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17 **Abstract**

18 A pantropic canine coronavirus (CCoV) strain (CB/05) has been recently associated to a fatal
19 outbreak of systemic disease in young dogs. We report the clinical, virological and serological
20 findings in dogs experimentally infected with strain CB/05. The dogs, three 2.5-month-old and two
21 6-month-old pups, were successfully infected, shedding viral RNA with their faeces for the entire
22 observation period (21 days) and displaying systemic clinical signs resembling those observed
23 during the course of natural infection. Leucopenia (acute lymphopenia) occurred in all infected
24 dogs, with values dropping below 60% of the initial counts. Considering the severity of the CB/05-
25 induced disease, two of the youngest pups were euthanized for ethical reasons at days 8 -9
26 postinfection, whereas the other pups underwent a slow but progressive improvement of their
27 clinical status with complete recovery. At postmortem examination, remarkable lesions were
28 observed in the internal organs of the euthanized pups, that tested positive for CCoV by real-time
29 RT-PCR and virus isolation on cell cultures. All pups seroconverted for CCoV, as shown by the
30 high optical density values and antibody titres detected by ELISA and virusneutralisation tests,
31 respectively. The present study confirms that strain CB/05 is highly pathogenic for dogs, being able
32 to induce a severe disease (and in some cases the death) even in experimental conditions.

33

34 **Keywords:** dog; pantropic coronavirus; experimental infection; systemic disease.

35 **1. Introduction**

36 Coronaviruses (CoVs) are enveloped, single-stranded RNA viruses which included in three
37 different antigenic groups. CoVs infecting dogs comprise canine enteric coronavirus (CCoV)
38 (Enjuanes et al., 2000) and the newly recognised canine respiratory coronavirus (CRCoV) (Erles et
39 al., 2003; Decaro et al., 2007a), belonging to group 1 and group 2 CoVs, respectively. Two CCoV
40 genotypes have been identified so far, namely CCoV type I and CCoV type II, which are
41 responsible for the occurrence enteritis in dogs and are frequently associated in mixed infections
42 (Pratelli et al., 2004; Decaro et al., 2005c). Although its tropism is restricted to the gastroenteric
43 tract, CCoV has been recently associated to systemic disease followed by fatal outcome in pups
44 (Buonavoglia et al., 2006). Severe clinical signs were observed in the affected pups, whereas
45 necropsy examination revealed remarkable gross lesions in lungs, liver, spleen, and kidneys.
46 Virological and bacteriological investigations failed to detect common canine pathogens.
47 Unexpectedly, CCoV type II RNA was detected at very high titres in the internal organs of the dead
48 pups and the virus (strain CB/05) was isolated on canine cell cultures. The association of strain
49 CB/05 to a severe, sometimes fatal disease of dogs, together with the isolation of the virus from
50 organs with severe lesions, strongly suggests that CCoV has changed its tropism, acquiring the
51 ability to spread from the enteric tract to the internal organs (Decaro et al., 2007b).

52 In this study, we report the results of the experimental infection with isolate CB/05 in pups
53 with different age, showing that in contrast with classical CCoVs, this virus is able to cause
54 systemic disease followed by fatal outcome in younger pups.

55

56 **2. Materials and methods**

57 **2.1 Cells and virus**

58 Canine fibroma A-72 cells were grown in Dulbecco's minimum essential medium supplemented
59 with 10% foetal calf serum. Strain CB/05 was isolated from the lungs of a dead pup (117/05-C) and
60 adapted to growth on A-72 cells. At the 3rd passage, the virus was titrated on cell cultures and

61 inocula containing $10^{6.25}$ TCID₅₀/ml of viral suspension were stored at -70°C. Contaminations by
62 other canine pathogens, such as canine parvovirus type 2 (CPV-2), canine distemper virus (CDV)
63 and canine adenoviruses (CAAdVs), were ruled out by specific molecular assays (Hu et al., 2002;
64 Decaro et al., 2005b; Elia et al., 2006).

65

66 **2.2. Experimental study**

67 The experimental study was performed according to the animal health and well-being
68 regulations and was authorised by the Ministry of Health of Italy (authorization no. 53/2005-C). Six
69 mixed-bred female dogs including four 2.5-month-old ($n = 1-4$) and two 6-month-old ($n = 5, 6$)
70 pups were housed at the “Infectious Disease Unit” of the Animal Hospital, Faculty of Veterinary
71 Medicine of Bari. The dogs had tested negative for CCoV RNA by a real-time RT-PCR assay
72 (Decaro et al., 2004) carried out on the faeces and for CCoV antibodies by an ELISA test (Pratelli et
73 al., 2002) carried out on serum samples. All dogs were housed individually in separate boxes, fed
74 twice daily with a commercial dry dog food and provided water *ad libitum*. After an acclimatization
75 period of 5 days, 5 animals ($n = 1, 2, 3, 5, 6$) were administered oronasally 3 ml of a viral
76 suspension of strain CB/05, with a titre of $10^{6.25}$ TCID₅₀ and 7.85×10^7 RNA copies per ml,
77 whereas one dog ($n = 4$), 2.5-month old, was maintained uninfected by oronasal administration of 3
78 ml of sterile saline solution.

79

80 **2.3. Clinical score, virus isolation and real-time RT-PCR**

81 The clinical condition of each dog was monitored daily for 21 days. A scoring system was
82 devised taking into account rectal temperatures, total white blood cell (WBC) counts, appearance of
83 clinical signs (vomiting, diarrhoea, depression, loss of appetite, dehydration), following the scheme
84 adopted in a previous study (Decaro et al., 2005a) and derived by Nakamura et al. (2004), with
85 some modifications (Table 1). Due to ethical reasons, dogs whose total clinical score reached a

86 value ≥ 15 were euthanized by intravenous administration of 10 mg/kg of body weight of Zoletil
87 100 (Virbac S.r.l., Italy) followed by 0.5 ml/kg body weight of Tanax (Intervet Italia, Italy).

88 EDTA-treated blood samples were collected daily for total and differential WBC counting
89 and for testing for CCoV RNAemia by real-time RT-PCR (Decaro et al., 2004). The presence of the
90 viral RNA in the blood was also evaluated at hours 3, 6, 9, 12 and 18 after inoculation. Plasma
91 samples were prepared daily to evaluate free viral RNAemia and weekly to determine CCoV
92 antibody titres by virus neutralisation (VN) and ELISA tests (Pratelli et al., 2002).

93 To evaluate the viral shedding in the faeces, the rectal swabs collected daily from the control dog
94 and from the dogs inoculated with strain CB/05 were subjected to RNA extraction using QIAamp®
95 Viral RNA Mini Kit (Qiagen S.p.A.). In addition, tissue samples from parenchymatous organs were
96 withdrawn from the two dead pups (Table 2). RNA was extracted from the WBC pellets using
97 QIAamp® RNA Blood Mini Kit (Qiagen S.p.A.), from the plasma samples using QIAamp® Viral
98 RNA Mini Kit and from the tissue samples using QIAamp® RNeasy Mini Kit. Attempts to isolate
99 the virus in A-72 cells were carried out on rectal swabs of all infected pups and on organs of the
100 sacrificed animals as described previously (Decaro et al., 2007b).

101 Real-time RT-PCR targeting the M gene of CCoV type II (GenBank accession number D13096)
102 was carried out on the RNA extracts as described elsewhere (Decaro et al., 2005c). The genotype-
103 specific RT-PCR assays were undertaken in an i-Cycler iQ™ Real-Time Detection System (Bio-
104 Rad Laboratories Srl, Milan, Italy) and the data were analyzed with the appropriate sequence
105 detector software (version 3.0). After reverse transcription, triplicates of the CCoV type II standard
106 dilutions and RNA templates were simultaneously subjected to real-time analysis. The 50 μ l-
107 reaction mixture contained 25 μ l of iQ™ Supermix (Bio-Rad Laboratories Srl), 600 nM of each
108 primers CCoV7-F (TAGTGCATTAGGAAGAAGCT) and CCoV7-R
109 (AGCAATTTTGAACCCTTC), 200 nM of probe CCoV7-Pb (FAM-CCTCTTGAAGGTGTGCC-
110 TAMRA) and 20 μ l of c-DNA. The thermal profile consisted of activation of iTaq DNA

111 polymerase at 95° C for 10 min, followed by 45 cycles of denaturation at 95° C for 15 s, annealing
112 at 48°C (type II-specific assay) for 30 s and extension at 60° C for 1 min.

113

114 **2.4. Evaluation of antibody response to CCoV**

115 Plasma samples from inoculated dogs were tested in parallel by virus neutralisation (VN) and
116 ELISA tests (Pratelli et al., 2002).

117 For VN test, duplicates of serial twofold dilutions of heat-inactivated plasmas (starting from
118 dilution 1:2) were mixed with 100 TCID₅₀ of the isolated strain CB/05 in 96-well microtitre plates.
119 After preincubation at room temperature for 90 min, 20,000 A-72 cells were added to each well.
120 The plates were read after 4 days of incubation at 37°C. VN titres were calculated with the Karber
121 method and expressed as the highest plasma dilution that was able to neutralise the virus.

122 For ELISA, microtitre plates were coated with CCoV antigen (enteric strain S-378) and, after
123 treatment with blocking solution (0.2% gelatin in carbonate buffer [15 mM Na₂CO₃, 35 mM
124 NaHCO₃, pH 9.6]) and repeated washing, the 1:50 dilutions of the plasma samples were added to
125 each well. Then the plates were incubated for 90 min at 37°C, washed four times and incubated for
126 60 min at 37°C with anti-dog IgG-goat peroxidase conjugates (Sigma-Aldrich srl, Milan, Italy).
127 After another washing cycle, 10 mg of freshly prepared substrate, 2,2'-azino-di-[3-
128 ethylbenzthiazoline sulfonate]diammonium salt (ABTS, Sigma-Aldrich srl) in 50 ml of 0.05 M
129 phosphate citrate buffer (pH 5.0) was placed in each well and the optical density at 405 nm (OD₄₀₅)
130 was determined. Positive and negative controls were used as described previously (Pratelli et al.,
131 2002).

132

133 **3. Results**

134 **3.1. Clinical signs and WBC counts**

135 Neither clinical signs or viral shedding were observed in the control dog, whose leukocyte counts
136 did not draw away from the baseline values. All the inoculated animals displayed severe clinical

137 signs similar to those observed in dogs infected naturally, although the outcome of the disease was
138 different on the basis of the age (Fig. 1). In fact, two out of the 3 youngest pups (dogs 1 and 3) had
139 to be euthanized for ethical reasons, at day 8 and 9 postinfection (p.i.), respectively, while both the
140 6-month-old pups recovered from the disease, albeit very slowly. Irrespective of the final outcome,
141 i.e, euthanasia or recovery, clinical signs were remarkably similar in all inoculated animals.

142 The 2.5-month-old dogs that underwent a fatal outcome (Fig. 1A) showed fever at days 1-2 p.i.,
143 with a peak of 39.9°C at day 2 p.i. (dog 1), and from day 1 to 6 p.i., with a peak of 40.0°C at day 3
144 p.i. (dog 3). Depression (day 3 to 8 p.i.), anorexia/dysorexia (day 3 to 9 p.i.), haemorrhagic
145 diarrhoea (day 2 to 7 p.i.), and vomiting (days 4-5 p.i.) also occurred. Leukopenia appeared at day 3
146 (dog 1) or 2 p.i. (dog 3), with total WBC counts remaining below 60% of the baseline values until
147 euthanasia. Acute lymphopenia was observed in both dogs, with lymphocyte numbers dropping
148 below 60% of the initial counts from day 3 p.i. until death (mean, 16.3%; 0.9×10^3 lymphocytes/ μ l
149 at day 8 p.i.). Postmortem examination revealed severe changes in the intestines and major organs,
150 which were very similar to those observed in dogs infected naturally (data not shown).

151 The 2.5-month-old dog that survived (Fig. 1B) displayed less severe symptoms, consisting of fever
152 (up to 39.2°C) from day 1 to 5 p.i. and at days 11-12 p.i., and mucoid diarrhoea (day 2 to 6 p.i.).
153 Total WBC counts dropped below the 60% of the initial counts from day 3 to 6 p.i., whereas severe
154 lymphopenia (below 60% of baseline values) was registered from day 1 to 8 p.i, with a peak at day
155 5 (19%; 1.6×10^3 lymphocytes/ μ l). Subsequently, the total number of peripheral blood lymphocytes
156 started to rise again and the clinical signs subsided. A mild loss of appetite occurred from day 1 to 6
157 p.i.

158 In the two 6-month-old pups (Fig. 1C), fever showed a biphasic course, with a first peak at day 2
159 p.i. of 39.8 and 40.1°C in dogs 5 and 6, respectively. A transient remission was observed from day
160 5 to 8 p.i. (dog 5) and at day 5 p.i. (dog 6), and a second episode of pyrexia occurred from day 9 to
161 14 p.i. with a peak of 39.5°C at day 10 p.i. (dog 5), and from day 6 to 8 p.i. with a peak of 39.4°C at
162 day 7 p.i. (dog 6). Depression was observed between days 3 and 8 p.i., whereas vomiting appeared

163 only sporadically in the same period (days 3, 4, 8 p.i. in dog 5; days 1, 3, 8 p.i. in dog 6), with 1-4
164 episodes per day. Both dogs displayed anorexia (days 3 to 7 p.i.), mucoid or fluid diarrhoea (days 3
165 to 10), and leucopenia, with WBC values dropping below 60% of the baseline from day 3 to 6 (dog
166 5) or 3 to 8 p.i. (dog 6). Lymphocytes dropped below 60% of the initial cell counts from day 3 to 6
167 p.i. in dog 5 and from day 3 to 8 p.i. in dog 6 (mean, 32.2%; 1.6×10^3 lymphocytes/ μl at day 3 p.i.).
168 Starting from day 10 (dog 5) or 9 p.i. (dog 6), the clinical conditions of the dogs improved
169 progressively, with a complete recovery at days 15-16 p.i.

170 The control (uninfected) pup (Fig. 1D) remained in a good clinical status during the entire
171 observation period and no variations in total WBC and lymphocyte counts were observed.

172

173 **3.2. Detection of CCoV RNA and CCoV antibodies**

174 The uninfected dog tested constantly negative for CCoV RNA. All the CCoV-infected dogs tested
175 negative for other common pathogens of dogs, including CCoV type I (Decaro et al., 2005c), CDV
176 (Elia et al., 2006), CAdVs (Hu et al., 2002) and CPV-2 (Decaro et al., 2005b).

177 The faecal shedding of the infected dogs followed the similar pattern, although higher viral RNA
178 titres were obtained from the two sacrificed animals in comparison to survivors (Fig. 1). The pups
179 that succumbed shed virus starting between days 1 and 3 p.i. and lasting until the day of death, with
180 a peak at day 6 (titre of 4.97×10^5 RNA copy numbers/ μl of template) or 9 (titre of 8.72×10^5 RNA
181 copy numbers/ μl of template). After their death, CCoV type II RNA was detected in the organs at
182 titres slightly lower than those observed in the dogs of the natural outbreak (Table 2). The 2.5-
183 month-old pup that recovered shed CCoV RNA starting from day 1 p.i. (titre of 2.97×10^1 RNA
184 copy numbers/ μl of template) and lasting for the entire observation period (21 days), with a peak at
185 day 6 p.i. (3.24×10^4 RNA copy numbers/ μl of template). Shedding of CCoV in the faeces of the
186 two 6-month-old dogs was observed from day 2 p.i. (mean titre, 1.40×10^3 RNA copy numbers/ μl

187 of template) to day 21 p.i. (last day of observation) reaching the maximal mean value of 6.79×10^5
188 RNA copy numbers/ μ l of template at day 10 p.i.

189 Surprisingly, CCoV RNA was never detected in the blood of the 6-month-old pups, as well as in the
190 euthanized animals, in whose organs remarkable viral RNA titres were found. Traces of CCoV
191 RNA were detected only in the blood of the survived 2.5-month-old pup between days 7 and 10 p.i.,
192 with plasma viral titres ranging from 5.54×10^0 to 9.30×10^1 RNA copies/ml of template.

193 The virus was successfully isolated on cell cultures from the rectal swabs of all inoculated dogs
194 (data not shown) and from some organs of the euthanized pups (Table 2).

195 All the infected animals seroconverted for CCoV, whereas antibodies were not detected in the
196 control dog (Fig. 2D). In the dogs that were euthanized the antibody titres were determined only at
197 day 7 p.i., with VN titres of 1:8 and OD values of 0.089 (geometric means, Fig. 2A). In survivors,
198 the maximal antibody titres were reached at days 14 and 21 p.i. by VN and ELISA test, respectively
199 (Fig. 2B and 2C).

200

201 **4. Discussion**

202 In a previous study, a pantropic variant of CCoV was associated to a fatal disease of dogs,
203 characterized by leucopenia, gastroenteritis and severe changes in the internal organs (Buonavoglia
204 et al., 2006). The disease induced by strain CB/05 in pups of the natural outbreak was reproduced in
205 dogs infected experimentally. All inoculated dogs were successfully infected, as shown by the
206 occurrence of faecal shedding, seroconversion and severe clinical signs. The viral excretion was
207 similar to those observed during enteric CCoV infection (Decaro et al., 2004, 2005c), but the course
208 of disease was more severe, as clinical signal characteristic of systemic infection were observed in
209 the infected dogs. The pantropism of the virus was confirmed by the presence of gross lesions in the
210 internal organs of the dead dogs, as well as by the detection of viral RNA in those tissues.
211 Interestingly, CCoV RNA was detected also in the brain of the dead dogs. In contrast, enteric CCoV

212 has been never associated to systemic infection, although the virus has been isolated previously
213 from some tissues (tonsils, lungs and liver) of experimentally infected pups (Tennant et al., 1991).
214 Two of the three inoculated 2.5-month-old pups had to be euthanized after few days postinfection,
215 whereas the other dogs (the remaining dog of the same age and the two dogs six-month of age)
216 recovered after a severe disease. Considering that dogs infected naturally were all between 45 and
217 56 days of age, it could be hypothesised that the age of the infected animals plays a role in
218 determining the fate of CB/05 infection, with a very severe clinical course in the youngest pups.
219 Moreover, in the experimental infection, the organs of the dead dogs contained lower CCoV RNA
220 titres with respect to dogs infected naturally, so that virus isolation was not obtained from all PCR-
221 positive tissues. Despite the drop of the WBC counts registered in all infected dogs and the
222 detection of the viral RNA in the internal organs of the sacrificed dogs, free or cell-associated
223 CCoV RNAemia was not found at any time either in euthanized or survived dogs, with the
224 exception of the recovered 2.5-month-old pup which showed very low RNA viral titres in the
225 plasma between days 7 and 10 p.i. Thus, at this moment, the mechanisms of lymphopenia and viral
226 spread to internal organs remained unknown. Albeit strange, our findings are compatible with those
227 obtained from cats experimentally infected with feline infectious peritonitis virus (FIPV). Cats
228 succumbed to FIPV display very high viral titres in the haemolymphatic tissues (Kipar et al., 2006),
229 in contrast with the low loads detected in the blood (De Groot-Mijnes et al., 2005). In our study,
230 CB/05-infected dogs were found to contain lower viral loads in the lymphoid tissues in comparison
231 to FIPV-infected cats, thus likely accounting for the undetected viral RNAemia in euthanized dogs.
232 In conclusion, we have confirmed the pantropism of strain CB/05, reproducing the natural disease
233 even in experimental conditions. Further studies will contribute to better understand the
234 epidemiological distribution and the pathogenetic mechanisms of the virus, including the possible
235 involvement of the different lymphocyte classes.

236

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242

243

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295
296**Table 1. Scoring system for clinical signs of CB/05 infection^a.**

Clinical signs	Daily score
Rectal temperature (°C)	
≤ 37.6 °C	3
37.7 - 38.7 °C	0
38.8 - 39.7 °C	1
39.8 - 40.0 °C	2
≥ 40.1 °C	3
WBC count ^b	
75-60	1
59-45	2
44-30	3
≤30	4
Diarrhoea	
Mucoïd	1
Fluid	2
Dysenteric	3
Haemorrhagic	4
Loss of appetite	
Dysorexia	1
Anorexia	3
Vomiting (episodes/day)	
1	1
>1	3
Depression	
Mild	1
Moderate	2
Severe	3
Dehydration	
Mild	1
Moderate	2
Severe	3

297
298^a Adapted from Decaro et al., 2005a.

299

^b Values are expressed as percentages of the WBC counts determined

300

prior to inoculation.

301

302

Table 2. CCoV RNA titres in the organs of dogs succumbed to natural or experimental infection with strain CB/05.

303

Tissue/sample	117/05-A ^a		117/05-B ^a		117/05-C ^a		Dog 1 ^b		Dog 3 ^b	
	RRT-PCR	VI								
Faeces	6.12 x 10 ⁵	+	9.24 x 10 ⁴	+	3.17 x 10 ⁵	+	5.14 x 10 ⁴	+	1.09 x 10 ⁵	+
Lung	1.08 x 10 ⁶	+	2.74 x 10 ⁴	+	2.32 x 10 ⁶	+	6.79 x 10 ³	-	2.99 x 10 ⁴	+
Spleen	4.46 x 10 ⁶	+	1.20 x 10 ⁵	+	6.87 x 10 ⁶	+	5.48 x 10 ⁴	+	3.07 x 10 ⁴	+
Liver	9.02 x 10 ⁴	+	5.98 x 10 ⁴	+	3.12 x 10 ⁵	+	1.93 x 10 ⁴	+	4.70 x 10 ³	-
Kidney	7.54 x 10 ⁵	+	1.40 x 10 ⁴	+	2.53 x 10 ⁵	+	5.11 x 10 ³	-	2.94 x 10 ³	-
Mesenteric lymph node	8.09 x 10 ⁴	+	2.01 x 10 ⁴	+	5.17 x 10 ⁴	+	2.19 x 10 ⁴	-	3.61 x 10 ⁴	+
Brain	5.23 x 10 ³	-	9.52 x 10 ²	-	1.25 x 10 ⁴	-	3.32 x 10 ²	-	6.00 x 10 ²	-

304

305 RRT-PCR, real-time RT-PCR. Results are expressed as CCoV type II RNA copy number/ μ l of template.

306 VI, virus isolation. Results are expressed as positive (+) or negative (-).

307 ^aDogs succumbed to natural infection (described by Decaro et al., 2007b).308 ^bDogs euthanized after experimental infection (this study).

309 **Figure legend**

310 **Fig. 1.** Disease progression in dogs infected experimentally with strain CB/05. A. 2.5-month-old
311 dogs that were euthanized (dogs 1 and 3, means); B. 2.5-month-old dog that survived (dog 2); C. 6-
312 month old dogs (dogs 5 and 6, means); D. control dog (dog 2). Dogs inoculated oronasally with
313 CB-05 strain were monitored for up to 21 days for total WBC, lymphocyte and polymorphocyte
314 counts (top graphs). In addition, fever, viral RNA shed in faeces and clinical score were
315 determined (bottom graphs). Total WBC, lymphocyte, and polymorphocyte counts are presented as
316 percentages of the cell counts determined at day 0. Viral RNA titres as determined by real-time RT-
317 PCR are expressed as log copy numbers (\log_{10}) per μl of template. Clinical scores were calculated
318 as shown in Table 1 and are reported for each day in correspondence of the temperature curves.

319

320 **Fig. 2.** Antibody responses in dogs infected experimentally with strain CB/05. A. 2.5-month-old
321 dogs that were euthanized (dogs 1 and 3, means); B. 2.5-month-old dog that survived (dog 2); C. 6-
322 month-old dogs (dogs 5 and 6, means); D. control dog (dog 4). Antibody responses are presented as
323 geometric means of virus neutralising (VN) titres and ELISA optical density (OD) values.

324

325

326



FIG. 1

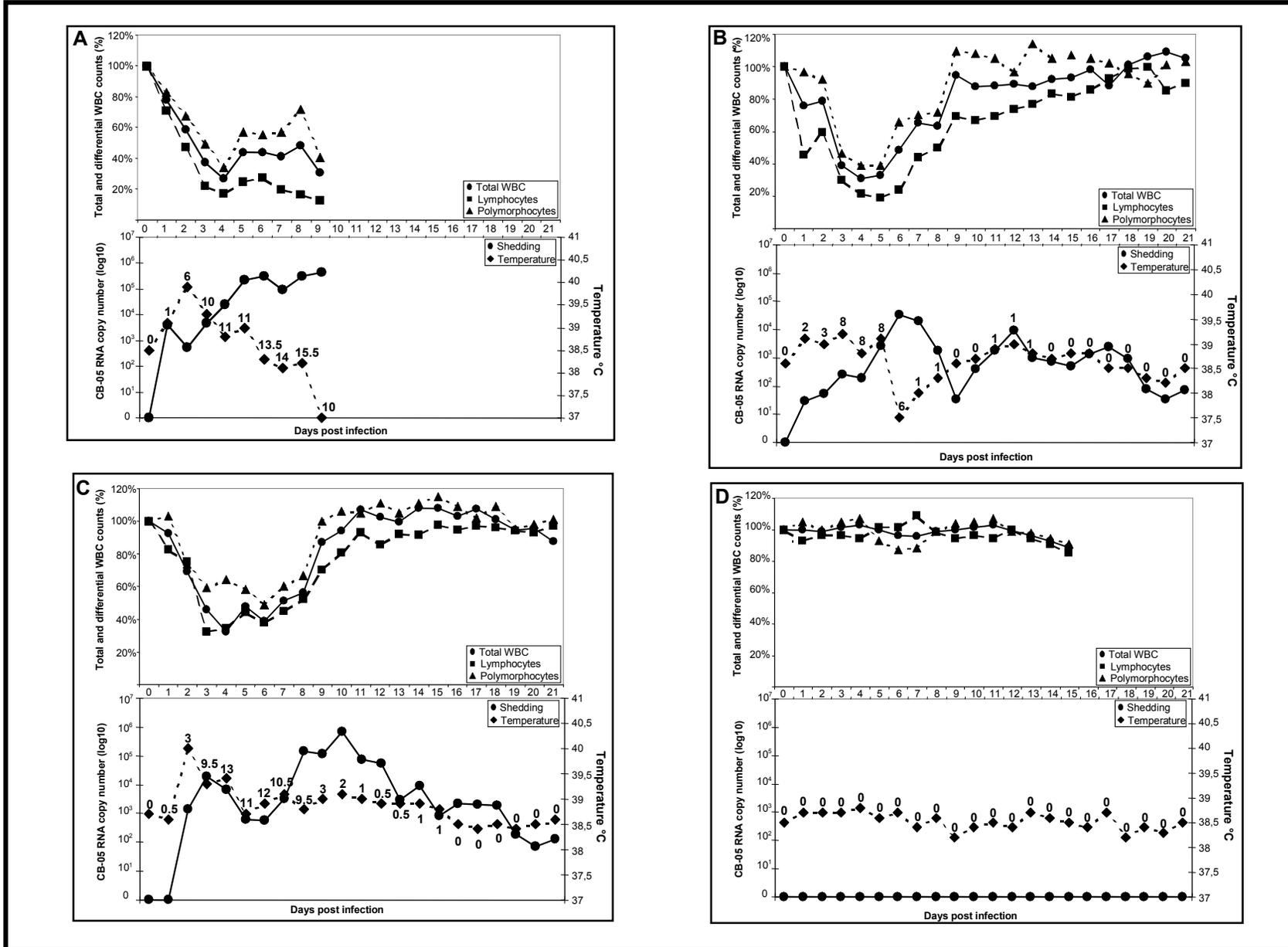




FIG. 2

