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3 Zoonosis.

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5 **Natural SARS-CoV-2 infection in kept ferrets, Spain**

6 **Authors:** Christian Gortázar¹, Sandra Barroso-Arévalo², Elisa Ferreras-Colino¹, Julio Isla³,

7 Gabriela de la Fuente³, Belén Rivera², Lucas Domínguez², José de la Fuente^{1,4}, José M. Sánchez-

8 Vizcaíno²

9

10 **Affiliations:**

11 1.- SaBio Instituto de Investigación en Recursos Cinegéticos IREC (CSIC-UCLM), Ciudad Real,

12 Spain (C. Gortázar, E. Ferreras-Colino, J. de la Fuente)

13 2.- Universidad Complutense de Madrid. VISAVET Center. Avda de Puerta de Hierro s/n, 28040

14 Madrid, Spain (J.M. Sánchez-Vizcaíno, L. Domínguez, S. Barroso. B.Rivera)

15 3.- Sabiotec, Ciudad Real, Spain (J. Isla, G. de la Fuente)

16 4.- Oklahoma State University, Stillwater, Oklahoma, USA (J. de la Fuente)

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19 **Abstract**

20 We found SARS-CoV-2 RNA in 6 of 71 ferrets (8.4%) and isolated the virus from one

21 rectal swab. Natural SARS-CoV-2 infection does occur in kept ferrets, at least under

22 circumstances of high viral circulation in the human population. However, small ferret

23 collections are probably unable to maintain prolonged virus circulation.

24 **Text**

25 Natural infection of animals with severe acute respiratory syndrome coronavirus 2
26 (SARS-CoV-2) has been reported in pet cats and dogs, zoo felids, and mustelids belonging to the
27 subfamily mustelinae [1]. Among mustelids, natural SARS-CoV-2 infections have been recorded
28 in farmed American mink (*Neovison vison*), and sporadically in a wild mink sampled close to an
29 infected farm in Utah¹ and in a kept pet ferret (*Mustela putorius furo*) from an infected
30 household in Slovenia². Ferrets are common laboratory models and experimental infections have
31 evidenced their susceptibility and ability to transmit the virus to other ferrets. SARS-CoV-2 is
32 shed up to 8 days post-infection (dpi) in nasal washes, saliva, urine, and feces and is effectively
33 transmitted to naive ferrets by direct contact and via the air [2, 3]. Experimentally infected ferrets
34 display either no clinical signs or exhibit elevated body temperature and loss of appetite [2, 4].

35 Ferrets are common pets^{3,4,5}, and are also used as work animals for rabbit control.
36 However, it remains unknown if SARS-CoV-2 circulates among kept ferret populations and if
37 ferrets, like farmed mink, could contribute to virus maintenance.

38 **The Study**

39 We studied 71 ferrets belonging to seven owners and used as working animals for rabbit
40 hunting in Ciudad Real province, central Spain. Group sizes ranged from four to 21 (mean 10).
41 Twenty ferrets belonging to groups 1 and 2 were re-sampled 66 days after initial sampling.
42 Sampling took place between August and November 2020. Animal sampling procedures had
43 been approved by the Madrid Animal Research Ethics Committee, ref. CM14/2020. One
44 oropharyngeal and one rectal swab (DeltaSwab® Virus 3ml, Deltalab S.L., Rubí, Spain) were
45 taken from each ferret for RNA extraction.

46 SARS-CoV-2-specific RNA was detected using a RT-qPCR assay. Briefly, RNA was
47 extracted using the KingFisher Flex System (Thermo Fisher, Waltham, MA, USA) according to
48 the manufacturer instructions. Detection of SARS-CoV-2 RNA was performed using the
49 envelope protein (E)-encoding gene and two targets (IP2 and IP4) of the RNA-dependent RNA
50 polymerase gene (RdRp) in an RT-PCR protocol established by the WHO according to the
51 guidelines ([https://www.who.int/emergencies/diseases/novel-coronavirus-2019/technical-](https://www.who.int/emergencies/diseases/novel-coronavirus-2019/technical-guidance/laboratory-guidance)
52 [guidance/laboratory-guidance](https://www.who.int/emergencies/diseases/novel-coronavirus-2019/technical-guidance/laboratory-guidance)) [5, 6]. Primer sets used are detailed in Table 1. The RT-qPCR
53 was carried out using the SuperScript III Platinum One-Step RT-qPCR Kit (ThermoFisher,
54 Massachusetts, USA), according to the manufacturer's protocol on a CFX Connect™Real-Time
55 PCR Detection System (BioRad, Berkeley, USA). The positive control for real-time RT-qPCR
56 was an *in vitro* transcribed RNA derived from the strain BetaCoV_Wuhan_WIV04_2019
57 (EPI_ISL_402124), loaned by the Pasteur Institute (Paris, France). Nuclease-free water was used
58 as negative control. A cycle threshold (Ct) cut-off of 40 cycles was used. A result was considered
59 positive when the sample showed a positive RT-qPCR for at least two of the three analyzed
60 targets.

61 Specimens considered positive by RT-qPCR were subjected to virus isolation in Vero E6
62 cells. Cells were cultured in RPMI 1640 medium supplemented with 10% fetal bovine serum
63 (FBS; Gibco, Madrid, Spain), 100 IU/ml penicillin, and 100 µg/ml streptomycin. Cells were
64 seeded in 96-well culture plates and cultured at 37°C with 5% CO₂ for 24 to 48 h. Then, cells
65 were inoculated with 10 µl of the direct sample (oronasal or fecal swabs). Mock-inoculated cells
66 were used as negative controls. Cultured cells were maintained at 37 °C with 5% CO₂, with a
67 daily observation of virus-induced cytopathic effect (CPE) and cellular death. After 6 days, cell
68 cultures were frozen, thawed, and subjected to three passages with inoculation of fresh Vero E6

69 cells with the lysates as described above. SARS-CoV-2 molecular detection was performed by
70 RT-qPCR on the supernatants from every passage to confirm the presence/absence of the virus in
71 the cell culture.

72 We found SARS-CoV-2 RNA in swab samples from 6 of 71 ferrets (8.4%) (Table 2),
73 belonging to four of seven investigated groups (57%). The likelihood of a swab testing positive
74 was unrelated with age class (under or over one year-old), sex and oral/rectal sample origin
75 (Fisher's two-tailed p values >0.2). RT-qPCR results were confirmed by sequencing the positive
76 PCR product. None of the 20 re-sampled ferrets was PCR-positive, including one individual that
77 had tested positive two months earlier (oropharyngeal swab; Ct = 35.38).

78 SARS-CoV-2 was isolated only from the rectal swab of one ferret (Ct in the original
79 sample = 34.5). Cell culture showed CPE and cellular death in the three passages. Virus recovery
80 was also confirmed by RT-qPCR (Ct value reduction from original inoculum to cell suspension
81 of third passage).

82 We conclude that natural SARS-CoV-2 infection in kept ferrets does occur in
83 circumstances of high viral circulation in the human population [7]. However, the high Ct values
84 observed, and the lack of positive ferrets at re-sampling, indicate that small ferret populations are
85 not as able to maintain prolonged virus circulation as large, farmed mink populations [8].

86 Specific guidance on SARS-CoV-2 in ferrets has been made available in the UK⁶.

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91 Spain.

92 **Conflicts of interest**

93 The authors have no conflicts of interest to declare.

94 **Author Bio**

95 Professor Christian Gortázar heads the SaBio research group at the Spanish Wildlife
96 Research Institute (IREC). His research interests include the epidemiology and control of
97 infections shared between wildlife, livestock, and human beings.

98 **Footnotes**

99 ¹ <https://promedmail.org/promed-post/?id=8015608>.

100 ² https://www.oie.int/wahis_2/public/wahid.php/Reviewreport/Review?page_refer=MapFullEven
101 [tReport&reportid=37289](https://www.oie.int/wahis_2/public/wahid.php/Reviewreport/Review?page_refer=MapFullEven).

102 ³ <https://www.vettimes.co.uk/app/uploads/wp-post-to-pdf-enhanced-cache/1/overview-of-ferrets->
103 [part-one-conditions-and-behaviour.pdf](https://www.vettimes.co.uk/app/uploads/wp-post-to-pdf-enhanced-cache/1/overview-of-ferrets-).

104 ⁴ <https://www.avma.org/resources-tools/reports-statistics/us-pet-ownership-statistics>.

105 ⁵ <https://www.mapa.gob.es/es/ganaderia/temas/produccion-y-mercados->
106 [ganaderos/20160222_informeestudioparapublicar_tcm30-104720.pdf](https://www.mapa.gob.es/es/ganaderia/temas/produccion-y-mercados-)

107 ⁶ <http://apha.defra.gov.uk/documents/guidance-sars-cov-2-ferrets.pdf>

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133 Address for correspondence: Christian Gortázar, SaBio research group at IREC Instituto de
134 Investigación en Recursos Cinegéticos (CSIC-UCLM), Ronda de Toledo 12, 13003 Ciudad
135 Real, Spain. email: christian.gortazar@uclm.es

136 Table 1. Primer sequences and amplified fragment sizes in base pairs.

Primer target	Sequence 5'–3'	PCR fragment size
Gene RdRp/ nCoV_IP2		
nCoV_IP2–12669Fw	ATGAGCTTAGTCCTGTTG	108 bp
nCoV_IP2–12759Rv	CTCCCTTTGTTGTGTTGT	
nCoV_IP2–12696b	AGATGTCTTGTGCTGCCGGTA	
Probe (+)	[5']Hex [3']BHQ–1	
Gene RdRp/ nCoV_IP4		
nCoV_IP4–14059Fw	GGTAACTGGTATGATTTCG	107 bp
nCoV_IP4–14146Rv	CTGGTCAAGGTTAATATAGG	
nCoV_IP4–14084	TCATACAAACCACGCCAGG	
Probe(+)	[5']Fam [3']BHQ–1	
Gene E/ E_Sarbeco		
E_Sarbeco_F1	ACAGGTACGTTAATAGTTAATAGCGT	125 bp
E_Sarbeco_R2	ATATTGCAGCAGTACGCACACA	
E_Sarbeco_P1	ACACTAGCCATCCTTACTGCGCTTCG	
	[5']Fam [3']BHQ–1	

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139 Table 2. RT-qPCR positive results by sample type.

Animal ID	Sample type	Ct value
G1-H6	Rectal swab	34.5
G1-H17	Nasal swab	37.29
G2-H5	Nasal swab	35.38
G5-H11	Nasal swab	39.83
G7-H7	Nasal swab	30.59
G7-H9	Nasal swab	38.91

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