

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

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19 **Keywords:** feline coronavirus; feline infectious peritonitis; tomcat reproduction; cattery
20 management; PCR; prevention

21 **Abstract**

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

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

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58 **Introduction**

59 Feline infectious peritonitis (FIP) is an immune-mediated disease of young cats. The
60 causative agent is the feline coronavirus (FCoV), generated by a mutation of the
61 widespread enteric pathotype, that gains the ability to replicate in macrophages, and
62 spreads through infected monocytes.¹ The course of the infection depends in part upon
63 the type and strength of the immune response of the host,²⁻⁴ but environmental factors
64 such as the level of stress and overcrowding also play a role.⁵ FCoV infection is very
65 common in cats; around 40% of the domestic cat population has been infected with
66 FCoV, and this figure may increase up to 90% in multi-cat households.^{6,7} Natural FCoV
67 infections are transient in ~70% of cats, but persistent infections can occur in ~13% of
68 cats,⁸ while around 5–10% of cats are believed to be resistant to FCoV infection. In
69 most cases, FCoV infection is asymptomatic, or results in only mild gastrointestinal
70 clinical signs; however, in a small percentage of cases, FCoV infection results in FIP.⁵
71 Asymptomatic FCoV infection was previously believed to be confined to the intestinal
72 tract, but it is now known that healthy FCoV-infected cats can have systemic infection,
73 albeit with lower viral loads than cats with FIP.⁹ These recurrent phases of intestinal
74 colonization and fecal shedding of the virus may lead to a transient localization in
75 several organs and are followed by seroconversion and by negativization at the
76 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

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

100

101 **Material and methods**

102 *Sample collection*

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

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

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

117 Immediately after collection, half testicle was frozen in plain tubes while the other half
118 was collected into 10% neutral-buffered formalin for histological and
119 immunohistochemical examination. For the two cats on which necropsy was performed,
120 tissue samples grossly affected by lesions were also collected into 10% neutral-buffered
121 formalin for histology and immunohistochemistry to reach a definitive diagnosis.
122 The study protocol was approved by the IACUC of the University of Milan (approval
123 number 109/2016).

124 *Semen collection*

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

131 *Serology*

132 Anti-FCoV antibodies titres were assessed using an indirect immunofluorescence test
133 performed on 10 multi-well slides produced at the University of Zurich according to
134 Osterhaus et al,²⁰ by coating each well with 4.5×10^3 PD-5 cells, half of which were
135 infected with swine transmissible gastroenteritis virus (serologically cross-reacting with
136 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 $15 \mu\text{L}$ 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 $300 \mu\text{L}$ 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: $50 \mu\text{L}$; 8-
154 $100 \mu\text{L}$) were centrifuged (5 min at $7000 \times g$) and the supernatant was discarded. The
155 resulting pellets were washed two times using $100 \mu\text{L}$ of phosphate buffer saline (PBS)
156 for 5 minutes at $7000 \times g$. 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 μ L 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 μ L 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 μ L 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 described²² 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,

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

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

228 **Discussion**

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

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

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

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

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

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

290 None of the semen samples were RT-PCR and RT-qPCR positive for FCoV. Only in
291 one cat for which both testicles and semen were available, results were discordant, with
292 positive RT-PCR on testicle but negative on semen. It cannot also be excluded that the
293 virus was present on the stromal or vascular tissues of the testicle and not in germinal
294 cells, leading to a negative PCR result on semen. Unfortunately, the negative results in
295 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.

323

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436 Table 1. Data on signalment, type of sample collected from the cats and included in this
437 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	3
42	ES	7	Testicle	
43	ES	6	Testicle	
44	ES	8	Serum, Testicle	1
45	ES	7	Serum, Semen, Testicle	1
46	ES	6	Blood, Testicle	1

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

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

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

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441 nRT-PCR: nested reverse transcriptase-polymerase chain reaction; RT-qPCR reverse

442 transcriptase-quantitative polymerase chain reaction; IHC: immunohistochemistry for

443 FCoV; na: specimen not available; neg: negative; pos: positive.

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