 minutes, 200 µL of chloroform for each ml of TRIzol were added
159 to each sample. After vortexing and incubating at room temperature for 3 minutes,
160 samples were centrifuged (15 min at 12000 x g at 4°C) and the resulting aqueous phase
161 was transferred in RNAse free tubes. Then, 500 µL of isopropyl alcohol every mL of
162 TRIzol were added to each sample followed by 10 minutes incubation at room
163 temperature. After centrifugation (10 min at 12000 x g at 4°C) the resulting supernatant
164 was eliminated and to each resulting pellet 1 mL of 75% ethanol was added. After
165 centrifugation (5 min at 12000 x g at 4°C), supernatant was discarded, and the sample
166 was dried for 10-15 minutes at room temperature. The pellet was then suspended in 30
167 µL of RNAse free water and incubated at 55 °C for 10 minutes. RNA samples were then
168 frozen at -80°C or immediately used for nRT-PCR.
169 A reverse transcription nested PCR (RT-nPCR) targeting a 177 bp product of the highly
170 conserved 3′ untranslated region (3′ UTR) of the genome of both type I and type II
171 FCoV was used.[10] RT-nPCR positive FCoV RNA from a cat with FIP was used as
172 positive control and RNase-free water as negative control. PCR products were
173 visualized under UV transilluminator on a 1.5 % agarose gel stained with ethidium
174 bromide.
175 Quantitative RT-qPCR targeting a 102 bp product of the 7b gene of FCoV was
176 performed on blood and semen samples as previously described[22] with minor

177 modifications. Threshold cycle (C_T) number was used as the measure of viral load. The
178 lower the C_T, the more virus is present in the sample.
179 *Histopathology and immunohistochemistry*
180 Formalin fixed samples were sent to the department of Comparative Biomedicine and
181 Food Science of the University of Padova for histology and immunohistochemistry
182 (IHC). Sections (3 μm) obtained from paraffine embedded samples were prepared and
183 stained with haematoxylin–eosin for histology with an automated stainer (Autostainer
184 XL, Leica Biosystems, Wetzlar, Germany). For IHC, 3 μm paraffin sections were
185 placed on surface-coated slides (Superfrost Plus). Slides were incubated at 37° C for 30
186 min before the immunostaining performed with an automatic immunostainer (Ventana
187 Benchmark XT, Roche-Diagnostics), which uses a kit with a secondary
188 antibody with a horseradish peroxidase (HRP)-conjugated polymer that binds
189 mouse and rabbit primary antibodies (ultraViews Universal DAB, Ventana Medical
190 System). All reagents were dispensed automatically except for the primary antibody,
191 which was dispensed by hand. A mouse monoclonal antibody against the feline
192 coronavirus was used as primary antibody (clone FIPV3-70 Serotec, Oxfork UK).
193 **Results**
194 *Caseload*
195 The caseload included 31 Domestic Shorthair cats, 6 Maine Coon, 3 Sphynx and 1 each
196 for the following breeds: Holy Birman, Chartreux, Norwegian Forest Cat, Persian,

197 Ragdoll, Scottish fold. The age ranged from 6 to 48 months (mean: 11,6; median: 7,5
198 months). The type of samples collected in the 46 cats included in this study is
199 summarized in table 1. Seventeen semen samples were collected: in all these cases
200 additional samples from the same cats were available (serum, blood and testicle in 7
201 cases; serum and blood in 3 cases; serum in 2 cases; blood and testicle in 2 cases; blood
202 in 2 cases; serum and testicle in 1 case).
203 A total of 39 testicles were collected, 24 of which were collected along with a blood and
204 serum sample. The remaining testicles were collected along with blood, serum and
205 semen (7 cats), with blood and semen (2 cats), alone (3 cats), with serum (1 cat), with
206 serum and semen (1 cat), with blood only (1 cat).
207 *Serology, PCR and immunoistochemistry*
208 Results obtained for each test are shown in table 2. Fourteen out of the 38 cats for which
209 serum was available were negative on serology, with an antibody titer lower than the
210 cut-off of 1:50, which is the threshold of positivity of our laboratory, while 7/38 cats
211 showed an antibody titer of 1:50. The remaining 17 cats showed variable antibody
212 titers: specifically the antibody titer was 1:100 in 7 cats, 1:200 in 6 cats, 1:400 in 3 cats
213 and 1:800 in 1 cat.
214 All the 17 semen samples were negative at both the nRT-PCR and the RT-qPCR for
215 FCoV. All the 39 blood samples were negative at the nRT-PCR and at the RT-qPCR,

except for one blood sample that was FCoV positive only using RT-qPCR, with a very high C_T value (C_T 38.9).

Regarding testicles, all the cats were negative at immunohistochemistry for FCoV, while six were positive at the nRT-PCR for FCoV. All the cats from which testicles were collected while alive, were healthy during orchiectomy, except for one cat (n° 43) which was affected by congenital portosystemic shunt. For two cats (n° 42 and 43) serum and blood were not available, therefore serology was not performed. Antibody titers of the remaining cats with PCR positive testicles were negative (cat n°5); 1:100 (cat n°18); 1:200 (cat n°15) and 1:400 (cat n°29). Interestingly, the only cat affected by FIP, as confirmed by positive immunohistochemistry for FCoV on brain and cerebellum, gave a negative result both with immunohistochemistry and PCR on testicles.

Discussion

FCoV RNA was never detected by nRT-PCR in the blood samples obtained from the cats examined in this study and only one out of 39 blood samples was identified as positive by RT-qPCR. The very high C_T value of the positive sample suggests that the concentration of viral RNA in the sample was extremely low. The RT-qPCR positivity resulted in a seronegative cat and this is in accordance with FCoV infection kinetic.[23] Antibody titers were variable even though with medium-low titers mostly, while titers higher than 1:200 were found only in few cases. Taken together, results of serology and

blood PCR suggest that the virus was present in the environment and stimulated transient seroconversion in some of the cats. Positive serology in cats without viral RNA in blood is in fact unlikely to be imputable to a low viral load in blood because samples were analyzed by RT-qPCR, which is a very sensitive method, and it is more likely that results are due to the characteristics of FCoV-host interactions.[4,10,24] It is also possible that an infected cat could not be identified with PCR on blood if the virus was present in the intestinal tract only. Unfortunately, our study design did not include fecal sampling and it is therefore impossible to confirm that seropositive and PCR-negative cats were shedding the virus with feces. However, positive serology demonstrates that the cats included in this study had been in contact with the virus, since cats may remain positive also after the clearance of the virus. In particular, antibodies against feline coronavirus are typically fluctuating and cats, especially those from multi-cat environments, alternate serological negativities and positivities, corresponding with reinfection episodes.[11,25] From this perspective, and considering that anti-FCoV antibodies are found in cats with viral RNA both in feces and tissues of healthy animals and in FIP affected cats, [11,26,27] the medium-high antibody titers recorded in some of the cats of the current study may indicate that these cats had been or still were FCoV infected at the moment of sampling, and therefore it is possible that they were harboring the virus in tissues. This hypothesis is supported by the finding that some testicles were RT-PCR positive, but always negative at immunohistochemistry. This is not surprising,

since PCR is characterized by a higher analytical sensitivity compared to immunohistochemistry.[28,29] On the other hand, RT-PCR is performed on homogenized samples, thus not allowing to determine which cellular line composing the testicle was infected.

It is important to highlight that only one of the cats with viral RNA in the testicle and with available serum was seronegative, while all the other cats with PCR-positive testicles had titers ranging from 1:100 to 1:400. On the light of what discussed above, this may be explained by two hypotheses. The first hypothesis is that the cats were viremic but with a blood viral load too low to be detected by standard PCR and the virus was present only in the vessels or in the plasma contained in the testicle, but the examination with RT-qPCR which is more sensitive than standard PCR makes this hypothesis unlikely as well as the fact that the only viremic cat, even if with a very low viral load, was PCR negative on testicles. Another hypothesis, as already demonstrated, is that the examined section for IHC did not include the cells infected by FCoV, which were present in the sections used for RT-PCR instead.[2,3] Anyway, the section used for PCR was carefully handled to avoid hematic contamination as much as possible and therefore it is unlikely that testicles were falsely positive due to contaminating FCoV genome. Also, the presence of FCoV in the testicular vessels would not explain why the same positivity was not found on blood, from which a larger amount of sample was used for RNA extraction. The most likely hypothesis is that the virus was isolated in the

testicular compartment through the blood-testis barrier, as already demonstrated with the blood-brain barrier, thus explaining the discordant results between peripheral blood and testicles.[30]

Interestingly, the only FIP affected cat resulted negative at RT-PCR on testicles. While it was not possible to perform serology and PCR on blood, several tissues of this cats were analyzed for diagnostic purposes. All the tissues examined were negative both at PCR and IHC, except for brain and cerebellum, which were the only organs harboring the typical FIP lesions among with intralesional antigen, and a mesenteric lymph node, which was positive at PCR only. This finding supports the evidence of a higher analytical sensitivity of RT-PCR but also the fact that positive PCR results does not allow to distinguish between FIP affected and FCoV infected healthy cats.[4,29] Moreover, the absence of typical histological lesions as well as of positive IHC demonstrates that genital involvement is rare during FIP, especially in non-effusive and localized forms, and probably also the testicle involvement in FCoV infected healthy cats.[17,18]

None of the semen samples were RT-PCR and RT-qPCR positive for FCoV. Only in one cat for which both testicles and semen were available, results were discordant, with positive RT-PCR on testicle but negative on semen. It cannot also be excluded that the virus was present on the stromal or vascular tissues of the testicle and not in germinal cells, leading to a negative PCR result on semen. Unfortunately, the negative results in IHC, likely due to the low amount of virus as hypothesized above, does not allow us to

296 further elucidate this aspect. It is important also to consider that the diagnostic
297 sensitivity of RT-PCR and RT-qPCR on feline semen is unknown; in this study we
298 applied the method of RNA extraction from semen that is described to have the best
299 analytical sensitivity in comparison with other methods.[31] Therefore, although unlikely,
300 since this method has been successfully used in other studies, the presence of false
301 negative results cannot be excluded.[32] Moreover, most of the cats from which semen
302 was tested, were also seronegative or with low antibody titers. Even though
303 seronegative cats cannot be considered free from infection for the already discussed
304 kinetics of both the virus and the antibody responses, it is possible that cats were not
305 viremic and that the virus was not systemically spread or localized in some organs at the
306 time of semen collection.[3] Unfortunately, for the only cat RT-qPCR positive on blood,
307 semen sample was not available.

308 **Conclusion**

309 Even if PCR positive results on testicles may suggest the venereal route as a potential
310 way of FCoV transmission, FCoV seems not to localize in the semen of tomcats,
311 therefore the venereal route as a way of transmission seems to be unlikely. Viral RNA
312 found in testicles could not be correlated with viremic phases, but this finding needs to
313 be confirmed. At the light of these results, AI seems safer than natural mating,
314 eliminating the contact between animals and diminishing the probabilities of fecal-oral
315 FCoV transmission. In light of the limited number of available semen samples and of

316 the fact that samples were obtained almost exclusively from healthy cats, it would be
317 useful to evaluate these data in a FCoV endemic population to have more chance to
318 detect viremic cats, which may possibly harbor FCoV also in semen. In addition, the
319 presence of higher antibody titers may allow to evaluate the potential use of serology as
320 an indicator of viral localization in tissue/semen. Therefore, further studies on a higher
321 number of samples and evaluating differences in semen and testicles of cats with higher
322 antibody titers or with positive RT-PCR on blood are needed.
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Table 1. Data on signalment, type of sample collected from the cats and included in this study.

N°	Breed	Age (months)	Samples	Total
1	Persian	12	Blood, serum, testicle	24
2	ES	6	Blood, serum, testicle	
3	ES	7	Blood, serum, testicle	
4	ES	6	Blood, serum, testicle	
5	ES	6	Blood, serum, testicle	
6	ES	8	Blood, serum, testicle	
7	ES	6	Blood, serum, testicle	
8	ES	6	Blood, serum, testicle	
9	ES	7	Blood, serum, testicle	
10	ES	6	Blood, serum, testicle	
11	ES	7	Blood, serum, testicle	
12	ES	7	Blood, serum, testicle	
13	ES	8	Blood, serum, testicle	
14	ES	7	Blood, serum, testicle	
15	ES	7	Blood, serum, testicle	
16	ES	9	Blood, serum, testicle	
17	ES	8	Blood, serum, testicle	

18	ES	7	Blood, serum, testicle	
19	ES	24	Blood, serum, testicle	
20	ES	6	Blood, serum, testicle	
21	ES	7	Blood, serum, testicle	
22	Birman	9	Blood, serum, testicle	
23	ES	36	Blood, serum, testicle	
24	Sphynx	11	Blood, serum, testicle	
25	Ragdoll	13	Blood, serum, semen, testicle	
26	ES	24	Blood, serum, semen, testicle	
27	Sphynx	11	Blood, serum, semen, testicle	
28	ES	6	Blood, serum, semen, testicle	7
29	Maine Coon	14	Blood, serum, semen, testicle	
30	Scottish gold	11	Blood, serum, semen, testicle	
31	ES	7	Blood, serum, semen, testicle	
32	Maine Coon	27	Blood, serum, semen	
33	Chartreux	9	Blood, serum, semen	3
34	Maine Coon	48	Blood, serum, semen	
35	Norwegian Forest cat	12	Serum, semen	2
36	Sphynx	10	Serum, semen	
37	Maine Coon	18	Blood, semen	2
38	Maine Coon	30	Blood, semen	
39	Maine Coon	25	Blood, semen, testicle	2
40	ES	7	Blood, semen, testicle	
41	ES	6	Testicle	
42	ES	7	Testicle	3
43	ES	6	Testicle	
44	ES	8	Serum, Testicle	1
45	ES	7	Serum, Semen, Testicle	1
46	ES	6	Blood, Testicle	1

438

439 Table 2. Results of the test performed on each cat involved in the study.

		RT-nPCR			RT-qPCR		
N°	Serology	Blood	Semen	Testicle	Blood	Semen	IHC

4	1:800	neg	na	neg	neg	na	neg
8	1:400	neg	na	neg	neg	na	neg
9	1:400	neg	na	neg	neg	na	neg
15	1:200	neg	na	pos	neg	na	neg
6	1:200	neg	na	neg	neg	na	neg
17	1:200	neg	na	neg	neg	na	neg
22	1:200	neg	na	neg	neg	na	neg
24	1:200	neg	na	neg	neg	na	neg
2	1:100	neg	na	neg	neg	na	neg
10	1:100	neg	na	neg	neg	na	neg
12	1:100	neg	na	neg	neg	na	neg
18	1:100	neg	na	pos	neg	na	neg
21	1:100	neg	na	neg	neg	na	neg
14	1:50	neg	na	neg	neg	na	neg
20	1:50	neg	na	neg	neg	na	neg
23	1:50	neg	na	neg	neg	na	neg
1	1:25	neg	na	neg	pos	na	neg
3	<1:25	neg	na	neg	neg	na	neg
5	<1:25	neg	na	neg	neg	na	neg
7	<1:25	neg	na	neg	neg	na	neg
11	<1:25	neg	na	neg	neg	na	neg
13	<1:25	neg	na	neg	neg	na	neg
16	<1:25	neg	na	neg	neg	na	neg
19	<1:25	neg	na	pos	neg	na	neg
29	1:400	neg	neg	pos	neg	neg	neg
27	1:100	neg	neg	neg	neg	neg	neg
25	1:50	neg	neg	neg	neg	neg	neg
26	1:50	neg	neg	neg	neg	neg	neg
28	1:50	neg	neg	neg	neg	neg	neg
30	1:50	neg	neg	neg	neg	neg	neg
31	<1:50	neg	neg	neg	neg	neg	neg
32	<1:25	neg	neg	na	neg	neg	na
33	<1:25	neg	neg	na	neg	neg	na
34	<1:25	neg	neg	na	neg	neg	na
36	1:200	na	neg	na	na	neg	na

35	<1:25	na	neg	na	na	neg	na
44	1:200	na	na	neg	na	na	neg
45	<1:50	na	neg	neg	na	neg	neg
37	na	neg	neg	na	neg	neg	na
38	na	neg	neg	na	neg	neg	na
39	na	neg	neg	neg	neg	neg	neg
40	na	neg	neg	neg	neg	neg	neg
41	na	na	na	neg	na	na	neg
42	na	na	na	pos	na	na	neg
43	na	na	na	pos	na	na	neg
46	na	neg	na	neg	neg	na	neg

nRT-PCR: nested reverse transcriptase-polymerase chain reaction; RT-qPCR reverse transcriptase-quantitative polymerase chain reaction; IHC: immunohistochemistry for FCoV; na: specimen not available; neg: negative; pos: positive.