ADVERTIMENT. L'accés als continguts d'aquesta tesi queda condicionat a l'acceptació de les condicions d'ús establertes per la següent llicència Creative Commons: https://creativecommons.org/licenses/?lang=ca

ADVERTENCIA. El acceso a los contenidos de esta tesis queda condicionado a la aceptación de las condiciones de uso establecidas por la siguiente licencia Creative Commons: https://creativecommons.org/licenses/?lang=es

WARNING. The access to the contents of this doctoral thesis it is limited to the acceptance of the use conditions set by the following Creative Commons license: https://creativecommons.org/licenses/?lang=en

SARS-CoV-2 infection in domestic and wildlife animals and its impact in the COVID-19 pandemic

Leira Fernández Bastit

Ph.D Thesis

Bellaterra, 2024

SARS-CoV-2 infection in domestic and wildlife animals and its impact in the COVID-19 pandemic

Doctoral thesis presented by **Leira – Paula Fernández Bastit** to obtain the Doctoral degree under the program of Animal Medicine and Health at the Faculty of Veterinary Medicine from *Universitat Autònoma de Barcelona*, under the supervision of **Joaquim Segalés Coma**, and **Júlia Vergara Alert.**

Bellaterra, 2024

El Prof. **Joaquim Segalés i Coma**, catedràtic del Departament de Sanitat i d'Anatomia Animals de la Facultat de Veterinària de la Universitat Autònoma de Barcelona i investigador adscrit a l'Institut de Recerca i Tecnologia Agroalimentàries – Centre de Recerca en Sanitat Animal (IRTA-CReSA) i la Dra. **Júlia Vergara i Alert**, investigadora de l'IRTA-CReSA,

Certifiquen:

Que la memòria titulada "SARS-CoV-2 infection in domestic and wildlife animals and its impact in the COVID-19 pandemic" presentada per **Leira - Paula Fernández Bastit** per a l'obtenció del grau de Doctora en Medicina i Sanitat Animals, s'ha realitzat sota la nostra direcció i tutoria, i n'autoritzem la seva presentació per tal de que sigui valorada per la comissió establerta.

I perquè així consti als efectes oportuns, signem la present declaració a Bellaterra (Barcelona), a 17 de Maig de 2024.

Prof. Joaquim Segalés i Coma	**Dra. Júlia Vergara i Alert**
Tutor i Director	Directora

Leira-Paula Fernández Bastit

Doctoranda

This Ph.D. thesis received partial funding from the BBVA Foundation as part of the project “Investigation on the potential role of pets as animal reservoirs for SARS-CoV-2” and the CBIG consortium (constituted by IRTA-CReSA, BSC and IrsiCaixa supported by Grifols pharmaceutical). The support also came from *El Consell de Col·legis Veterinaris de Catalunya* (CCVC), veterinary clinics from Catalonia and Valencian Community, HIPRA Laboratories, *Centre de Recuperació de Fauna Salvatge de Torreferrussa*, *Agència de Salut Pública de Barcelona*, and the company Vet & Wildlife.

Leira-Paula Fernández Bastit was partially supported by an IRTA’s fellowship and the crowdfunding initiative #Yomecorono.

Per al meu avi

"No hi ha res a témer a la vida, només comprendre-ho.

Ara és temps per comprendre més per témer menys."

Maria Salomea Skłodowska-Curie

Table of contents

LIST OF ABBREVIATIONS 1

ABSTRACT 5

RESUM 9

RESUMEN 13

CHAPTER 1. GENERAL INTRODUCTION 17

1.1 EMERGING ZOONOTIC DISEASES 19

1.2 EMERGING ZOONOTIC CORONAVIRUSES 19

1.3 SEVERE ACUTE RESPIRATORY SYNDROME CORONAVIRUS 2 (SARS-COV-2) 22

1.3.1 EMERGENCE OF A NOVEL CORONAVIRUS IN 2019: SARS-COV-2 22

1.3.2 TAXONOMY 23

1.3.3 GENOME ORGANIZATION, REPLICATION, AND VIRAL GENE EXPRESSION 23

1.3.4 VIRION STRUCTURE 25

1.3.5 VIRUS REPLICATION CYCLE 27

1.3.6 VIRUS EVOLUTION AND GENETIC VARIANTS 27

1.3.7 HOST CELL TROPISM AND DISEASE IN HUMANS 29

1.4 SARS-COV-2 INFECTION IN ANIMALS 30

1.4.1 SARS-COV-2 ORIGIN 30

1.4.2 EXPERIMENTAL INFECTIONS BY SARS-COV-2 IN ANIMAL SPECIES 32

1.4.3 ANIMAL MODELS FOR SARS-COV-2 RESEARCH 41

1.4.4 NATURAL INFECTIONS BY SARS-COV-2 IN ANIMAL SPECIES ____ 46
1.5 ONE HEALTH APPROACH ____ 54

CHAPTER 2. HYPOTHESES AND OBJECTIVES ____ 59

2.1 HYPOTHESES ____ 61
2.2 OBJECTIVES ____ 63

CHAPTER 3. STUDY 1 SARS-COV-2 infection and hummoral responses against different variants of concern in domestic pets and stray cats from North-Eastern Spain (2020 – 2022) ____ 65

3.1 INTRODUCTION ____ 67
3.2 MATERIAL AND METHODS ____ 68
3.3 RESULTS ____ 74
3.4 DISCUSSION ____ 87
3.5 APPENDIX 3.1 ____ 99

CHAPTER 4. STUDY 2 Survey of SARS-COV-2 in captive and free-ranging wildlife from Spain ____ 109

4.1 INTRODUCTION ____ 111
4.2 MATERIAL AND METHODS ____ 113
4.3 RESULTS ____ 123
4.4 DISCUSSION ____ 125

CHAPTER 5. STUDY 3 Monitoring SARS-COV-2 infection in urban and peri-urban wildlife species from Catalonia (Spain) ____ 133

5.1 INTRODUCTION ____ 135

5.2 MATERIAL AND METHODS 137
5.3 RESULTS 142
5.4 DISCUSSION 143

CHAPTER 6. STUDY 4 Susceptibility of domestic goat (C*apra aegagrus hircus*) to experimental infection with SARS-CoV-2 B.1.351/Beta variant 149

6.1 INTRODUCTION 151
6.2 MATERIAL AND METHODS 153
6.3 RESULTS 158
6.4 DISCUSSION 166

CHAPTER 7. STUDY 5 Comparison of three commercial ELISA kits for detection of antibodies against SARS-COV-2 in serum samples from different animal species 171

7.1 INTRODUCTION 173
7.2 MATERIAL AND METHODS 176
7.3 RESULTS 181
7.4 DISCUSSION 193

CHAPTER 8. GENERAL DISCUSSION 211

CHAPTER 9. CONCLUSIONS 227

REFERENCES 231

LIST OF SCIENTIFIC PUBLICATIONS BY LEIRA FERNÁNDEZ BASTIT 271

List of abbreviations

ACE2	angiotensin converting enzyme 2
ARDS	acute respiratory distress syndrome
CoVs	coronaviruses
COVID-19	coronavirus disease 2019
CPE	cytopathic effect
dpi	days post inoculation
E	envelope
EID	emerging infectious disease
ELISA	enzyme-linked immunosorbent assay
ER	endoplasmic reticulum
EZD	emerging zoonotic disease
gRNA	genomic RNA
hACE2	human angiotensin converting enzyme 2
hCoVs	human coronaviruses
HIV	human immunodeficiency virus
ID_{50}	infectious dose 50
IHC	immunohistochemistry
IM	intramuscular
IN	intranasal
IT	intra-tracheal
IV	intravenous
LRT	low respiratory tract
M	membrane
MERS-CoV	Middle East respiratory coronavirus
MM	*Mus musculus*
N	nucleocapsid

nAbs	neutralizing antibodies
NHP	non-human primates
NSPs	non-structural proteins
NT	nasal turbinate
ORF	open reading frame
RBD	receptor binding domain
RdRp	RNA-dependent RNA polymerase
RLU	relative light unit
PRNT	plaque reduction neutralization test
pp1a	polyprotein 1a
pp1ab	polyprotein 1ab
pVNT	pseudovirus neutralization test
RN	*Rattus norvegicus*
RR	*Rattus rattus*
RT-qPCR	reverse-transcription quantitative PCR
RTC	replication and transcription complex
RZ	reverse zoonotic
S	spike
S1	subunit 1 of the spike protein
S2	subunit 2 of the spike protein
SARS-CoV	severe acute respiratory syndrome coronavirus
SARS-CoV-2	severe acute respiratory syndrome coronavirus 2
sgRNA	subgenomic RNA
$TCID_{50}$	tissue culture dose 50
TMB	tetramethyl benzidine
TMPRSS2	transmembrane protease serine 2
TRS-B	transcription-regulating sequence
TRS-L	transcription leader sequence
URT	upper respiratory tract

VNT	virus neutralization test
VOC	variant of concern
VTM	viral transport media
VUM	variant under monitoring
WHO	World Health Organization
WT	wild-type
WTD	white-tailed deer

Abstract

The severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) has caused the coronavirus disease 2019 (COVID-19) pandemic, significantly impacting health, economies, and social stability. The virus emerged in late 2019 in China and rapidly spread worldwide, making evident the interconnectedness of the current globalized world. The high transmission capacity of the virus led to the emergence of several SARS-CoV-2 variants that caused major threats to human health. Importantly, SARS-CoV-2 has a potential zoonotic origin, likely stemming from *Rhinolophus* bats, and it is believed that jumped to humans through an unidentified intermediate host. In February 2020, the first cases of SARS-CoV-2 infections in animals were documented, triggering significant concerns regarding the virus crossing species barriers and posing major challenges for controlling its transmission. This *PhD Thesis* started in early 2020 with the aim to study the potential susceptibility of various animal species and assessing their potential role in the epidemiology of the COVID-19 pandemic.

In April 2020, we began a monitoring study in North-Eastern Spain to evaluate the presence of SARS-CoV-2 in companion animals (*Study 1*). In our study, which lasted until January 2022, we detected acute and/or past infections of SARS-CoV-2 and its variants in pet cats, dogs, and ferrets. The frequency of detection of active SARS-CoV-2 infection was low (0.3%) but detection of antibodies was relatively frequent in animals that had previous contact with infected owners (25% in cats, 10% in dogs, and 40% in ferrets). Additionally, we detected SARS-CoV-2 antibodies in stray cats, although with a significantly lower percentage (2.34%) compared to pet cats (7.65%). Moreover, this study demonstrated that companion animals were able to neutralize different SARS-CoV-2 variants, with cats exhibiting higher

neutralizing antibody (nAbs) titers than the other species across almost all studied variants.

On the other hand, we conducted SARS-CoV-2 surveillance studies (from 2020 to 2023) in both captive and free-ranging wildlife animals from Spain (*Study 2*), as well as in urban/peri-urban species, including rodents and wild boars, in Catalonia (*Study 3*). Our studies demonstrated no evidence of SARS-CoV-2 acute and/or past infections in these groups of animals, except for one bottlenose dolphin (*Tursiops truncatus*) in a zoological park. This dolphin exhibited antibodies against the nucleoprotein of SARS-CoV-2 by an enzyme-linked immunosorbent assay (ELISA), and low titers of nAbs (38.2%) by a pseudoneutralization assay (pVNT).

The emergence of SARS-CoV-2 variants during the pandemic also triggered concerns regarding their impact on animal populations. In this *PhD Thesis*, we investigated the susceptibility of domestic goats (*Capra aegagrus hircus*) to a SARS-CoV-2 variant with an expanded host range (B.1.351 variant) (*Study 4*). Goats, like other common livestock species, demonstrated limited susceptibility to SARS-CoV-2 experimental infection, supporting the notion that they played a negligible role in virus transmission so far.

Finally, since this *PhD Thesis* used three different ELISA kits to study humoral responses against SARS-CoV-2 in animal species, we evaluated their correlation and compared them using the pVNT as a reference (*Study 5*). ELISA-1 (used in *Studies 1, 3 and 4*), which targets receptor binding domain (RBD) nAbs of SARS-CoV-2, had excellent diagnostic performance, and demonstrated to be a reliable tool for initial screenings for high-throughput animal studies. ELISA-2 (targeting RBD nAbs) and ELISA-3 (targeting nucleoprotein antibodies) were used in *Study 2* and demonstrated lower sensitivity for detecting seropositive animals.

Overall, our results indicated that animals did not significantly influence the epidemiology of the COVID-19 pandemic. However, we evidenced that regular human-animal interactions pose risks of SARS-CoV-2 exposure in animal species. This *PhD Thesis* aligns with the One Health concept, emphasizing the need of robust multidisciplinary collaboration, integrating expertise from diverse fields, to manage global health threats effectively.

Resum

El coronavirus 2 de la síndrome respiratòria aguda greu (SARS-CoV-2), responsable de la pandèmia de la malaltia per coronavirus 2019 (COVID-19), ha causat un impacte significatiu sobre la salut, l'economia i l'estabilitat social. El SARS-CoV-2 té un potencial origen zoonòtic; es creu que es va originar en ratpenats del gènere *Rhinolophus,* i va saltar als humans a través d'un hoste intermediari no identificat. Al febrer del 2020, es van documentar els primers casos de SARS-CoV-2 en animals, desencadenant preocupacions sobre la capacitat del virus de travessar barreres entre espècies i plantejant grans reptes per controlar-ne la transmissió. Aquesta tesi doctoral es va iniciar a principis del 2020 amb l'objectiu d'estudiar la potencial susceptibilitat de diverses espècies animals i avaluar el seu possible paper en l'epidemiologia de la COVID-19.

El mes d'abril de 2020 vam iniciar un estudi de monitoratge al nord-est d'Espanya per avaluar la presència de SARS-CoV-2 en animals de companyia (*Estudi 1*). L'estudi, fins al gener del 2022, va detectar infeccions agudes i/o passades de SARS-CoV-2 i les seves variants en gats, gossos i fures. La freqüència de detecció d'infeccions agudes va ser baixa (0,3%), però la detecció d'anticossos va ser relativament freqüent en animals amb contacte previ amb propietaris infectats (25% en gats, 10% en gossos i 40% en fures). També es van detectar anticossos en gats ferals, amb un percentatge més baix (2,34%) comparat amb els gats domèstics (7,65%). Els animals de companyia van mostrar la capacitat de neutralitzar diferents variants de SARS-CoV-2, essent els gats els que mostraren nivells més alts de títols d'anticossos neutralizants (nAbs) respecte les altres espècies en gairebé totes les variants estudiades.

Aquesta tesi doctoral inclou estudis de vigilància de SARS-CoV-2 (des de 2020 fins a 2023) en animals salvatges, tant en captivitat com en llibertat, a Espanya (*Estudi 2*), així com en espècies urbanes/periurbanes a Catalunya (*Estudi 3*). Els estudis no varen evidenciar infeccions agudes o passades en aquests grups d'animals, exceptuant un dofí mular (*Tursiops truncatus*) en un parc zoològic. Aquest dofí va presentar anticossos contra la nucleoproteïna de SARS-CoV-2 mitjançant un assaig immunoenzimàtic (ELISA), i baixos nivells de nAbs (38,2%) mitjançant un assaig de pseudoneutralització (pVNT).

L'emergència de variants de SARS-CoV-2 durant la pandèmia va generar preocupacions sobre el seu impacte en poblacions animals. Aquesta tesi doctoral inclou la investigació de la susceptibilitat de la cabra domèstica (*Capra aegagrus hircus*) a una variant de SARS-CoV-2 amb un espectre d'hostes ampliat (variant Beta (B.1.351)) (*Estudi 4*). Les cabres van demostrar una susceptibilitat limitada a la infecció experimental per SARS-CoV-2, suggerint un paper insignificant en la transmissió del virus.

Finalment, vàrem avaluar la correlació de tres kits ELISA utilitzats en aquesta tesi doctoral i els vàrem comparar utilitzant el pVNT com a referència (*Estudi 5*). L'ELISA-1 (utilitzat en els *Estudis 1, 3 i 4*), que detecta nAbs contra el domini d'unió al receptor (RBD) de SARS-CoV-2, va demostrar tenir un excel·lent rendiment diagnòstic, essent una eina fiable per a estudis d'animals a gran escala. L'ELISA-2 (orientat a detectar nAbs contra RBD) i l'ELISA-3 (orientat als anticossos contra la nucleoproteïna), utilitzats a l'*Estudi 2,* van mostrar baixa sensibilitat per detectar animals seropositius.

En general, els nostres resultats van indicar que els animals no van influir significativament en l'epidemiologia de la pandèmia de la COVID-19. No obstant això, les interaccions regulars entre humans i animals suposen

riscos d’exposició a SARS-CoV-2 en espècies animals. Aquesta tesi doctoral s’alinea amb el concepte Una Sola Salut, destacant la necessitat d’una col·laboració multidisciplinària sòlida per gestionar les amenaces per a la salut global.

Resumen

El coronavirus 2 del síndrome respiratorio agudo severo (SARS-CoV-2), responsable de la pandemia de la enfermedad por coronavirus 2019 (COVID-19), ha causado un impacto significativo en la salud, la economía y la estabilidad social. El SARS-CoV-2 tiene un posible origen zoonótico; se cree que surgió de murciélagos del género *Rhinolophus*, y que saltó a los humanos a través de un huésped intermediario no identificado. En febrero del 2020, se documentaron los primeros casos de infecciones por SARS-CoV-2 en animales, generando preocupaciones significativas sobre la posibilidad de que el virus cruzara las barreras de especies y planteara grandes desafíos para controlar su transmisión. Esta tesis doctoral comenzó a principios del 2020 con el objetivo de estudiar la posible susceptibilidad de varias especies animales y evaluar su papel potencial en la epidemiología de la pandemia de la COVID-19.

En abril de 2020, iniciamos un estudio de monitoreo en el noreste de España para evaluar la presencia de SARS-CoV-2 en animales de compañía (*Estudio 1*). El estudio, que duró hasta enero del 2022, detectó infecciones agudas y/o pasadas de SARS-CoV-2 y sus variantes en gatos, perros y hurones domésticos. La frecuencia de detección de infecciones agudas fue baja (0,3%), pero la detección de anticuerpos fue relativamente frecuente en animales que habían tenido contacto previo con propietarios infectados (25% en gatos, 10% en perros y 40% en hurones). También se detectaron anticuerpos en gatos callejeros, aunque con un porcentaje significativamente menor (2,34%) comparado con los gatos de compañía (7,65%). Los animales de compañía demostraron la capacidad de neutralizar diferentes variantes de SARS-CoV-2, siendo los gatos los que mostraron títulos más altos de

anticuerpos neutralizantes (nAbs) comparado con las otras especies en casi todas las variantes estudiadas.

Por otro lado, esta tesis doctoral incluye estudios de vigilancia del SARS-CoV-2 (desde 2020 hasta 2023) en animales salvajes tanto en cautiverio como en libertad de España (*Estudio 2*), así como en especies urbanas/periurbanas en Cataluña (*Estudio 3*). Los estudios no demostraron evidencia de infecciones agudas o pasadas de SARS-CoV-2 en estos grupos de animales, exceptuando un delfín mular (*Tursiops truncatus*) en un parque zoológico. Este delfín mostró anticuerpos contra la nucleoproteína de SARS-CoV-2 mediante un ensayo inmunoenzimático (ELISA) y títulos bajos de nAbs (38,2%) mediante un ensayo de pseudoneutralización (pVNT).

La aparición de variantes de SARS-CoV-2 durante la pandemia generó preocupaciones importantes sobre su impacto en las poblaciones animales. En esta tesis doctoral, investigamos la susceptibilidad de las cabras domésticas (*Capra aegagrus hircus*) a una variante de SARS-CoV-2 con un rango de huésped ampliado (variante Beta (B.1.351)) (*Estudio 4*). Las cabras demostraron una susceptibilidad limitada a la infección experimental por SARS-CoV-2, sugiriendo un papel insignificante de este huésped en la transmisión del virus.

Finalmente, evaluamos la correlación de tres kits ELISA utilizados en esta tesis doctoral y los comparamos utilizando el pVNT como referencia (*Estudio 5*). El kit ELISA-1 (utilizado en los Estudios 1, 3 y 4), que detecta nAbs contra el dominio de unión al receptor (RBD), demostró un rendimiento diagnóstico excelente, siendo una herramienta confiable para estudios de animales a gran escala. ELISA-2 (enfocado en nAbs contra RBD) y ELISA-3 (enfocado en anticuerpos de nucleoproteína), utilizados en el Estudio 2, demostraron baja sensibilidad para detectar animales seropositivos.

En general, nuestros resultados indicaron que los animales no influenciaron significativamente la epidemiología de la pandemia de COVID-19. Sin embargo, las interacciones regulares entre humanos y animales suponen riesgos de exposición a SARS-CoV-2 en especies animales. Esta tesis doctoral se alinea al concepto de Una Sola Salud, destacando la necesidad de una colaboración multidisciplinaria sólida para gestionar eficazmente las amenazas a la salud global.

CHAPTER 1. GENERAL INTRODUCTION

Chapter adapted from the following review article:

Fernández-Bastit, L., Vergara-Alert, J. & Segalés, J. Transmission of severe acute respiratory syndrome coronavirus 2 from humans to animals: is there a risk of novel reservoirs? *Curr. Opin. Virol.* **63**, 101365 (2023).

1.1 Emerging zoonotic diseases

Emerging zoonotic diseases (EZDs) are infections caused by pathogenic agents that jump from a non-human animal reservoir to human population and potentially spread globally causing threats for public health, economy, and social stability [1]. Due to the current globalized world, EZDs have been on the rise in the recent decades, accounting for approximately 75% of the emerging infectious diseases (EIDs) in humans to date [2]. Bacteria, viruses, prions, parasites, and fungi can cause EZDs. Nonetheless, viruses, especially RNA viruses, such as the human immunodeficiency virus (HIV), H1N1 influenza virus, Ebola virus, Zika virus, and three coronaviruses (CoVs), including the severe acute respiratory syndrome CoV (SARS-CoV), the Middle East Respiratory Syndrome CoV (MERS-CoV), and the severe acute respiratory syndrome CoV-2 (SARS-CoV-2), have been responsible for the most notable recent zoonotic epidemics and/or pandemics [3,4].

1.2 Emerging zoonotic coronaviruses

SARS-CoV, MERS-CoV, and SARS-CoV-2 are the most pathogenic human CoVs (hCoVs) identified so far, being characterized for their capacity to induce respiratory illnesses ranging from mild to severe, and even leading to fatal consequences [5]. The four remaining hCoVs, HCoV-229E, HCoV-OC43, HCoV-NL63, and HCoV-HKU1, circulate worldwide and typically result in symptoms resembling common cold [4]. These seven hCoVs are zoonotic viruses, originating from other mammalian hosts, including bats and rodents, where they are usually not harmful. It is believed that these viruses were transmitted to humans through either known or yet unidentified intermediate hosts (**Figure 1.1**) [6,7]. While the low pathogenic hCoVs continue to spread among humans, transmission of the highly pathogenic SARS-CoV

and MERS-CoV has been more restricted, leading experts to consider humans as a dead-end host [7]. SARS-CoV emerged in 2002 in China's Guangdong Province, spreading to different countries and continents and resulting in 8,096 infections with a lethality rate of around 10% before the epidemic was contained [7]. Apparently, SARS-CoV jumped from masked palm civets *(Paguma larvata)* to humans at a wild game food market [8]. On the other hand, the dromedary camel (*Camelus dromedarius*) is the main animal reservoir for MERS-CoV and the primary source of transmission to humans [7]. This particular hCoV was first identified in the Arabian Peninsula in 2012 [9]. Intermittent outbreaks of MERS-CoV still occur in the Middle East due to zoonotic transmission. Currently, there have been 2,609 human infections with 939 associated deaths across 27 countries, resulting in an approximately 36% case-fatality. Saudi Arabia alone has reported 2,200 cases and 858 deaths related to MERS-CoV [10].

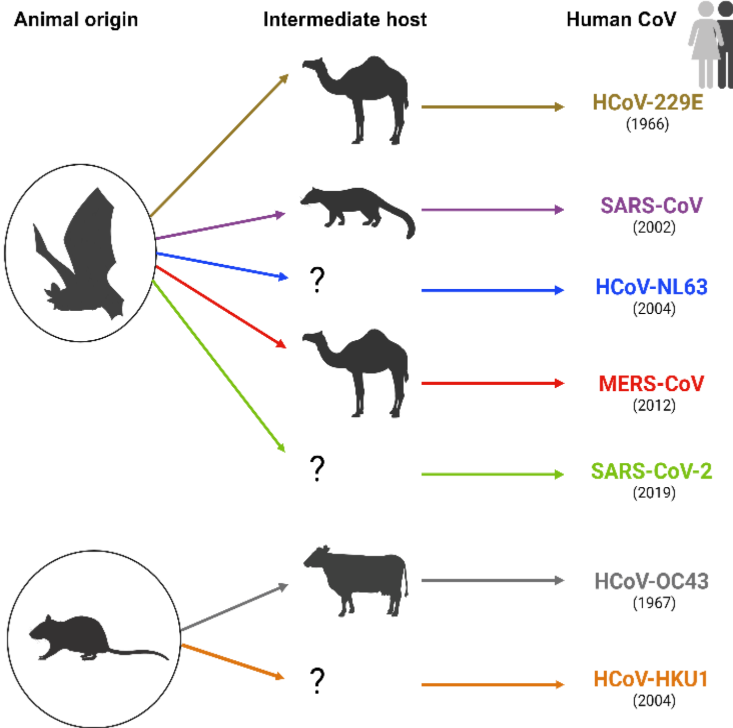

Figure 1.1 Animal origin and intermediate hosts of the seven human coronaviruses (hCoVs). Bats species are the origin of HCoV-229E, SARS-CoV, HCoV-NL63, MERS-CoV, and SARS-CoV-2, while rodent species are the origin of HCoV-OC43 and HCoV-HKU1. Dromedary camels are recognized as intermediate hosts for HCoV-229E and MERS-CoV, and palm civets and bovines are identified as intermediate hosts for SARS-CoV and HCoV-OC43, respectively. The potential intermediate host for HCoV-NL63, SARS-CoV-2, and HCoV-HKU1 is still unknown. Figure created with Biorender.com.

1.3 Severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2)

1.3.1 Emergence of a novel coronavirus in 2019: SARS-CoV-2

In December 2019, an outbreak of severe pneumonia of unknown etiology was reported in Wuhan City, Hubei Province of China [11]. By January 7, 2020, the causative agent isolated from throat swab samples was recognized as a novel CoV, which was initially named 2019-nCoV [12]. Upon genome sequencing, the virus was characterized and named as SARS-CoV-2 and was determined to be the causative agent of the coronavirus disease 2019 (COVID-19) [11,12]. This virus shares 79% of its genome sequence with SARS-CoV and exhibits 50% similarity to MERS-CoV [11].

SARS-CoV-2 rapidly spread globally, leading the World Health Organization (WHO) to declare COVID-19 a pandemic on March 11, 2020 [13]. From then on, the virus triggered a significant public health crisis, placing an immense pressure on healthcare systems and medical resources worldwide. As of March, 31, 2024, SARS-CoV-2 is responsible for 774,889,074 million infections and 7,038,623 million deaths [14]. Additionally, the pandemic resulted in significant economic impacts and disruptions to education due to lockdowns, travel restrictions, and business shutdown. Since virus discovery, to develop an effective vaccine and treatment for COVID-19 was considered of urgency to decrease virus spread and the severity of the disease. Currently, 70.6% of the population has received at least one dose of available COVID-19 vaccines, with 64.9% fully vaccinated [15]. This has led to a significant reduction in transmission, infection, and mortality rates [16], prompting the WHO to declare the end of the pandemic on May 5, 2023 [13]. However, new cases of infections continue to be reported worldwide, indicating ongoing SARS-CoV-2 circulation. Notably, only 32.7% of people

living in low-income countries have received at least one vaccine dose, in contrast to 79.9% in high-income countries [15].

1.3.2 Taxonomy

SARS-CoV-2 is classified within the *Orthocoronavirinae* subfamily of the *Coronaviridae* family, which is part of the *Cornidovirineae* suborder within the *Nidovirales* order [17]. Within the *Orthocoronavirinae* subfamily, there are four genera: *Alphacoronavirus* (*Alpha-CoV*), *Betacoronavirus* (*Beta-CoV)*, *Deltacoronavirus* (*DeltaCoV)*, and *Gammacoronavirus* (*Gamma-CoV*). SARS-CoV-2 specifically belongs to the *Beta-CoV* genus and the *Sarbecovirus* subgenus [17]. The *Alpha*- and *Beta-CoV* genera are known to infect mammals, while the *Gamma*- and *Delta-CoVs* mainly infect birds, cetaceans, and other mammals [18].

1.3.3 Genome organization, replication, and viral gene expression

Similar to other hCoVs, SARS-CoV-2 contains a single-stranded, positive-sense RNA (ssRNA (+)) of ≈29.9 kilobases. The viral genome has a 5' cap and 3' poly trail and comprises 15 open reading frames (ORFs) that encode 29 proteins (**Figure 1.2**). At the 5'-end, there are the ORF1a and ORF1ab, which represent almost two-thirds of the entire genome. ORF1a and ORF1ab encode two polyproteins (pp1a and pp1ab) that generate 16 non-structural proteins (NSPs: nsp1 – 16) [19]. These NSPs have multiple enzymatic functions involving regulation of genome replication and transcription, forming the replication and transcription complex (RTC) [19]. NSPs include the RNA-dependent RNA polymerase (RdRp), also known as nsp12, which serves as the core of the RTC[20]. The remaining one-third of the genome, located downstream, encodes four structural proteins, the spike (S),

nucleocapsid (N), envelope (E) and membrane (M) proteins, and nine accessory proteins (ORF3a, ORF3b, ORF6, ORF7a, ORF7b, ORF8b, ORF9b, ORF9c, and ORF10) [21,22]. These accessory proteins play crucial roles in virus-host cell interactions, immune evasion, and pathogenesis [22].

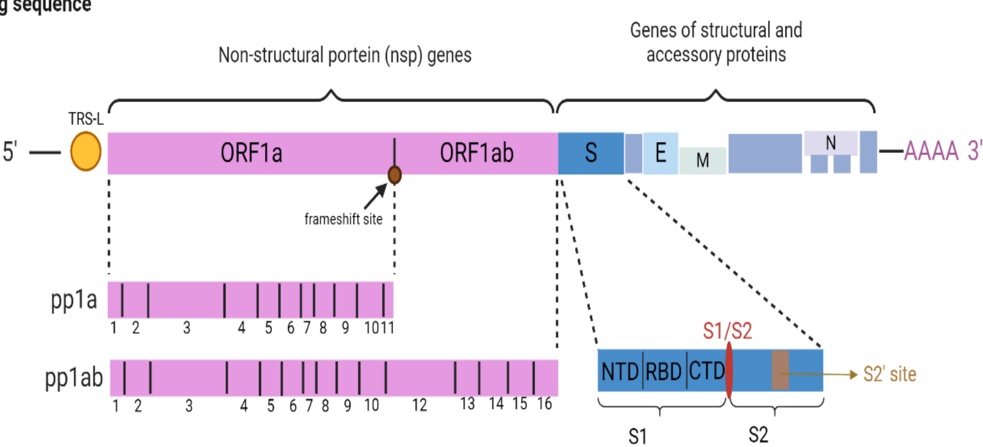

Figure 1.2 Scheme of SARS-CoV-2 RNA genome organization. The genome is constituted by the 5'-cap and 3'-polyA tail and codifies for 16 non-structural protein (nsp) genes, 4 structural proteins (spike [S], membrane [M], envelope [E], and nucleocapsid [N]), and nine accessory protein genes. The 1-16 nsp genes are directly translated from the ORF1a and ORF1ab (both highlighted in pink) resulting in two polyprotein chains: pp1a and pp1ab. Figure created with Biorender.com.

SARS-CoV-2 RNA contains a transcription leader sequence (TRS-L) at the 5'-end, with a transcription-regulating sequence (TRS-B) located upstream of most ORFs in the structural and accessory gene region (**Figure 1.3**). The entire genomic RNA (gRNA) of the virus serves as a template for the synthesis of new copies of gRNA, and for discontinuous transcription in subgenomic RNAs (sgRNA), both via an intermediate antisense RNA (RNA-)[19,21]. TRS-B regions serve as attenuation signals for sgRNA synthesis. During the synthesis of the negative-strand RNA, the RTC encounters TRS-B and restarts transcription at the TRS-L site at the 5'-proximal region,

leading to a base-pairing interaction between TRS-B (sgRNA-) and the TRS-L (coding sequence). Subsequently, the anti-TRS-L is synthetized in the sgRNA- and the whole minus strand serves as a template for transcription of sgRNA [22]. Structural and accessory proteins are translated from 11 viral individual positive sgRNA [19,21].

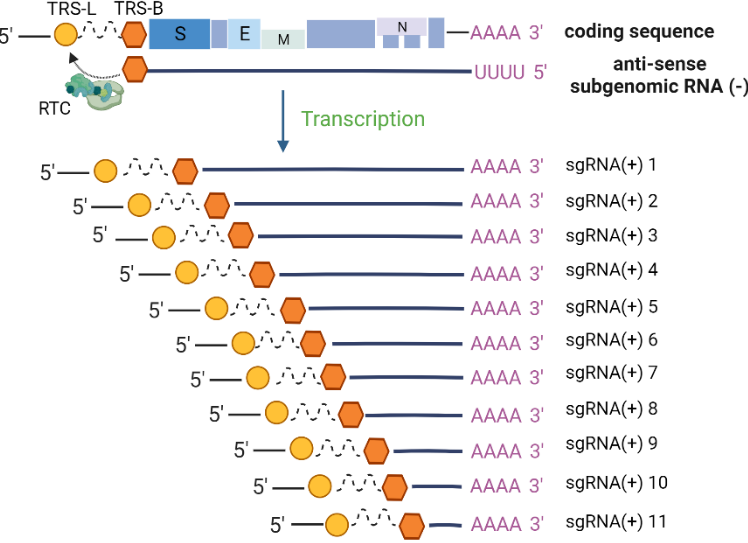

Figure 1.3 Discontinuous transcription of subgenomic RNA (sgRNA) of SARS-CoV-2 carried out by the replication and transcription complex (RTC). Figure created with Biorender.com.

1.3.4 Virion structure

SARS-CoV-2 consists of enveloped viral particles that are spherical in shape with a diameter of 80 nm [23]. Within the viral envelope, M, S, and E structural proteins play a pivotal role in virion formation (**Figure 1.4A**) [21]. The M protein, the most abundant structural protein of SARS-CoV-2, supports the viral envelope and interacts with the remaining structural viral

proteins displaying a central role in virus assembly [21]. The S protein, a transmembrane trimeric glycoprotein, form the viral "crown" and facilitates the virus's entry into host cells. It determines the viral host range, tissue tropism and is the primary target for neutralizing antibodies (nAbs) [24]. The S protein contains two subunits (S1 and S2), which mediate viral attachment to the host cell surface receptors and fusion of viral and host cell membranes to facilitate viral entry (**Figure 1.4B**)[22]. The S1 subunit folds into four domains: the N-terminal domain (NTD), the receptor binding domain (RBD), responsible for interaction with host cell receptors, and two carboxy-terminal domains (CTD1 and CTD2) (**Figure 1.2**)[24]. The E protein, a small integral protein, forms an ion channel in the viral membrane and modulates the virion release [22]. Lately, the N protein binds to the gRNA and leads its packaging into a ribonucleoprotein complex within the assembled virus progeny [19]. This protein is highly immunogenic eliciting the production of antibodies by the infected host [24].

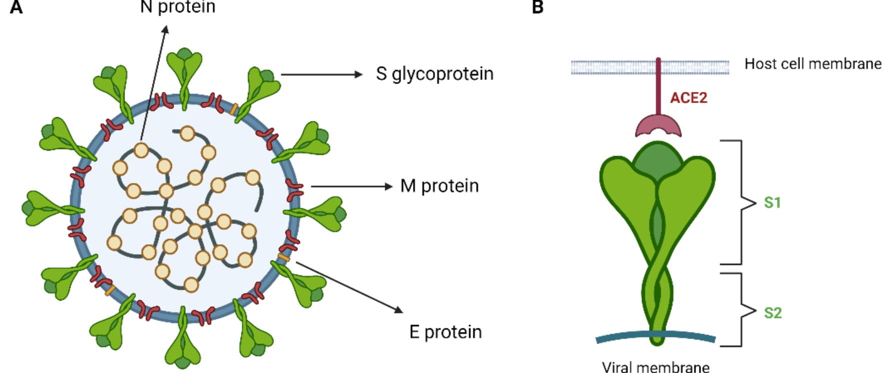

Figure 1.4 A) Virion structure of SARS-CoV-2. There are four structural proteins including the nucleocapsid (N), the spike (S), the membrane (M), and the envelope (E) proteins. **B)** Structure of the spike glycoprotein of SARS-CoV-2. It is composed of two subunits, the subunit 1 (S1), which interacts with the angiotensin converting enzyme 2 (ACE2) host cell receptor, and the subunit 2 (S2) that is responsible for fusion of viral and host cell membranes. Figure created with Biorender.com.

1.3.5 Virus replication cycle

The life cycle of SARS-CoV-2 includes viral entry into host cells, replication and transcription, assembly, and release. The infection begins when SARS-CoV-2 recognizes its host cell receptor, the angiotensin converting enzyme 2 (ACE2), by using the RBD within the S1 region of the S protein [25]. Upon binding to the ACE2, furin-like proteases activate the S protein by the cleavage of the polybasic furin cleavage site (RRAR) at the S1 and S2 boundary[26]. This is an essential step for initiating the fusion of virus and cell membranes [26]. Subsequently, the host cell transmembrane protease serine 2 (TMPRSS2) cleaves a second cleavage site (S2' site), facilitating viral entry [27]. A part from TMPRSS2, the cathepsin L is also a protease involved in the activation of the S protein during endosomal entry [26]. Inside the host cell, the RNA is released, and cell ribosomes start translating the virus genetic code giving place to the pp1a and pp1ab polyproteins, followed by the synthesis of the NSPs [22,28]. NSPs generate double membrane vesicles (DMV) from the endoplasmic reticulum (ER), where new gRNAs are created and sgRNA are processed to produce the structural and accessory proteins that will constitute the new viral particles [22,28]. The M, S and E proteins migrate to the intermediate compartment between the ER and Golgi apparatus (ERGIC) and interact with the N protein that packages the RNA [28]. Subsequently, all proteins migrate to the Golgi apparatus, where new viral particles buds and virions are released from the host cells by exostosis [22].

1.3.6 Virus evolution and genetic variants

Initial SARS-CoV-2 genetic sequences led to define two distinct lineages named as lineages A and B, with the latest emerging as the dominant lineage worldwide [29]. The high transmission capability of the virus triggered genetic changes leading to the emergence of numerous viral variants.

Early in the pandemic (January-February 2020), the B.1 variant carrying the D614G mutation (amino acid Aspartic [D] changed by a Glycine [G] in position 614 of the S2 subunit) emerged [30]. This variant surpassed the wild-type (WT) strain of SARS-CoV-2, which was linked to higher infectivity but not increased disease severity, becoming the predominant variant worldwide [31]. The D614G mutation has been conserved in all major subsequent variants of SARS-CoV-2 that appeared to date. Most of these variants acquired specific mutations in the RBD, and exhibited enhanced affinity for the host cell receptor and increased viral infectivity compared to the ancestral one [30,32].

Those variants that are characterized by its higher transmission capability, virulence, and/or increased immunologic escape capacity are referred as variants of concern (VOC) [33]. Five SARS-CoV-2 VOCs have so far been identified to cause diverse epidemiological waves at various phases of the pandemic: the Alpha (B.1.1.7), identified in United Kingdom (UK) on September 2020, the Beta (B.1.351), first detected in South Africa on September 2020, the Gamma (P.1), first recognized in Brazil on September 2020, the Delta (B.1.617.2), first identified in India on March 2021, and Omicron (B.1.1.529), first reported in South Africa in November 2021 [33]. The Omicron (B.1.1.529) variant is known to be the one from which a larger number of sub-lineages have emerged. The Omicron parent lineages are designated as BA.1 (B.1.1.529.1), BA.2 (B.1.1.529.2), BA.3 (B.1.1.529.3), BA.4 (5.1.1.529.4), and BA.5 (5.1.1.529.5) (https://cov-lineages.org). As of March 2023, BA.2, BA.4, and BA.5 were regarded as de-escalated VOCs [33]. As of May 2024, the circulating variants, no longer considered VOCs but now variants of interest (VOI), belong to Omicron (B.1.1.529) sub-lineages, including BA.2.86, EG.5, JN.1, and XBB[14].

1.3.7 Host cell tropism and disease in humans

Upon SARS-CoV-2 infection, there is an incubation period of 3-10 days, in which an infected individual can transmit the virus to others through direct or indirect contact [34,35]. Although fecal-oral transmission can also happen, airborne droplets and aerosol are the main routes to spread the virus among people [35].

The main determinants of SARS-CoV-2 infection capacity are the presence, tropism, and expression levels of the ACE2 receptor in human cell tissues [27,36]. The ACE2 is a transmembrane protein of type I that plays a role in regulating the renin-angiotensin-aldosterone system, which is responsible for controlling blood pressure, fluid balance, and electrolyte homeostasis [37]. In humans, ACE2 is mainly expressed in the small intestine, heart, kidneys, testis, thyroid, and adipose tissue, with moderate expression levels in the lungs, colon, liver, bladder, and adrenal glands [38,39]. Therefore, although the primarily route of infection for SARS-CoV-2 is through the upper respiratory tract (URT), it can also cause damage to other organs and tissues [40,41].

The specific expression of ACE2 expression in different cell tissues can part elucidate the varying susceptibility to SARS-CoV-2 infection and the clinical manifestations of COVID-19 [38,42]. SARS-CoV-2 can lead to either asymptomatic (40-45%) or symptomatic infections; most common symptoms comprise headache, fever, dry cough, dyspnea, diarrhea, and vomiting [35,39]. While majority of patients experience mild COVID-19 symptoms, some individuals may develop severe to fatal complications [42], particularly affecting the lower respiratory tract (LRT). Such outcome includes severe pneumonia and acute respiratory distress syndrome (ARDS), potentially leading to organ failure, septic shock, and even death [40,42]. The progression to severe COVID-19 is also associated to a phenomenon known as "cytokine

storm", which is a consequence of an hyperinflammatory response, where immune cells secrete high levels of pro-inflammatory cytokines after infection [35,43]. The fatality rate for SARS-CoV-2 infection is 2.3% [35].

Numerous risk factors have been associated with a higher impact on the severity and increased mortality of COVID-19. These include older age (≥50), male gender, the presence of comorbidities (*e.g.,* diabetes, obesity, hypertension, cardiovascular diseases, and chronic kidney disease), and weakened immune defenses of individuals (*e.g.,* cancer or HIV patients) [42,43]. The probabilities of infection and disease progression are also influenced by demographic, socioeconomic factors, and ethnicity [42].

Differences in disease severity are also influenced by the specific variant causing the infection. For instance, the Delta (B.1.617.2) variant has been associated to a higher risk of severe disease and hospitalization compared to earlier variants [44,45]. On the other hand, the Omicron (B.1.1.529) variant and its sub-variants have evidenced to cause less severe disease than other previous variants, despite the significant number of mutations accumulated in the S protein that provide the virus with a notable immune evasion capability [46]. This latter fact has raised concerns due to the reduced effectiveness of previous acquired immune responses generated by previous infection or vaccination [47].

1.4 SARS-CoV-2 infection in animals

1.4.1 SARS-CoV-2 origin

Since the beginning of the COVID-19 pandemic, uncovering the origin of SARS-CoV-2 has been the target of numerous scientific investigations and has sparked debates among scientific and non-scientific

communities [48]. It has been proposed a laboratory origin of SARS-CoV-2, suggesting that the virus was constructed *de novo* or was cultured and adapted from bat-origin CoV in a laboratory [48]. However, based on scientific data and the lack of convincing evidence, this hypothesis might be unlikely [29,48]. Conversely, considering that most of initial cases of SARS-CoV-2 infections were linked to the Huanan seafood Wholesale Market in Wuhan, China, where a high-density population had contact with live animals, the hypothesis of an animal origin for SARS-CoV-2 has gained significant support [11,12,29]. Importantly, a range of mammal species susceptible to SARS-CoV-2 were traded at the Wuhan's Market towards the end of 2019, supporting the idea of a spillover event where the virus jumped from wild animals to humans [49].

Given the past outbreaks of SARS-CoV and MERS-CoV originating from bats (**Figure 1.1**), bats have been highlighted as a potential source of future zoonoses. This concern was further reinforced by the emergence of SARS-CoV-2, as the major whole genome sequence identity has been found in CoVs from Asian horseshoe (*Rhinolophus*) bats, specifically RaTG13 (96.1%) and BANAL-52 (96.8%) [11,50]. Bats are known to harbor a wide variety of mammalian CoVs (*Alpha*- and *Beta-CoVs*) [51], suggesting the possible circulation of the precursor lineage of SARS-CoV-2 in this species [48]. However, the genetic difference (≈ 4%) between identified bat CoVs and SARS-CoV-2, along with their limited ability to bind the human ACE2 (hACE2) receptor, raises suspicions of an intermediate host between bats and humans [52,53].

SARS-CoV-2 related CoVs have been identified in pangolins from Guangdong (China) (PDCoV-GD), showing a higher amino acid sequence identity in the RBD (91.2%) of SARS-CoV-2, compared to the identified bat-CoVs [29,52]. Despite this, they share an overall sequence similarity of 91.2% with SARS-CoV-2. Pangolin-CoVs show a stronger binding to hACE2 than

SARS-CoV-2 and have the capacity to infect human cell cultures, supporting a considerable potential for human adaptation [29,52]. One hypothesis suggests a possible recombinant event between bat-CoV and pangolin-CoV as the origin of SARS-CoV-2 [29,52,54,55].

However, considering that various animal species have demonstrated to be susceptible to SARS-CoV-2 infection both experimentally and naturally, the potential for an alternative intermediate host cannot be ruled out [56].

1.4.2 Experimental infections by SARS-CoV-2 in animal species

The potential zoonotic nature of SARS-CoV-2 together with the widespread and sustainment of SARS-CoV-2 among human population have raised the need to study the susceptibility of several animal species to prevent cross-species transmission events and the establishment of new animal reservoirs. Additionally, the identification of susceptible species contributes to find new animal models for SARS-CoV-2 studies [57].

Different factors can determine the susceptibility of animal species to SARS-CoV-2. These factors include: (i) the presence, expression, and tropism of the ACE2 receptor in cell tissues, (ii) the binding affinity between RBD and ACE2 receptor, (iii) the intracellular environment of hosts, and (iv) the presence of effective mechanisms of immune system, among others [58–60]. Additionally, the presence, expression levels, and tropism of TMPRSS2 may also impact susceptibility to viral infection, serving as a crucial factor for viral entry.

The ACE2 gene is present in vertebrates and exhibits a highly conserved sequence among mammals [37,61]. Twenty-five amino acids within

the ACE2 protein were identified as critical determinants for SARS-CoV-2 binding, with six of these residues (Ser19, Lys26, Thr27, Asp30, Leu79, and Met82) being strongly associated to viral host susceptibility [37,61]. Accordingly, through comparative genomic, evolutionary, and structural analyses of ACE2 and its critical residues across humans and non-human vertebrates, a broad host range for SARS-CoV-2 was predicted in the early stages of the pandemic [37,59,61]. Certain species such as non-human primates (NHP), cervids, and cetaceans were categorized as being at high to very high risk of infection, while common livestock species and felids were considered to have a moderate risk of infection [37,59]. Other vertebrate species including fish, birds, or reptiles were grouped in the very low risk of infection category [37,59]. Comparative genomic analyses of TMPRSS2, along with its expression levels and cell-tissue tropism, yielded similar results to as those observed for ACE2 [58,61].

Although *in silico* studies supplies important insights of the potential risk of infection of animal species, confirmation is warranted applying *in vitro* (*e.g.,* cell cultures and organoid models) and *in vivo* studies [62]. Consistently, many domestic and wildlife animal species already demonstrated its susceptibility upon experimental infections with more or less extend (**Figure 1.5**). Among highly susceptible animal species, there are both domesticated animals and wildlife.

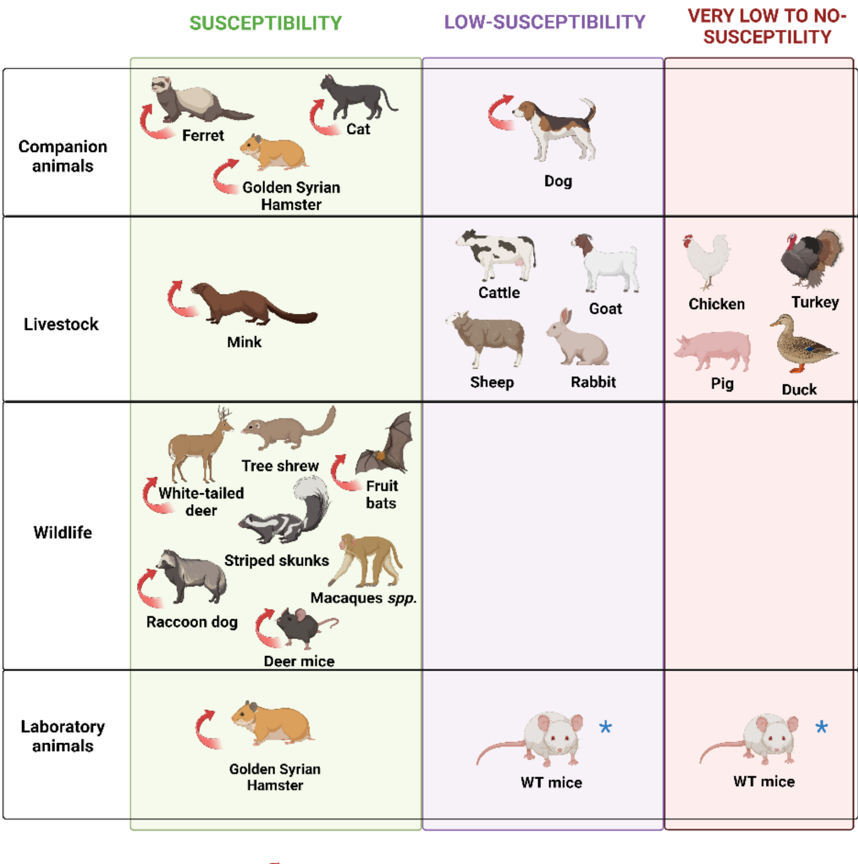

Figure 1.5 Susceptibility degree of companion animals, livestock, wildlife, and laboratory animals to SARS-CoV-2 under experimental conditions. Susceptible animals (left column), low-susceptible animals (middle column), and very-low or no susceptible animals (right column) are shown separately. *Wild-type (WT) mice, which is represented twice, is not susceptible to the ancestral variant but to the Alpha, Beta, Gamma, and Omicron. The red arrows indicate the animal species with the ability to transmit SARS-CoV-2 to co-housed animals. Figure created with Biorender.com and retrieved from Fernández-Bastit et al., 2023 with some modifications.

1.4.2.1 Companion animals

1.4.2.1.1 Domestic cat

Domestic cats (*Felis catus*) are highly susceptible to SARS-CoV-2 infection (10^5 – $7x10^5$ pfu) through various routes of administration (nasal, oral, tracheal, and ocular) (**Figure 1.5**) [63]. Cats typically exhibit high viral loads in nasal and oral swabs (up to 10^6 pfu/mL), with slightly lower loads in rectal swabs. The virus mainly replicates in the respiratory tract and intestine, resulting in shedding of infectious virus in the URT [64–66]. Consequently, these animal hosts can transmit the virus via direct or indirect contact [64–67], albeit with limited sustained cat-to-cat transmission [68]. This limitation is attributed to reduced SARS-CoV-2 transmissibility and virulence observed after the virus undergoes serial passaging between cats [68]. Additionally, domestic cats develops an effective humoral immune response that can provide protection from reinfection, although it may not offer complete sterilizing immune protection [64,65,69].

Cats usually remain asymptomatic or exhibit mild clinical signs, with some cases showing mild to moderate histopathological lesions in the URT and LRT. However, experimental infection in juvenile cats (1-3 months old) resulted in severe clinical disease and histopathological changes in the URT, LRT, and small intestine [63,66]. Additionally, intra-tracheal (IT) SARS-CoV-2 inoculation caused severe clinical and histopathological disease, mainly with the Delta (B.1.617.2) variant and to a lesser extent with the ancestral (B.1) variant, consistent with observations in acute and severe cases of COVID-19 in humans [70,71]. The Omicron BA.1.1 variant has shown lower virulence in cats compared to the D614G and Delta (B.1.617.2) variants [72].

1.4.2.1.2 Domestic ferrets

The domestic ferret (*Mustela putorius furus*) is also considered a highly susceptible host to SARS-CoV-2 (**Figure 1.5**). This animal can be infected with SARS-CoV-2 through IN inoculation (10^5 – 6x10^5 tissue culture infectious dose 50 ($TCID_{50}$)), and can efficiently transmit the virus through direct or indirect contact [66,73,74]. After challenge, ferrets can shed the virus in nasal secretions, saliva, urine, and feces [73]. Efficient viral replication is restricted to the URT without causing severe disease, and ferrets develop a specific antibody response to SARS-CoV-2 [66,73]. This species usually exhibit asymptomatic infection or mild clinical signs including fever, acute bronchiolitis, and loss of appetite [73]. It is worth noting that the Delta (B.1.617.2) variant demonstrated higher transmission and replication capabilities than the Alpha (B.1.1.7) variant in ferrets, with the latest showing a clear fitness advantage over the D614G variant [75,76]. This findings align with observations in human populations [75]. In contrast, the Omicron (BA.1) variant did not establish a productive infection when IN inoculated (10^5 $TCID_{50}$); the animals remained clinically healthy after infection, did not shed virus, and did not seroconvert at 21 days post inoculation (dpi) [75,77].

1.4.2.1.3 Golden Syrian hamsters

Golden Syrian hamsters (*Mesocricetus auratus*) are highly susceptible to SARS-CoV-2 infection and can develop moderate- to severe COVID-19 (**Figure 1.5**) [78,79]. IN inoculation with SARS-CoV-2 (10^4 – $10^{5.8}$ $TCID_{50}$/animal) or its viral variants in hamsters leads to progressive weight loss, lethargy, and clinical signs resembling URT and LRT infections observed in humans [78–80]. Consistently, SARS-CoV-2-inoculated hamsters exhibit high viral loads (10^5 – 10^7 $TCID_{50}$/g) in URT and LRT, as well as relatively high viral loads in intestine samples [79,81]. After a primary SARS-

CoV-2 infection in hamsters, humoral immunity provides protection against lung disease in a secondary infection, although it may not confer a sterilizing immunity [78]. The Omicron variant can also infect hamsters experimentally, albeit with lower virulence compared to earlier variants [80,82].

1.4.2.1.4 Dog

Dogs *(Canis lupus familiaris)* experimentally infected via IN inoculation (10^5 pfu/animal) with the ancestral variant of SARS-CoV-2 manifested no clinical signs, limited viral replication, and low levels of nAbs, demonstrating a low susceptibility to SARS-CoV-2 (**Figure 1.5**) [64,66]. In a recent study, dogs inoculated with the Delta (B.1.617.2) or Omicron (BA.1) variants demonstrated the ability to transmit the virus to co-housed animals, unlike previous infections with ancestral variant (**Figure 1.5**) [83]. Also, the authors described the development of severe pneumonia upon infection with the Omicron (BA.1) variant. This study used a higher viral dose (10^6 $TCID_{50}$/animal) for inoculation compared to previous ones [83].

1.4.2.2 Livestock animals

1.4.2.2.1 Mink

Minks (*Neovison vison*) have demonstrated high susceptibility upon IN and IT inoculation (10^5 – 10^6 $TCID_{50}$) with SARS-CoV-2 [84,85]. Ancestral (B.1 lineage) and Alpha (B.1.1.7) variants caused severe respiratory disease evidenced by clinical manifestations, radiographic findings, and histopathology (**Figure 1.5**) [84–86]. After inoculation, the animals displayed significant weight loss, dull mention, shivering, lethargy, increased respiratory efforts, and nasal discharge. High viral loads and infectious virus were detected in all inoculated animals mainly in the URT and LRT, and several of them developed lung lesions that resembled severe COVID-19 in

humans [84]. Similarly, in minks, the Omicron variant ($4x10^4$ pfu/animal) also led to clinical disease, and nasal and pulmonary pathology [86]. Minks are able to transmit the virus to naïve animals, as reported by some groups [85,86].

1.4.2.2.2 Cattle, sheep and goats

Cattle (*Bos Taurus*), goat (*Capra aegagrus hircus*), and sheep (*Ovis aries*) demonstrated to be minimally permissive to SARS-CoV-2 infection and its variants (**Figure 1.5**) [87–89]. Further details about their susceptibility, especially regarding goats, are described and discussed in *Chapter 6* of this *PhD Thesis*.

1.4.2.2.3 Other livestock species

Rabbits (*Oryctolagus cuniculus*) IN inoculated with SARS-CoV-2 at dosages of 10^5 $TCID_{50}$ or 10^6 $TCID_{50}$, but not lower doses, demonstrated productive infection [90]. However, due to the asymptomatic nature of the infections, viral RNA was only occasionally detected in fecal samples, and throat samples, but not in lung tissue samples (**Figure 1.5**) [87,90].

Multiple studies investigated the susceptibility of pigs *(Sus scrofa domesticus*) to SARS-CoV-2 infection, and demonstrated non-susceptibility upon IN, IT, intramuscular (IM), or intravenous (IV) inoculation ($10^5 – 10^6$ $TCID_{50}$ /animal) with the ancestral variant (**Figure 1.5**) [66,91,92]. Remarkably, *Pickering et al.,* described any evidence of infection in pigs inoculated IN with 10^6 $TCID_{50}$ [93]. According to Vergara-Alert and collaborators, pigs are a useful animal model for immunogenicity studies, since they seroconvert and produce nAbs upon IM and IV inoculation [92].

Some poultry species (chicken [*Gallus gallus domesticus*], ducks [*Anas platyrhinchos domesticus*] and turkeys [*Meleagris gallopavo*]) also

shown to be non-susceptible to SARS-CoV-2 (**Figure 1.5**) in agreement with previous predictive *in silico* studies [37,66,91,94].

1.4.2.3 Wildlife animals

1.4.2.3.1 White-tailed deer (WTD)

The white-tailed deer (WTD; *Odocoileus virgnianus*) has been shown highly susceptibility to SARS-CoV-2 following IN inoculation with the ancestral virus and its variants (10^5 – $10^{6.3}$ $TCID_{50}$/ml) [95–97]. Challenged WTD develop a subclinical infection but still carry the virus, shedding it to large amounts mainly through oral and nasal secretions; the virus is also present in their lymphoid tissues [96,97]. The virus shows a broad tropism in these animals, affecting their URT, LRT, lymphoid tissues, and central nervous system (CNS) [95–97]. Additionally, SARS-CoV-2 is efficiently transmitted from infected to non-infected animals through both direct and indirect contact. There is also evidence of vertical transmission from doe to fetus [95–97]. WTD also develop strong neutralizing immune response against the virus [95–97].

1.4.2.3.2 Non-human primates (NHP)

Different species of NHP including rhesus macaques (*Macaca mulatta*), cynomolgus macaques (*Macaca fascicularis*), and African green monkeys (*Chlorocebus aethiops*), have demonstrated to be susceptible to SARS-CoV-2, resulting in mild to moderate disease (**Figure 1.5**) [98–101]. Rhesus macaques, when inoculated via IN route ($\approx 1 \times 10^4 – 1 \times 10^5$ $TCID_{50}$) developed respiratory disease, including mild to moderate interstitial pneumonia, needing approximately two weeks to fully recover [98–100]. These animals exhibited viral dissemination in the respiratory and gastrointestinal

tracts with high viral loads, triggering a robust immune response, which protected against subsequent infections [98–100].

1.4.2.3.3 Other wildlife species

Fruit bats (*Rousettus aegyptiacus*), raccoon dogs (*Nyctereutes procyonoides*), deer mice (*Peromyscus maniculatus*), striped skunks (*Mephitis mephitis*), and tree shrew (*Tupaia belangeris*) have all shown to be susceptible to SARS-CoV-2 upon experimental infections. Some of these animals were even able to transmit the virus to sentinel contact animals [102–105].

1.4.2.4 Laboratory animals

1.4.2.4.1 WT mice

WT mice are resistant to SARS-CoV-2 ancestral variant (including D614G), but they are susceptible to different variants that emerged subsequently, including Alpha (B.1.1.7), Beta (B.1.351), Gamma (P.1), and Omicron (BA.1.1) (**Figure 1.5**) [106]. These variants cause subclinical infection in WT mice when they are exposed to a moderate virus dose (IN inoculation; $\approx 10^3$ $TCID_{50}$/animal). The virus can be detected in the URT (nasal turbinate) and LRT (lungs) for 4 to 7 dpi [106,107]. However, there is no evidence of viral transmission between mice inoculated with the Alpha (B.1.1.7) variant [107]. Alpha (B.1.1.7), Beta (B.1.351), Gamma (P.1), and Omicron (BA.1.1) variants contain an specific mutation in the S protein (N501Y), which has been linked to an increased affinity to the murine ACE2 receptor, thus expanding the host range of the virus to murine species [107]. In contrast, previous variants such as Delta (B.1.617.2) and the ancestral SARS-CoV-2 variants lack the N501Y mutation, potentially explaining the non-productive infection observed in laboratory WT mice infected by these variants [106,107].

1.4.3 Animal models for SARS-CoV-2 research

Based on the varying of animal species demonstrated through experiments, a diverse range of animal species have been used extensively as models in SARS-CoV-2 research [62,108]. Across them, the spectrum of disease outcomes ranges from mild to severe, with some species capable of transmitting the virus and developing robust immune responses with the potential to confer protection against reinfection [62,108]. These results underscore the necessity to carefully select animal models based on specific research objectives including vaccine and therapy development, viral transmission, and disease progression [109,110]. Understanding the strengths and limitations of each animal model is crucial for expanding our knowledge on SARS-CoV-2 and COVID-19 [109,110]. The most commonly used animal models in SARS-CoV-2 research include golden Syrian hamsters, ferrets, NHP, and mice [62,108]. The advantages, disadvantages, and suitability of these animal models for SARS-CoV-2 studies are outlined in the **Table 1.1**.

The golden Syrian hamster is one of the models which better recapitulates COVID-19 in human, showing a progressive weight loss of 5% to 20%, as well as clinical signs, such as anosmia, limited neurotropism, and severe interstitial pneumonia [79,108,111]. Additionally, this model is utilized to study transmission dynamics, immune responses, and potential treatments and vaccines against SARS-CoV-2 [79,108,110,111]. Lastly, studies with hamsters helped in understanding the sex-related differences in SARS-CoV-2 infection. Similar to the human population, male hamsters tend to exhibit more severe lung disease and weaker antibody responses compared to females. Additionally, older hamsters often experience a more severe progression of the disease than younger ones [112].

The ferret model, while not fully recapitulates the severe COVID-19 outcomes observed in humans, serves as a valuable model to study asymptomatic and mild SARS-CoV-2 infection. Moreover, it is useful to study age-related differences in SARS-CoV-2 infection dynamics and viral replication [113]. Older ferrets tend to be more susceptible to infection compared to younger ones, displaying higher viral shedding and replication in the URT[113]. Because ferrets prone to SARS-CoV-2 infection, they facilitate assessment of viral transmission dynamics and vaccine efficacy in preventing infection and reducing viral shedding [73,77,113]. In addition, this animal model allows performing comparative studies on the pathogenesis and transmissibility of the different SARS-CoV-2 variants [77].

NHP share genetic, immunological, and physiological similarities with humans, making them valuable models for studying human diseases [62]. Rhesus macaques and cynomolgus macaques have served as pivotal models for testing the efficacy of COVID-19 vaccines and treatments currently available in the market [109,114]. Studies conducted in NHP have enabled the assessment of the immune response to different SARS-CoV-2 variants and the protection against reinfection by homologous and heterologous variants from the first infection [114]. This knowledge is crucial for evaluating immunization strategies and guiding the development of vaccines and therapies against novel emerging variants [114].

Considering that mice were initially resistant to SARS-CoV-2 infection, two main strategies were developed for the use of mice as a model organism: (i) mice-adapted-SARS-CoV-2 strains and (ii) transgenic, knock-in or viral-vector transduced mice expressing the hACE2 receptor [110]. Among these models, one of the most employed so far is the K18-hACE2 mice (**Table 1.1**). In this animal model, the human epithelial-cell keratin 18 (Krt18) promoter regulates their expression of the hACE2 receptor [108,115].

Interestingly, there is a direct correlation between the infectious dose and the severity of disease progression in K18-hACE2. Specifically, lower-doses of SARS-CoV-2 infection (2 x 10^1 – 2 x 10^2 pfu) resemble some aspects of the non-severe COVID-19, whereas higher-doses of infection (2 x 10^3 – 2 x 10^4 pfu) result in mimicking certain outcomes of the severe COVID-19 [115,116]. Following the administration of high doses of SARS-CoV-2, K18-hACE2 mice develop lethal encephalitis and neurological infections, as well as damage in multiple tissues, which are partially comparable to post-mortem samples from COVID-19 patients [115,116]. Notably, K18-hACE2 mice inoculated with lower viral doses were protected against reinfection with high viral doses (2 x 10^4 pfu), demonstrating a robust humoral response [115]. Additionally, this animal model is useful for comparing the pathogenicity caused by the different variants of SARS-CoV-2 [106]. These findings confirm that K18-hACE2 mice are a valuable animal model for pathogenicity studies as well as for the development of antivirals and vaccines against SARS-CoV-2 infection [108,109].

Other non-traditional animal models have also been considered for SARS-CoV-2 studies, such as cats and minks [117]. Depending on the route of viral administration, cats exhibit different disease progression [70,117]. IN inoculation tends to generate asymptomatic to mild respiratory disease, whereas IT inoculation represents a model for more severe disease, similar to the clinical profile observed in hospitalized COVID-19 patients [70,117]. Besides, minks offer potential as an animal model for investigating severe and fatal SARS-CoV-2 infections. The finding of an animal model recapitulating completely the severe COVID-19 form of humans is one of the primary unmet needs in the animal model development [84,117]. Important clinical manifestations in minks include weight loss and respiratory disease

with severe pneumonia [84]. Nonetheless, determining the optimal dose and administration route for this animal model remains to be establish [84]

Table 1.1 List of the main animal models for SARS-CoV-2 research.

Animal model	SARS-CoV-2 studies	Advantages	Disadvantages	References
Golden Syrian hamsters (*Mesocricetus auratus*)	Transmission, disease pathogenesis, therapeutics and vaccine development.	• Low cost and easy to handle • Develop mild to moderate respiratory disease • Viral shedding and transmission	• They not fully recapitulate human disease severity	[79,111]
Domestic ferrets (*Mustela putorius furus*)	Transmission, therapeutics and vaccine development.	• Anatomy of URT and LRT very similar to that of humans • Develop mild clinical signs similar to humans • Viral shedding and transmission	• Lack of replicative virus in LRT • They not recapitulate human disease severity • Higher cost and handling compared to rodent species	[73,77,113]
NHP (*Macaca mulatta; Macaca fascicularis*)	Disease pathogenesis and immunity, therapeutics and vaccine development.	• Close genetic relationship to humans • Close physiological and immunological similarity to humans • Allow for detailed study of immune response and pathology	• High cost • Long gestation and maturation periods • Ethical concerns • Small population size	[98,99,118]
hACE2 mice (K18-hACE2)	Disease pathogenesis, therapeutics and vaccine development.	• Low cost and cost-effective reproduction. Easy to handle	• Do not naturally replicate human disease	[106,115,116]

1.4.4 Natural infections by SARS-CoV-2 in animal species

As of May, 2024, there is evidence indicating that 29 different animal species across 36 countries in the Americas, Africa, Asia, and Europe have been naturally infected by SARS-CoV-2 [119]. This resulted in 776 reported SARS-CoV-2 outbreaks, where either SARS-CoV-2 RNA (indicating acute infection) and/or antibodies (suggesting viral exposure) have been detected in an epidemiological unit (*e.g.,* farm or household) (htpps://vis.csh.ac.at/sars-ani/). These cases include both domestic and wildlife species (in captivity and free range) [120]. Notably, most of the natural infections in animals have been associated with direct or indirect contact with SARS-CoV-2 infected humans, indicating a potential reverse zoonotic (RZ) transmission (human to animal transmission) (**Figure 1.6**) [121].

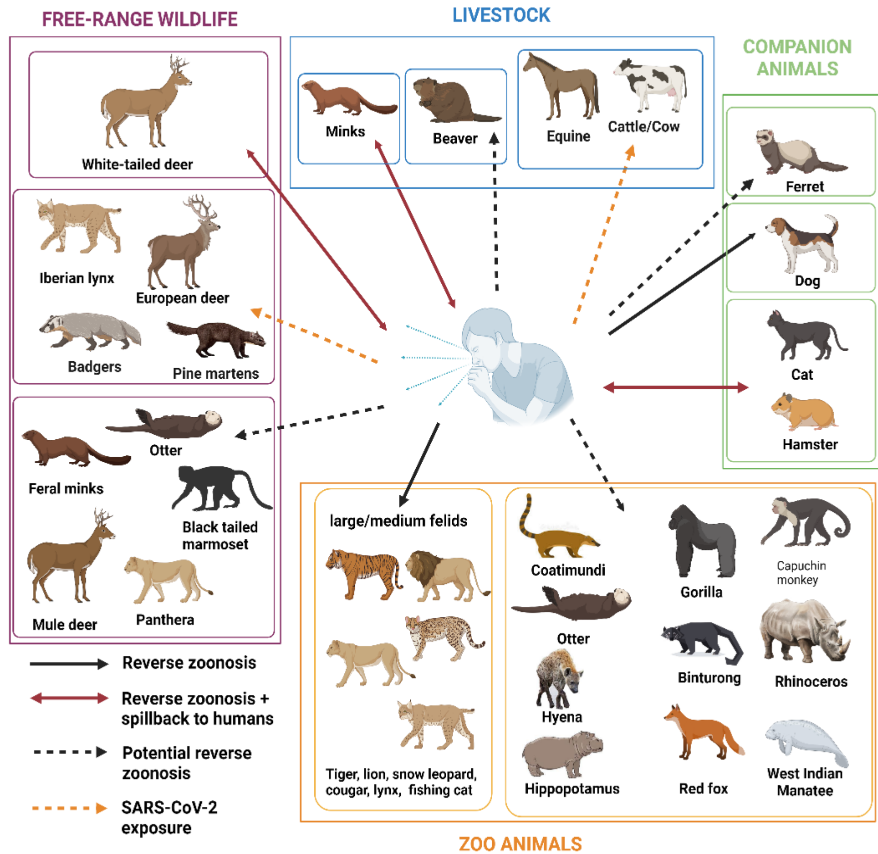

Figure 1.6 Natural infection and/or exposure to SARS-CoV-2 in free-range wildlife (purple square), livestock (blue square), companion animals (green square), and zoo (orange square) animals, which was associated with SARS-CoV-2 infected humans. Different arrows represent the route of transmission between animals and humans: black and red solid arrows indicate human-to-animal and animal-to human transmission, respectively, evidenced by sequencing analysis; black dashed arrows indicate those cases in which human-to animal transmission was not evidenced by sequencing analysis but suggested by epidemiological data; orange dashed arrows indicate the viral exposure of animal species probably by contact with SARS-CoV-2 infected humans. Figure created with Biorender.com and retrieved from Fernández-Bastit et al., 2023 with some modifications.

1.4.4.1 Companion animals

The first case of SARS-CoV-2 animal infection was described in Hong Kong on February 2020 in an asymptomatic dog with underlying health conditions [122]. The animal tested positive for the virus in nasal and oral swabs by reverse-transcription quantitative PCR (RT-qPCR) two days after its owner was diagnosed of COVID-19 [122]. A RZ transmission was strongly suspected since viral genetic sequences from both the animal and human were identical [122]. Subsequently, on March 2020, a cat living with an owner diagnosed with COVID-19 also tested positive for SARS-CoV-2 in nasopharyngeal swab samples and vomit or feces over a period of fifteen days [123]. This cat exhibited severe respiratory, gastrointestinal, and general clinical signs (*e.g.,* pronounced lethargy and loss of appetite leading to anorexia) apparently related to SARS-CoV-2 infection [123]. Thereafter, cases of SARS-CoV-2 and its variants infecting companion animals, mainly cats and dogs but also ferrets and hamsters, have been consistently reported worldwide, often linked to human-animal interactions (**Figure 1.6**) [124,125,125–130]. While most cats and dogs show no clinical signs following natural SARS-CoV-2 infections, cases in which clinical signs have been observed, typically involve the respiratory tract, including coughing, sneezing, respiratory distress, and congestion [63,131]. Digestive signs such as diarrhea and vomiting, as well as non-specific clinical signs such as lethargy or lack of appetite can also occur in infected animals [63,131–133].

Large-scale studies performed in different countries (*e.g.,* USA, China, Italy, Switzerland, Spain, and France) demonstrated a higher risk of infection in pets living with owners affected by COVID-19 compared to those where contact with an infected individual was not determined [134–140]. Additionally, multiple studies have demonstrated a positive correlation between the number of COVID-19 patients in a household and viral loads

after infection with an increased likelihood of transmission to companion animals [136,141]. Besides, although stray and shelter cats have limited human contact, they have also been exposed to and/or infected by SARS-CoV-2 in different countries [142–144].

Instances of pet-to-human transmission have been sporadically documented: once in hamsters at a pet shop in Hong Kong, resulting in subsequent human-to-human transmission [145], and once in cats transmitting SARS-CoV-2 to its veterinarian in Thailand (**Figure 1.6**) [146].

Further details on exposure and infection by SARS-CoV-2 and its variants in companion animals and stray cats are described in *Chapter 3* of this *PhD Thesis*.

1.4.4.2 Livestock

1.4.4.2.1 Farm minks (Neovison vison)

In April 2020, the Netherlands reported increased mortality in two mink farms, in which animals developed severe interstitial pneumonia caused by the SARS-CoV-2 [147]. Until November 2020, SARS-CoV-2 was spread and detected in 68 out of 126 mink farms from the whole country [148]. Additionally, SARS-CoV-2 infection was reported in hundreds of Danish mink farms in June 2020 [149]. The introduction of different viral strains in minks was confirmed in both countries by genetic analysis of viral sequences from the animals and from associated SARS-CoV-2 human cases along with epidemiological data, with humans being the primary source (**Figure 1.6**) [149–151]. Animal-to-animal transmission within farms was confirmed, facilitating viral host adaptation and the emergence of new SARS-CoV-2 strains [148,151,152]. In November 2020, a mink-derived lineage of SARS-CoV-2 recognized as C*luster 5 variant*, was identified in humans in Denmark. This variant resulted

in around 4,000 cases in humans through further human-to-human transmission [152]. As a result, the Danish government ordered the culling of millions of minks by mid-June 2020 [152], leading to the disappearance of the *Cluster 5* after 2020 [152]. Mink-to-human transmission of different SARS-CoV-2 variants was also described on multiple occasions in the Netherlands [148,151]. Many other countries including the United States, Canada, France, Greece, Italy, Spain, Sweden, Poland, and Lithuania also reported SARS-CoV-2 outbreaks in mink farms, which were associated with RZ transmission and subsequent zoonotic transmission [153–157].

Specific and recurrent mutations observed in naturally infected minks have been linked with fitness advantages of SARS-CoV-2 in mustelids (mainly minks and ferrets). These mutations include N501T, F486L, Y453F, L452M, L219V, and G37E [148,154,157]. Mutations N501T an Y453F have also been detected in ferrets experimentally inoculated with SARS-CoV-2 [158]. N501T, F486L, Y453F mutations are placed in the RBD of SARS-CoV-2 and are known to improve the affinity of the virus for the ACE2 receptor in mustelids [148,159]. Especially, the Y453F mutation was present within the *Cluster 5 variant*, and has been related with mink-to-human transmission in different investigations [158]. Additionally, the Y543F mutation exhibited the ability to reduce viral neutralization in blood samples from convalescent human patients, raising concern about the efficacy of both existing vaccines and the acquired humoral response from previous infections [160]. However, this mutation has demonstrated to attenuate the viral replication in human airway epithelium, which could partially explain why *Cluster 5* did not propagate further after the culling of minks [158,159].

1.4.4.2.2 Other livestock species

There have been no documented cases of natural acute infection with SARS-CoV-2 common livestock species such as cattle, goat, sheep, pigs, and horses. However, serological analyses have occasionally revealed the presence of nAbs against SARS-CoV-2 in bovine [161,162] and in one equine [163], suggesting exposure in these animal species after contact with COVID-19 positive humans (**Figure 1.6**).

1.4.4.3 Wildlife

1.4.4.3.1 Wild captive animals

Various large felid species, including lions (*Panthera leo*) and tigers (*Panthera tigris*) but also pumas (*Puma concolor*), snow leopards (*Panthera pardus),* and lynxes (*Lynx spp.*), have been naturally infected by SARS-CoV-2 and its variants in zoological parks across different countries during the pandemic (**Figure 1.6**) [164–170]. Infections in these felids have generally resulted in mild-to-moderate respiratory clinical signs, accompanied by signs such as loss of appetite, anorexia, vomiting and diarrhea, and occasionally leading to fatalities [165,167,170,171], in contrast to the predominantly subclinical infections reported in domestic cats [131]. The Delta (B.1.617.2) variant has been notably linked to increased mortality rates among large felids [172]. In this context, RZ transmission has played a key role due to the close interaction between zookeepers and the animals [165–168,170]. Also, alongside significant viral shedding in the URT of large felids, there has been evidence of prolonged fecal shedding containing infectious virus, thereby underlining the risk of transmission between animals and from animals to keepers [171,173]. Other documented infections within zoos include NHP [174,175], numerous non-

felid carnivore species, and various terrestrial and semi-aquatic mammals (**Figure 1.6**) [176–178].

1.4.4.3.2 Free-ranging wildlife animals

1.4.4.3.2.1 WTD

The most concerning SARS-CoV-2 spillover event from humans to wildlife involves free-ranging WTD (**Figure 1.6**) [121]. Episodes of SARS-CoV-2 exposure and/or acute infection in these animal hosts have been described in multiple locations in the United States (*e.g.,* Illinois, Michigan, New Work, Pennsylvania, Texas, Ohio, and Iowa) and Canada, where they are considered urban and peri-urban animals [179–181]. In agreement with experimental and computational studies, natural infection in WTD has resulted in subclinical infections with high viral loads and the presence of infectious virus primarily in nasal swabs, providing evidence of viral shedding and high natural susceptibility [180,182]. Sequencing analysis has confirmed separate events of RZ transmission, related with SARS-CoV-2 and its variants such as Alpha (B.1.1.7), Delta (B.1.617.2), and Omicron (B.1.1.529) [182–184]. Additionally, specific mutations have been consistently found in viral sequences from deer but not in sequences derived from humans, supporting deer-to-deer transmission and viral persistence within their population (**Figure 1.6**) [182,185]. In Ontario, Canada, a divergent lineage of SARS-CoV-2, designated as lineage B.1.641, was identified in WTD as a result of viral host evolution and adaptation [185]. The lineage B.1.641 shares a common ancestor with sequences from minks and humans in Michigan (the USA), suggesting a potential spillover event from humans to deer, possibly involving minks as intermediate host [185]. A human spillback of the B.1.641 was also suspected, although recurrent deer-to-human transmission or human-to-human transmission of B.1.641 was not evidenced [185]. As a matter of fact, the B.1.641 variant was efficiently neutralized by sera from

vaccinated or convalescent human individuals, indicating a non-significant impact on immune evasion capacity of SARS-CoV-2 in humans [185]. The widespread of SARS-CoV-2 in WTD indicates that it may be considered as a potential animal reservoir of the virus [182,183,185].

Although rarely, the SARS-CoV-2 Delta (B.1.617.2) variant has also been detected in mule deer (*Odocoileus hemionus*) in Utah (USA) [119], and viral exposure was suspected in free-ranging fallow deer (*Dama dama*) and red deer (*Cervus elaphus*) in sub-urban and urban areas from Spain (**Figure 1.6**) [186]**.** This is the first study that describes seropositivity against SARS-CoV-2 in European deer, as other survey studies conducted in Germany, Austria, UK, and Belgium yielded negative results [186].

1.4.4.3.2.2 Other free-range wildlife species

Other wildlife animals, belonging mainly to the family *Mustelidae*, have also been infected or exposed to SARS-CoV-2 (**Figure 1.6**). Aguiló-Gisbert et al., reported two positive free-ranging minks that were caught in the wild in the Valencian Community (Eastern Spain). These animals did not appear to have escaped from any nearby mink farm [187]. A generalized outbreak of a COVID-19-like condition among mink populations in that geographic area was highly unlikely since the remaining 11 out of 13 trapped minks of the study tested negative [187]. SARS-CoV-2 was also found in a wild Eurasian otter (*Lutra lutra*) living far away from the locations where infected minks were found in the Valencian Community [188]. In another study, SARS-CoV-2 exposure was described in 11 free wild American minks in Utah (United States), which were presumed to be domestic escapees from a fur farm where outbreaks of SARS-CoV-2 had occurred previously [189]. Also, 3 out of the 11 antibody-positive minks tested positive by RT-qPCR [189]. Mink farms are considered a potential source of infection of other susceptible

species, such as free-ranging animals that could have access to the farms and have direct contact with infected minks or their feces, feed, or breeding. This is the case of infected stray cats that were found within the surroundings of mink farms, from which sequencing analysis strongly supports mink-to-cat transmission [190]. Other species belonging to the family *Mustelidae* have also been exposed to SARS-CoV-2 in the wild, including pine martens (*Martes martes*) and European badgers (*Meles meles*) from Brittany, France (**Figure 1.6**) [191].

Furthermore, cases of SARS-CoV-2 infection have been described in a free-ranging feline species (*Panthera pardus fusca)* in India [192], and in a free-ranging NHP, a black-tailed marmoset (*Mico melanurus*) from an urban area in Mid-West Brazil on March 2022 (**Figure 1.6**) [193].

1.5 One Health approach

The emergence of SARS-CoV-2 as a novel CoV with a potential wildlife origin has highlighted the interactions between animals, humans, and the environment [2,194]. The modern era characterized by industrialization, urbanization, and increasing human activity, has brought animals and humans closer together, thereby facilitating the bidirectional transmission of pathogens (**Figure 1.7**) [195]. Climate change also displays a significant role in global health. Alterations in climatic factors (*e.g.,* temperature or humidity) can influence the migration patterns of animals, thus, promoting the geographical distribution of pathogens and their exposure to previously non-exposed human and animal populations [196,197]. Human activities such as global mobility and the trade of exotic animals also contribute significantly to the spread of EZD. Besides, intensive farming methods, industrial livestock production, wildlife exploitation, and wildlife habitat destruction create ideal conditions for zoonotic transmissions and disease propagation [195].

Urbanization and changes in land usage intensify the pressure on animal species, such as rodents, to adapt and develop new patterns for thriving in human environments [195,198]. Rodent species, as well as bats, are among the most studied animal reservoirs of zoonotic pathogens, and as previously mentioned, all hCoVs so far seem to have originated from these animals [7,198].

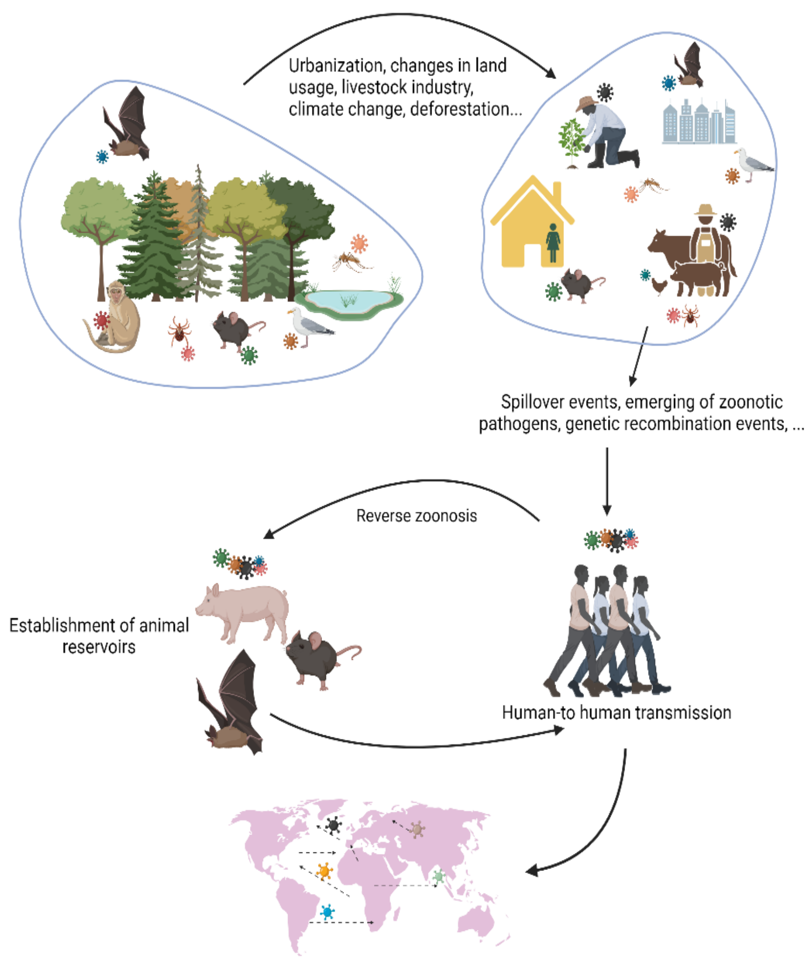

Figure 1.7 Increasingly human activity (*e.g.*, frequent changes in landscape, deforestation, urbanization, and livestock industry) and the climate change facilitate the proximity between humans and animals and enhance the emergence of zoonotic pathogens and further spillover events. Figure created with Biorender.com.

Given all these factors, human, animal, and environmental health should be considered equally important when developing EZD management strategies. Preventive measures should be implemented to control their spread, cross-species transmission, and the establishment of novel animal reservoirs [199]. This principle establishes the pillars of the One Health approach, which gained popularity in the early 21st century [199]. The One Health strategy supports a multisectorial collaboration among professionals from medicine, veterinary science, public health, environmental science, and related fields to address the complex interactions between human, animal, and environmental health factors and to achieve a global health (**Figure 1.8**) [194,199].

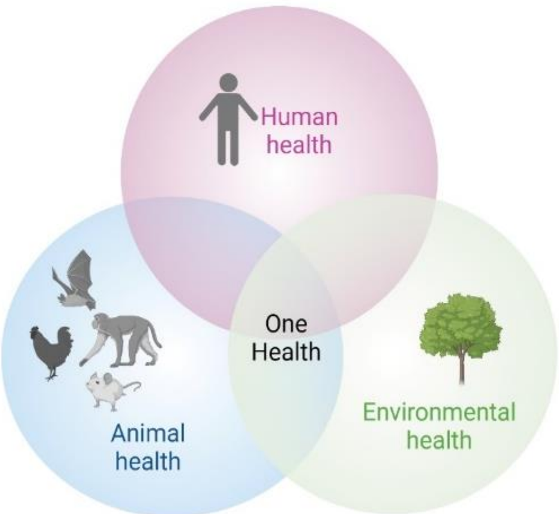

Figure 1.8 One Health concept. Multisectorial collaboration between human, animal, and environmental sectors is crucial to achieve an optimal global health. Figure created with Biorender.com.

Different initiatives have been established to design joint One Health plans to better manage global threats and avert possible future pandemics: on an international scale, the One Health Initiative was created by four global

organizations including the Food and Agriculture Organization of the United Nations (FAO), the United Nations Environment Programme (UNEP), the WHO, and the World Organization for Animal Health (WOAH) [200]. Also, the European Commission founded the One Health European Joint Programme [201], and in Spain, the Strategic Health and Environment Plan (PESMA) based on the One Health concept was approved at the end of 2021 [202].

SARS-CoV-2 is a great example on the need for implementation of the One Health strategy [194]. CoVs can infect a multitude of animal species, and their ability to cross-species barriers has been evidenced in multiple times. Among other strategies, investigations on the SARS-CoV-2 epidemiology in animal species may provide us with valuable knowledge into the SARS-CoV-2 distribution, potential spillover events, and the establishment of animal reservoirs. Altogether may contribute to better control the disease and prevent significant further impacts not only to the global health, but also to economies and social stability [2,194].

CHAPTER 2. HYPOTHESES AND OBJECTIVES

2.1 Hypotheses

Once SARS-CoV-2 was identified as the causative agent behind multiple cases of pneumonia and thousands of human infections and fatalities, concerns arose regarding the virus's animal host range [11,53]. Previous instances of SARS-CoV originating from bats and jumping to humans through palm civets as intermediate hosts [8], along with evidence of susceptibility in various animal species such as cats, ferrets, golden Syrian hamsters, mice, raccoon dogs, and NHP [203–208], supported the hypothesis that SARS-CoV-2 could infect a wide range of animal species. With SARS-CoV-2 sharing 79% of its genome sequence with SARS-CoV and both viruses using the S protein to enter cells via the ACE2 host cell receptor [11,209], the susceptibility of animals to SARS-CoV-2 infection became a focus point of research efforts.

In February 2020, approximately one month after the emergence of SARS-CoV-2, the first natural infections were reported in companion animals[122,210]. These animal infections were related to previous contact of the animals with humans affected by SARS-CoV-2 [122,210]. Also, predictive computational studies suggested a broad host range of SARS-CoV-2 and the potential for infection in mammals, particularly carnivores [37,59,211]. Subsequently, experimental infections early in the pandemic confirmed the susceptibility of a variety of animal species (*e.g.,* cats, hamsters, ferrets, fruit bats, and NHP) [64,66,91,99]. The potential risk of SARS-CoV-2 infection in animal species was not only based on genomic sequences, and the presence and expression levels of ACE2, but also on the regular interaction of animals with the human population. Thus, the focus of the present *PhD Thesis* was primarily on evaluating the potential risk of SARS-CoV-2 infection in companion animals, livestock species, and captive animals.

Considering the increasing interaction between domestic and wildlife species because of human activities, the possibility of the potential introduction of SARS-CoV-2 into wildlife animal populations could not be ruled out. In addition, the potential wildlife origin of the virus in bats [11,50], supported the hypothesis of other wildlife animals being infected or exposed to SARS-CoV-2. Therefore, to determine the presence of SARS-CoV-2 in wildlife species was also of particular interest, as it could significantly impact the epidemiology of the COVID-19 pandemic. This scenario may lead to the establishment of animal reservoirs for SARS-CoV-2 and the emergence of novel variants that could have more severe effects on human health.

Importantly, urban and peri-urban animal species are well-adapted to human environments, with regular direct or indirect contact with humans, human-produced waste, and also domestic animals [212]. At the beginning of the pandemic, the transmission capacity of SARS-CoV-2 through fomites or contaminated food and water was still unknown. Therefore, the potential exposure to SARS-CoV-2 of urban species, such as rodents, and the increasing population of species such as wild boar was also deemed worthy of further investigation [213,214]. The hypothesis of these animals being exposed to or infected by SARS-CoV-2 was reinforced when new viral variants emerged and demonstrated its ability to infect previous non-susceptible species including murine ones [106,107].

The novel variants of SARS-CoV-2 raised special concerns due to their heightened transmission, immune evasion and/or increased virulence in humans [30]. Accordingly, their impact on animal species was of particular interest during the pandemic. Goats, which are a common livestock species worldwide, were predicted to have a moderate risk of SARS-CoV-2 infection according to *in silico* studies [37,59]. In addition, *in vitro* studies exhibited SARS-CoV-2 infection and replication in goat cells, similar to the outcomes

observed in cells from susceptible species [215]. Later, goats demonstrated to be permissive to infection by the SARS-CoV-2 ancestral variant, although with limited susceptibility [87]. Given that other variants including Alpha, Beta, Gamma, and Omicron already exhibited their capacity to expand their host range [106,107], we considered the possibility of higher susceptibility of goats to these variants compared to the ancestral one. Additionally, findings of higher susceptibility of goats to SARS-CoV-2 variants could contribute to establish new animal models for SARS-CoV-2 research.

2.2 Objectives

The primary objective of this *PhD Thesis* was to determine the potential role of domestic and wildlife animal species in the epidemiology of the COVID-19 pandemic (from 2020 to 2023). To achieve this goal, various specific objectives were established:

1. To determine the prevalence and seroprevalence of SARS-CoV-2 in companion animals and stray cats from North-Eastern Spain and evaluate their capacity to neutralize the different viral VOCs emerged during the pandemic (*Chapter 3*).
2. To assess acute SARS-CoV-2 infection and/or past exposure in free-ranging and captive wildlife species potentially susceptible to the virus during the COVID-19 pandemic (*Chapter 4*).
3. To monitor SARS-CoV-2 acute infection and/or exposure in common urban and peri-urban species from Catalonia, Spain, including rodent species (mice and rats) and wild boar, during the whole course of the pandemic (from 2020 to 2023) (*Chapter 5*).

4. To investigate the susceptibility of the domestic goat, a common livestock species worldwide, to a SARS-CoV-2 variant with expanded host range (Beta (B.1.351) variant) (*Chapter 6*).
5. To evaluate the diagnostic performance of various Enzyme-Linked Immunosorbent Assays (ELISAs) used during this *PhD thesis* to study humoral responses in animal species against SARS-CoV-2 (*Chapter 7*).

CHAPTER 3.
STUDY 1

SARS-CoV-2 infection and humoral responses against different variants of concern in domestic pets and stray cats from North-Eastern Spain (2020 – 2022).

Chapter adapted from the article:

Fernández-Bastit, L. *et al.* Severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) infection and humoral responses against different variants of concern in domestic pet animals and stray cats from North-Eastern Spain. *Transbound Emerg Dis* **69**, 3518–3529 (2022)

3.1 Introduction

To date, it is strongly believed that SARS-CoV-2 is a zoonotic virus that emerged from SARS-like CoVs from bats [11,50]. Although a direct ancestor has not been detected in the wild yet, the closest genome sequences have been identified in Horseshoe bats (*Rhinolophus spp*.) from South-east Asia [11,50]. In addition, genomic analysis suggested that transmission from bat to humans likely occurred through an unidentified intermediate host [216,217]. The susceptibility of different animal species (domestic, captive, and wildlife) to SARS-CoV-2 infection has been demonstrated through experimental and natural infections, suggesting their potential role in the epidemiology of the disease [56,110]. Of particular concern is the potential susceptibility of those animals that are frequently in contact with the human population, such as companion animals.

Previous experimental *in vivo* studies performed in domestic cats, ferrets, and golden Syrian hamsters demonstrated viral replication in respiratory and gastrointestinal tracts, as well as RNA shedding as detected by RT-qPCR in swabs [64,66,218]. These animal species are able to transmit the virus to co-housed animals [64,66,218]. In contrast, dogs appeared to be less susceptible to SARS-CoV-2 infection experimentally with no apparent ability to transmit the virus [64,66]. Nevertheless, the ability of SARS-CoV-2 to infect dogs has been evidenced under natural conditions, and confirmed for cats, hamsters, and ferrets [57,126]. The majority of these animal infections have been associated with the contact with COVID-19-affected humans, suggesting a RZ transmission of SARS-CoV-2 [56]. Stray cats have also been exposed to SARS-CoV-2, although with less proportion of animals compared to domestic pets [140,143,144]. Besides, pet-to-human transmission has been described only in two different occasions. One case occurred in Hong Kong,

related to an outbreak in a pet-shop, in which hamsters transmitted the virus (Delta (B.1.617.2) variant) to humans [145]. The other case occurred in Thailand, where a cat transmitted the virus to a veterinarian clinician [146]. For all abovementioned facts, it is crucial to investigate the role of pets in the epidemiology of COVID-19 and to determine their susceptibility to infection by SARS-CoV-2 and the different variants recognized as VOCs during the pandemic.

According to the Council of Veterinary Colleges of Catalonia [219], a total of 1,531,002 pet animals were registered in 2021 in Catalonia, corresponding to 253,860 cats, 1,264,795 dogs, and 5,601 ferrets. Thus, the present work aimed to determine the prevalence of SARS-CoV-2 infection and the seroprevalence of cats (stray and pet), dogs, and ferrets from the North-Eastern of Spain (Catalonia and Valencian Community). The study included pets from COVID-19 positive households, pets with no evidence of contact with COVID-19 affected owners, and pets with no information about their COVID-19 environment. In addition, since the present work included samples from the beginning of the pandemic (April 2020) until January 2022, levels of nAbs against different VOC identified to date were investigated for a first time in a large number of pet animals.

3.2 Material and methods

Sample collection

A total of 1,009 animals were included in the study: 564 dogs (*Canis familiaris*), 381 cats (*Felis catus*; 253 pet cats and 128 stray cats), and 64 ferrets (*Mustela putorius furo*). Nasopharyngeal or oropharyngeal swabs (n = 987), rectal swabs (n = 929), and serum samples (n= 879) were taken from most of these animals from April 2020 to January 2022. At least one type of

sample from each animal was obtained. Nasopharyngeal, oropharyngeal, and rectal swabs were collected using sterile dry swabs or DeltaSwab Virus 2 mL contained in viral transport media (VTM) (Deltalab, S.L., Catalunya, Spain). Such sampling was performed by veterinarians from multiple veterinary clinics (Catalonia and Valencian Community, North-Eastern Spain), from the Veterinary Clinical Hospital of the Autonomous University of Barcelona (UAB, Barcelona, Spain), as well as from the Veterinary Pathology Diagnostic Service (SDPV) of the UAB. Lung tissue (n = 236) was also available from most of those animals necropsied at the SDPV. Samples from stray cats were obtained from veterinary clinics having permissions to work with these populations from the corresponding municipalities.

Pets were classified according to a questionnaire filled in by the owners, in which clinical signs (digestive and respiratory) of the animals were described, if any, as well as whether if they had contact or not with a COVID-19 affected human. Thus, animals were divided within three main categories: 1) those from households with current or previous COVID-19 affected owners [COVID-19(+) group], 2) those pets with no evidence of contact with COVID-19 affected owners [COVID-19(-) group], and 3) pets from households from which no information on COVID-19 environment was available (Unknown COVID-19 group). In addition, the following data were recorded when possible: breed, gender (female/male), and age.

All samples were obtained from veterinary clinicians using conventional sampling protocols in compliance with the guidelines by the Code of Research Ethics of IRTA. Samples from stray cats were obtained from two different veterinary clinics with the authorization of the local government from Palamós, Girona (reference number 14869) and Barcelona city (project license 21001495). Samples were subsequently sent to IRTA-CReSA for SARS-CoV-2 investigations by a transport company under the

regulations stated in the UN3373 regulation. Owners/keepers were duly informed regarding the purpose of the study and the data protection policy and granted their consent for each pet.

RNA extraction and detection of SARS-CoV-2 by RT-qPCR

A total of 992 out of 1,009 animals were tested for the detection of SARS-CoV-2 RNA: 380 cats (252 pet cats, 128 stray cats), 550 dogs, and 62 ferrets. First, sterile dry oral/nasal and rectal swabs were transferred into cryotubes containing 500 µL Dulbecco's Modified Eagle Medium (DMEM) (Lonza, Basel, Switzerland) supplemented with 100 U/mL penicillin, 100 µg/mL streptomycin, and 2 mM glutamine (all from Gibco Life Technologies, Madrid, Spain) and finally vortexed. DeltaSwabs Virus with VTM were directly vortexed. Regarding lung tissue samples, a portion of approximately 0.2 mg was placed into cryotubes with 500 µL of supplemented DMEM with a single zinc-plated, steel 4.5-mm beads. Tissues were mechanically homogenized at 30 Hz for 1 min using a TissueLyser II (QIAGEN GmbH, Hilden, Germany) and centrifuged for 3 min at 10,000 rpm. All samples were subjected to viral RNA extraction using the Indimag Pathogen Kit (Indical Biosciences, Leipzig, Germany) on a BioSprint 96 workstation (Qiagen, Hilden, Germany), according to the manufacturer's instructions. Then, SARS-CoV-2 RNA was quantified by RT-qPCR using a previously described protocol, which targets the envelope protein (E)-encoding gene [220] with some modifications [78]. Briefly, RT-qPCR was performed using AgPath-IDTM One-Step RT-PCR Reagents (Applied Biosystems, Life Technologies, Waltham, MA, USA) and amplification was achieved using a 7500 Fast Real-Time PCR System (Applied Biosystems, Life Technologies, Waltham, MA, USA). Samples with a Cq value <40 were

considered positive for SARS-CoV-2 genomic detection. Positive samples were re-analyzed by two different RT-qPCR assays, targeting the RdRp gene specific for SARS-CoV-2 and the N gene [220], following a previously published protocol [130].

SARS-CoV-2 genome sequencing

Viral RNA from all positive samples was converted to cDNA with the PrimeScript ™ RT reagent kit (Takara Bio Europe SAS, Saint-Germain-en-Laye, France), as previously described [221]. Then, samples were sequenced following a previously described procedure [165]. Briefly, cDNA was used for DNA synthesis using the ARTIC-CoV v3 PCR protocol followed by Illumina sequencing [222]. Raw data analysis was performed by viralrecon pipeline (https://github.com/nf-core/viralrecon, accessed on: 4 July 2022) while consensus sequence was called using samtools/ivar at the 75% frequency threshold. All high-quality genomic sequences were deposited in GISAID repository.

RNA inhibition ELISA (ELISA-1)

Blood samples were centrifuged at 1800 x g for 10 min at 4ºC, and the obtained sera were inactivated at 56ºC for 1 h and stored at -20ºC until further use. For the analysis, samples were previously thawed and vortexed. nAbs targeting SARS-CoV-2 RBD were measured in available serum samples (n = 789; 444 dogs, 298 cats [170 pet cats, 128 stray cats] and 47 ferrets) using the GenScript cPass™ SARS-CoV-2 Neutralization Antibody Detection Kit (Genscript, the Netherlands; referred as ELISA-1 in this *PhD Thesis*), following the manufacturer's protocol. Briefly, serum samples were diluted 1:10 and mixed with equal volumes of recombinant HRP-conjugated RBD. After an incubation period at 37ºC for 30 min, 100 µL of each diluted

sample was transferred to 96-well plates pre-coated with a recombinant hACE2 protein. Non-nAbs or any unbound HRP-RBD were bound to the plate and nAbs were removed during washing steps. After washing, the tetramethyl benzidine (TMB) substrate solution was incubated at room temperature for 15 min. The stop solution was added, and Optical Densities (OD) were read at 450 nm on a microtiter plate reader. Results were expressed as percentage of inhibition, which was determined using the following formula: % Inhibition = (1 - [OD_{450} sample/OD_{450} negative control]) x 100. Samples and controls were included in duplicate (SD ≤ 10%). Inhibition of ≥ 30% was considered as a positive neutralization.

Neutralization assay of SARS-CoV-2 pseudoviruses expressing the spike protein of different VOCs

Serum samples that tested positive (n=40) by the ELISA-1 were also analyzed with a pseudovirus-based neutralization test (pVNT) against different SARS-CoV-2 VOCs following a previously described protocol [223]. Briefly, HIV reporter pseudoviruses expressing SARS-CoV-2 S protein (from the ancestral virus and the Alpha, Beta, Delta, and Omicron BA.1 VOCs) and luciferase were generated. Pseudoviruses expressing a VSV-G protein instead of the S glycoprotein were generated and used as control of specificity as previously described [224]. For the neutralization assay, 200 $TCID_{50}$ of pseudovirus were pre-incubated with three-fold serial dilutions (from 1/20 to 1/43,740 for the Omicron BA.1 variant, and from 1/60 to 1/43,740 for all the other variants) of heat-inactivated sera samples. Then, hACE2 overexpressing HEK293T cells (10^5 cells/mL) were added onto mixed samples. After 48 h, cells were lysed with Britelite Plus Luciferase reagent (Perkin Elmer, Waltham, MA, USA) and luminescence was measured

for 0.2 s with EnSight multimode late reader (Perkin Elmer, Waltham, MA, USA).

The neutralization capacity of the sera samples was calculated by comparing the experimental relative light unit (RLU) calculated from infected cells treated with each serum to the maximal RLUs (maximal infectivity calculated from infected untreated cells), and minimum RLUs (minimal infectivity calculated from uninfected cells) and expressed as percentage of neutralization: % Neutralization = $(RLU_{max} – RLU_{experimental})/(RLU_{max} – RLU_{min})$ x 100. ID_{50} (infectious dose 50) values were calculated by plotting and fitting neutralization values and the log of plasma dilution to a 4-parameters equation in Prism 9.0.2 (GraphPad Software, San Diego, CA, USA). All ID_{50} values are reported as reciprocal dilution.

Statistical analyses

Chi-square with Yate's correction test was used to compare differences in SARS-CoV-2 RNA detection and antibody prevalence among studied groups. The relationship between the antibody presence, households' conditions and gender were also analyzed. *P*-values lower than 0.05 were considered statistically significant. Relative risk (RR) ratios and 95% confidence intervals (95% CI) were determined to evaluate the risk of exposure to SARS-CoV-2 in cats and dogs from positive and negative COVID-19 households.

Wilcoxon test with Bonferroni's correction test was used to compare titers of nAbs against different SARS-CoV-2 VOCs in each species. A Kruskall Wallis test with Dunn's multiple comparison test was used to compare titers of nAbs against different VOCs in different collection periods of the COVID-19 pandemic in all seropositive animal samples. On the other

hand, comparison of the titers of nAbs against VOCs between species was also evaluated. Tests with *P*-values lower than 0.05 were considered statistically significant.

Spearman's correlation test was used to evaluate the existence of a positive relationship between the ELISA-1 (% of Inhibition) and the pVNT (ID_{50}) assays.

All results were analyzed with GraphPad Prism 9.0 Software (La Jolla, CA, USA).

3.3 Results

Sample data

The total number of pet cats, dogs, and ferrets included in the study are displayed in **Table 3.1**, classified into three main categories: COVID-19(+), COVID-19(-), and Unknown households. Besides, 128 stray cats were also included in the study.

Table 3.1 Pet cats, dogs, and ferrets distributed in three main categories according to COVID-19 household environment conditions: pets that had contact with COVID-19 affected owners [COVID-19 (+)], pets with no evidence of contact with COVID-19 affected owners [COVID-19 (-)], pets with unknown COVID-19 household environment [Unknown COVID-19].

Households	Pet cat	Dog	Ferret	Total
COVID-19 (+)	47 (18.58%)	196 (34.75 %)	6 (9.38 %)	249 (28.26%)
COVID-19 (-)	101 (39.92%)	218 (38.65 %)	42 (65.63 %)	361 (40.98%)
Unknown COVID-19	105 (41.50%)	150 (26.7 %)	16 (25.00 %)	271 (30.76%)
Total	253 (100%)	564 (100%)	64 (100%)	881 (100%)

Both female (n = 357) and male (n = 357) animals were represented in the study. However, gender information was not available from some of the animals (n = 295). **Table 3.2** shows the total of samples analyzed by RT-qPCR and by the ELISA-1 according to the gender within animal species.

Table 3.2 Number of animals tested by RT-qPCR and by the ELISA-1 were grouped according to the animal gender and species (cat, dog, and ferret).

	Gender	SARS-CoV-2 RT-qPCR	ELISA-1
Pet cats	Female	72	69
	Male	78	74
	Unknown	102	27
	Total	**252**	**170**
Stray cats	Female	48	48
	Male	46	46
	Unknown	34	34
	Total	**128**	**128**
Total cats	Female	120	117
	Male	124	120
	Unknown	136	61
	Total	**380**	**298**
Dogs	Female	206	207
	Male	201	203
	Unknown	143	34
	Total	**550**	**444**
Ferrets	Female	22	17
	Male	24	23
	Unknown	16	7
	Total	**62**	**47**
All species	Female	348	341
	Male	349	346
	Unknown	295	102
	Total	**992**	**789**

SARS-CoV-2 RNA detection and SARS-CoV-2 sequencing

A total of 992 animals were analyzed by RT-qPCR and only three of them tested positive (0.30%; 95% CI = [0.00% - 0.64%]) for SARS-CoV-2 RNA: one pet cat (C1) (1/380; 0.26%; 95% CI: [0.00% - 0.78%]) and two dogs (D1, D2) (2/550; 0.36%: 95% CI: [0.00% - 0.87%]). No statistically significant differences (Chi-square with Yates' correction, $p > 0.8832$) in RNA prevalence were observed between cats and dogs.

C1 was a 4-year-old male European x Persian crossbred cat, D1 corresponded to a male Schnauzer dog, and D2 was a 13-year-old female Breton dog. Specific epidemiological and clinical investigations about the infection of C1 and D2 are described in a previously published case report [130] and in the **Appendix 3.1,** respectively. All of them were living in a COVID-19 positive household with previous affected owners. C1(No. 1 in **Supplementary Table 3.1**) tested positive in nasal swab for the UpE (Cq = 33.69), RdRp (Cq = 34.01), and the N (Cq = 35.1) genes and resulted negative for all mentioned genes in rectal swab. D1 (No. 11 in **Supplementary Table 3.1**) tested positive in nasal swab for the UpE (Cq = 13.21), RdRp (Cq = 19.39), and N (Cq = 19.83) genes, and in rectal swab for the UpE (Cq = 24.68), RdRp (Cq = 29.73) and N (Cq = 30.92) genes. D2 (No. 19 in **Supplementary Table 3.1**) tested positive in oral swab for the UpE (Cq = 34.36) and RdRp (Cq = 35.77) genes but negative for the N gene (Cq>40) and for all the genes in the rectal swab. One week after the first positive result, both C1 and D2 tested negative by RT-qPCR; D1 was a dog received at the SDPV and the cause of euthanasia was renal failure due to an overdose of anti-inflammatory non-steroidal drugs. None of studied ferrets resulted positive by RT-qPCR.

SARS-CoV-2 genome sequencing was performed in all positive samples and a specific SARS-CoV-2 variant was identified in each case. C1 was infected with an early epidemic SARS-CoV-2 variant (B.1 PANGO lineage, D614G) (Global Initiative on Sharing Avian Influenza Data, GISAID acc. EPI_ISL_482820), viral genome from nasal and rectal swabs of D1 was consistent with the Alpha (B.1.1.7) VOC (nasal swab: EPI_ISL_13608276, Rectal swab: EPI_ISL_13608277), while D2 was infected with the Delta (B.1.617.2) VOC (GISAID acc. EPI_ISL _6344510).

Detection of SARS-CoV-2 nAbs targeting the RBD

A total of 789 serum samples were evaluated by ELISA-1. RBD nAbs were detected in 16/298 (5.36%; % 95 CI = [2.81% - 7.93%]) cats, 22/444 (4.95%; 95% CI = [2.94% - 6.97%]) dogs, and 2/47 (4.26%; 95% CI = [0.00% - 10.03%]) ferrets (**Table 3.3**). Within the 16 positive samples from cats, 13 samples corresponded to pet cats (13/170, 7.65%; 95% CI = [3.65% - 11.64%]) and 3 samples were from stray cats (3/128, 2.34%; 95% CI = [0.00% - 4.96%]). Of the total of 40 positive samples, 29 (72.5%) corresponded to pets from COVID-19(+) households, being specifically, 10/16 (62.5%) cats, 17/22 (77.27%) dogs, and 2/2 (100%) ferrets. However, nAbs were also detected in pet cats and dogs from the other groups (COVID-19(-) and Unknown COVID-19) (**Table 3.3**).

Along the positive feline serum samples, one corresponded to the C1 (No. 1 in **Supplementary Table 3.1**), which exhibited a 96.24% of RBD inhibition one week after testing positive for RT-qPCR. C1 was living with another cat mate that also tested positive with an inhibition of 96.59%, although it tested negative by RT-qPCR (No. 2 in **Supplementary Table 3.1**)[130]**.** On the other hand, D2 had RBD nAbs with an inhibition of 67.93% (No. 19 in **Supplementary Table 3.1**) 21 days after the initial respiratory and digestive clinical signs and 67.60% two and a half months after the display of clinical signs **(Appendix 3.1)**. Unfortunately, it was not possible to obtain serum sample from the D1 since it was euthanized due to its clinical condition (severe necrosis of the bilateral renal papilla, mitral and tricuspid endocardiosis as well as of edema and pulmonary congestion). Regarding the positive canine serum samples, one corresponded to a dog with an inhibition of 85.51% (No. 15 in **Supplementary Table 3.1**), which was living with another dog mate that exhibited an inhibition of 30.62% (No. 14 in **Supplementary Table 3.1**). They were included in the COVID-19 positive

household group and were sampled at the same time point (June 2021). Finally, both seropositive ferrets (No. 20 and No. 21 in **Supplementary Table 3.1**) were from the same household. No other animals came from the same household.

Cats from COVID-19 positive households [COVID-19(+)] were significantly more likely to seroconvert against SARS-CoV-2 (Chi-square with Yates' correction, $p < 0.0001$) with a higher risk of SARS-CoV-2 exposure (RR = 11.67; 95% CI = [2.67 – 50.97]) compared to those that did not have any contact with a COVID-19 affected human or no evidence was determined [COVID-19(-)] (**Table 3.3**). Similar results were observed in dogs (Chi-square with Yates' correction, $p = 0.0030$; RR = 4.77, 95% CI = [1.63 – 14.92]). Both positive ferret samples were living in a COVID-19 household positive.

In addition, the seroprevalence between females and males in each species was also compared. No significant link between seropositivity and the gender of animals, nor for cats (Chi-square with Yates' correction, $p = 0.6005$) or for dogs (Chi-square with Yates' correction, $p = 0.3462$), was observed (**Table 3.3**). The two positive ferrets belonged to the male gender.

Table 3.3 Results obtained by the ELISA-1. Seroprevalence of each species (cat, dog, and ferret) according to the COVID-19 environment household and gender. *P*-value determined by Chi-square Yate's correction test to analyze the relationship between seroprevalence and household, and seroprevalence and gender within each species.

Seroprevalence/ Households	Cats		Dogs	Ferrets	Total
	Pet cats	Stray cats			
COVID-19(+) %	**23.80** (10/42)	-	**8.99** (17/189)	**40.00** (2/5)	**12.28** (29/236)
COVID-19(-) %	**2.04** (2/98)	-	**1.89** (4/212)	**0.00** (0/35)	**1.74** (6/345)
P-value	$p < 0.0001$		$p = 0.0030$		
Unknown Covid-19 (%)	**3.33** (1/30)	-	**2.32** (1/43)	**0** (0/7)	**2.50** (2/80)
Total population %	**7.65** (13/170)	**2.34** (3/128)	**4.95** (22/444)	**4.26** (2/47)	**50.63** (40/790)
	5.36 (16/298)				
Seroprevalence/gender					
Female %	**4.27** (5/117)		**3.86** (8/207)	**0.00** (0/17)	**3.81** (13/341)
Male %	**6.66** (8/120)		**6.40** (13/203)	**8.70** (2/23)	**6.65** (23/346)
P-value	$p = 0.6005$		$p = 0.3462$		
Gender non-determined	**4.91** (3/61)		**2.94** (1/34)	**0.00** (0/7)	**3.92** (4/102)

Neutralizing responses against SARS-CoV-2 spike variants

Positive samples (n=40) from the ELISA-1 were then tested by the pVNT to evaluate their neutralization capacity against (i) ancestral (B.1), (ii)

Alpha (B.1.1.7), (iii) Beta (B.1.351), (iv) Delta (B.1.617.2), and (v) Omicron (BA.1) variants.

Almost all serum samples positive by the ELISA-1 were able to neutralize all variants, except few of them from which no nAbs were detected (ID_{50} <60 WH1, Alpha, Beta, Delta; ID_{50} <20 Omicron BA.1) (**Figure 3.1**). Thus, D2, which was infected by the Delta (B.1.617.2) variant, was able to neutralize all the other variants **(Appendix 3.1).** On the other hand, C1 demonstrated to neutralize the ancestral lineage, from which was infected. However, it was not tested against the other variants due to a limited volume of sera. The cat mate of C1 was able to neutralize all variants. No statistically significant differences were observed between titers of nAbs against ancestral (B.1), Alpha (B.1.1.7), Beta (B.1.351), and Delta (B.1.617.2) variants (according to the Wilcoxon test with Bonferroni's correction test) in either cats or dogs (**Figure 3.1**). In contrast, both cats and dogs had statistically lower titers against the Omicron (BA.1) variant compared to all the other variants. In the case of ferrets, statistical analyses of humoral responses could not be performed since only two samples were positive.

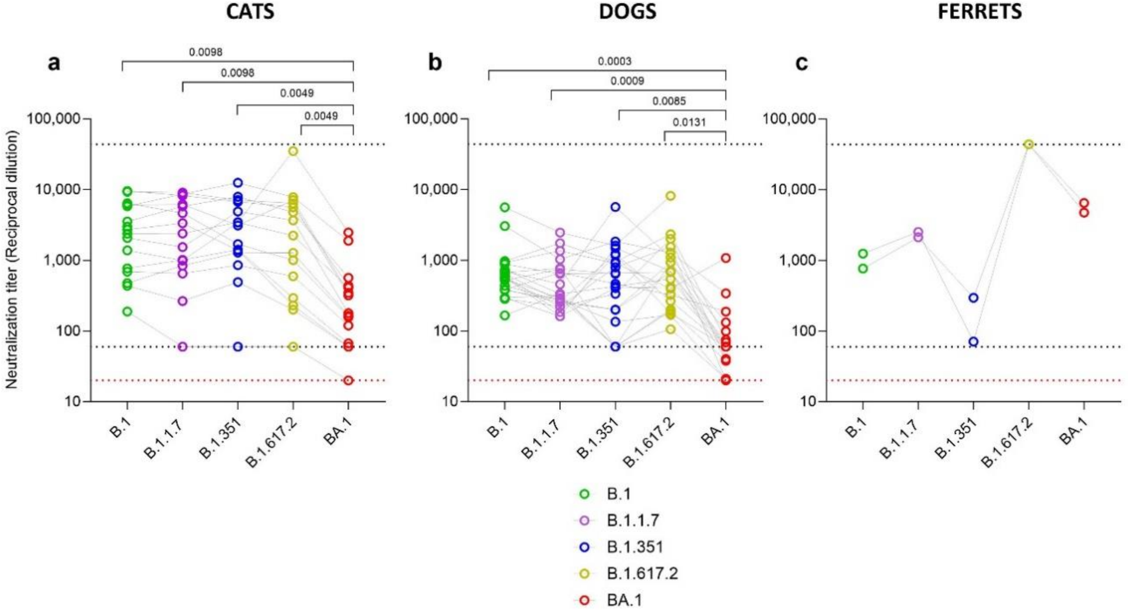

Figure 3.1 Neutralization titers against ancestral (B.1), Alpha (B.1.1.7), Beta (B.1.351), Delta (B.1.617.2), and Omicron (BA.1) variants of SARS-CoV-2 in **a)** cats, **b)** dogs, and **c)** ferrets. Three-fold serial dilutions of sera samples were performed to test all variants (1/20 – 1/43,740 BA.1 variant; 1/60-1/43,740 for the other variants). nAbs titers against different VOCs are represented as empty colored circles. Grey lines connect the nAbs titers against different VOCs of individual samples. Black discontinuous lines indicate the maximum and minimum limits of quantification of the assay for all the variants; red discontinuous lines indicate the minimum limit of quantification for Omicron variant. Wilcoxon test with Bonferroni's correction test was used to compare titers of nAbs against the different variants in each species. Significant *P*-values (< 0.05) indicated in each plot. Neutralization titers were expressed in ID_{50} (reciprocal dilution).

Additionally, we compared titers of nAbs against different VOCs from all positive samples in different collection periods established according to the main pandemic waves in Catalonia (Spain) [225] (**Figure 3.2**). The first period was established from March 2020 to December 2020, mainly

dominated by the ancestral (B.1) variant; the second period was considered from January 2021 to July 2021, when the Alpha (B.1.1.7) variant was the most prevalent; Finally, the third period was dated from June 2021 to January 2022 mainly dominated by the Delta (B.1.617.2) variant and also by the Omicron (BA.1) variant at the end of this period (from November to December 2021 onwards). The second and third periods were overlapped since Alpha (B.1.1.7) and Delta (B.1.617.2) variants predominated together in Spain during June and July 2021. Serum samples were grouped according to the period in which they were collected (first period N = 9; second period N = 14; third period N = 28). Eleven samples were collected between June and July 2021, thereby, they were considered within both the second and third period.

Sera exhibited significant lower titers of nAbs against the Omicron (BA.1) variant compared to titers against the ancestral (B.1) and Beta (B.1.351) variants in the first period (March 2020 – Dec 2020) (**Figure 3.2**). Additionally, lower titers of nAbs against Omicron (BA.1) were observed compared to all other variants in both the second period (January 2021 – July 2021) and the third period (June 2021 – January 2022). No additional statistically significant differences were observed in nAb titers among the different variant within each period.

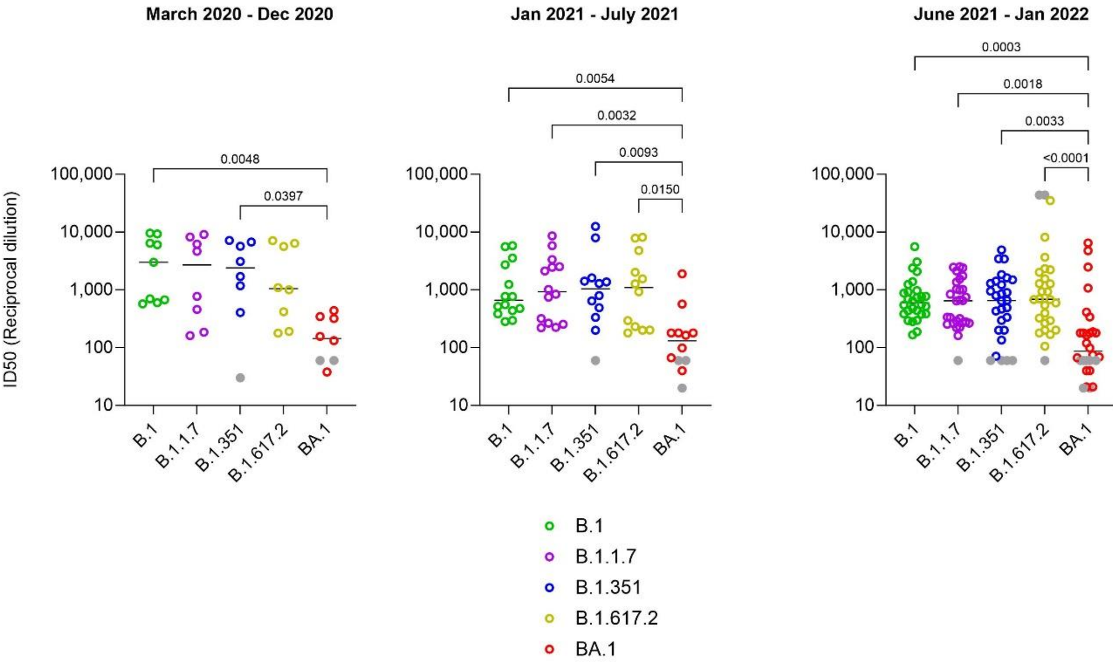

Figure 3.2 Comparison of neutralization titers against the ancestral (B.1), Alpha (B.1.1.7), Beta (B.351), Delta (B.1.617.2), and Omicron (BA.1) variants in three different periods established according to the pandemic waves in Catalonia: March 2020 – December 2020 (dominated by the Ancestral variant; N = 9/40); January 2021 – July 2021 (dominated mainly by the Alpha variant; N = 14); June 2021 – January 2022 (dominated mainly by the Delta variant and also Omicron variant at the end of the period; N=28). nAbs titers against different VOCs are represented as empty colored circles. Filled grey circles indicate samples out of the minimum or maximum limit of quantification. Bars indicate the media titer of each group. A Kruskall Wallis test with Dunn's multiple comparison test was used to compare nAbs titers within each period (*P*-values < 0.05 were considered as significant and are indicated). Neutralization titers were expressed in ID_{50} (reciprocal dilution).

Next, we compared titers of nAbs between species (**Figure 3.3**). Cats showed significantly higher neutralizing titers against all variants compared to dogs, except for the Delta (B.1.617.2) variant. Ferrets showed significant lower titers for the Beta (B.1.315) VOC compared to cats and higher titers against the Delta (B.1.617.2) and Omicron (BA.1) VOCs compared to dogs.

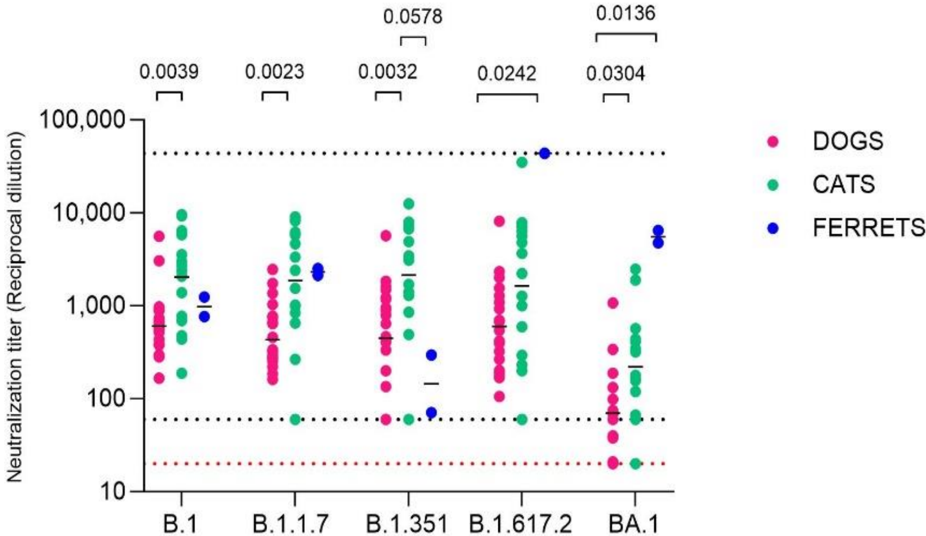

Figure 3.3 Titers of neutralizing antibodies for ancestral (B.1), Alpha (B.1.1.7), Beta (B.1.351), Delta (B.1.617.2), and Omicron (BA.1) in dogs (n = 22), cats (n = 16) and ferrets (n = 2). Discontinuous lines indicate the maximum and minimum limit of quantification of the assay for all the variants; red discontinuous line indicates the minimum limit of quantification for Omicron variant. Bars indicate the geometric mean titer in each group. *P*-values show the significant differences of titers of nAbs among species (Kruskall-Wallis test with Dunn's multiple comparison test).

Correlation analyses were performed using the results obtained from the ELISA-1 (% Inhibition) and the results obtained from the pVNT (ID_{50}) (**Figure 3.4**). A significant positive correlation between the percentage of inhibition and the neutralization titers was observed using all different pseudoviruses expressing the S protein of the ancestral (B.1, *r=0.7775*), Alpha (B.1.1.7, *r=0.7251*), Beta (B.1.351, *r=0.7078*), Delta (B.1.617.2, *r=0.6159*), and Omicron (BA.1, *r=0.6253*) variants.

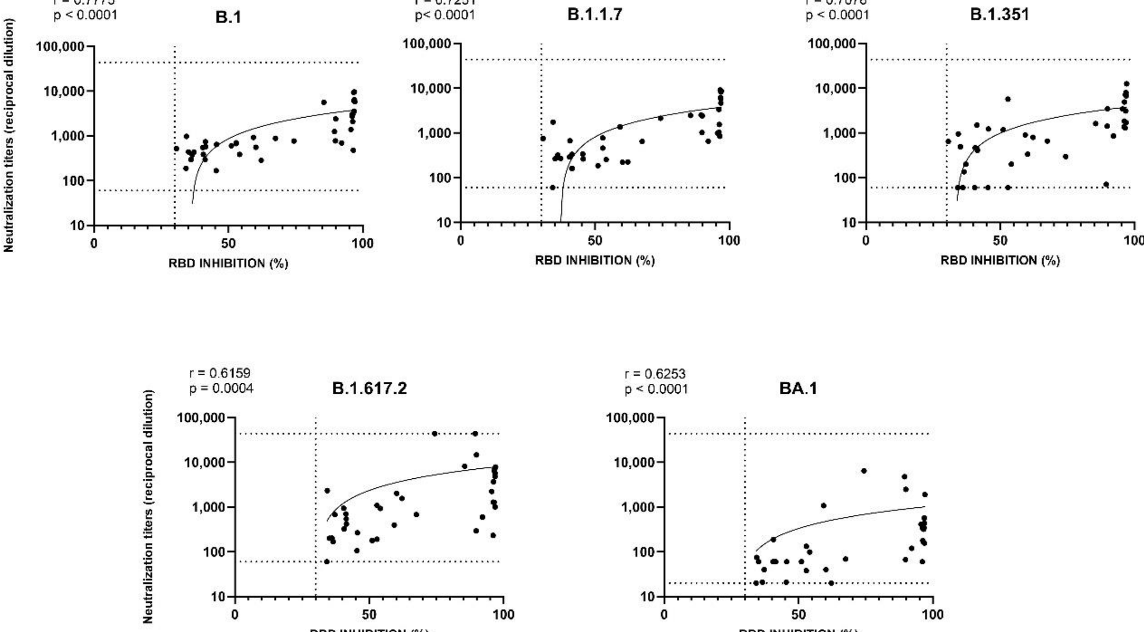

Figure 3.4 Correlation between the ELISA-1 (RBD Inhibition %) and the pseudoviral neutralization assay (ID_{50}) performed for each variant (B.1, B.1.1.7, B.1.351, B.1.617.2, and BA.1). Correlation was assessed by Spearman's correlation test (correlation coefficient and *P*-value indicated in the figure). Discontinuous lines show the cut-off of each assay.

3.4 Discussion

Since the beginning of the COVID-19 pandemic, many studies have been performed to determine the incidence of infection and seroprevalence in pets, as well as to know their role in the epidemiology of the disease [134,139,226–229]. The present work stands out since it is the first large-scale study on SARS-CoV-2 infection in pets and stray cats performed in the North-Eastern of Spain. Additionally, this work included for the first time the study of the humoral immune response of a large series of stray cats and pet animals against different VOCs.

In our study, a very low percentage of SARS-CoV-2 actively infected animals was found (0.3%), corresponding to one pet cat (C1) and two dogs (D1 and D2). Interestingly, we determined that C1 was infected on April 2020 with the D614G variant [130], D1 was infected on February 2021 with the Alpha (B.1.1.7) variant, whereas D2 was infected on July 2021 with the Delta (B.1.617.2) variant **(Appendix 3.1).** The period in which animals were infected was in accordance with the period in which each variant causing infection was the predominant variant in Spain [225]. Since there is evidence that all of them were living with COVID-19 affected owners, SARS-CoV-2 RZ transmission was strongly suspected [130] (**Appendix 3.1**). These results are consistent with previous reports, since the majority of natural infections in pets have been described in animals living in COVID-19 positive households [122,123,230]. In some cases, human-to-pet transmission have been evidenced by genomic and sequencing analysis [231–233], as the case of C1, included in this case series [130]. Reverse transmission has also been shown in other animal species, such as in zoo animals as large felines and NHP [165,167], farmed minks [151], and in wild animals such as the WTD [183]. Of note, evidence of SARS-CoV-2 adaptation and the appearance of new SARS-CoV-2 strains occurred in farmed minks and in WTD, and these variants were subsequently

transmitted to humans [151,183]. RT-qPCR positive animals in the present study corresponded to pet animals that were not in contact with other animals, except for C1 which was in contact only with another cat mate, suggesting that no further spread of SARS-CoV-2 was possible.

Considering the large number of samples in our study, we confirmed a similar low incidence of SARS-CoV-2 infection in cats (0.26%) and dogs (0.36%), consistent with other references [228,234]. Although none of the ferrets tested positive by RT-qPCR in the present study, the detection of SARS-CoV-2 RNA has already been demonstrated under natural conditions in kept ferrets [126]. Another study performed in Spain showed higher viral RNA prevalence of infection in cats and dogs, albeit still low, with values of 1.63% and 2.59%, respectively [226]. It is important to highlight that, in our study, a higher number of animals were exposed to SARS-CoV-2 in the past, based on serological results, than those detected as currently infected. A large number of animals that were positive for nAbs might have already cleared the virus by the time of sampling, in agreement with previous findings indicating a short RNA shedding period [235]. This also may partially explain the low viral load observed in C1 and D2, suggesting that these animals, were likely in the recovery phase of infection when the samples were collected [130]. In contrast, high viral loads were found in nasal and rectal swabs of D1, although viral isolation was not successful. However, SARS-CoV-2 isolation has been achieved from swabs from cats, dogs, and ferrets collected during natural and experimental infections, demonstrating infectious viral shedding in these species [66,73,126,135,226]. Furthermore, given that pets often experience subclinical infections [73,135,139,230], it is rather difficult to suspect the right timing of active SARS-CoV-2 infection. Consequently, obtaining samples precisely during the infectious phase becomes more challenging. In some cases, SARS-CoV-2 infections in pets caused mild clinical signs, mainly

respiratory (coughing, sneezing) and digestive (diarrhea, vomiting) [134,232], as we observed in the case of D2 (**Appendix 3.1**). While comorbidities contribute to the development of moderate or severe disease in humans [236], this correlation has not been demonstrated in pets. In fact, C1 and D1 were sacrificed due to their worsening clinical status [130], but probably not associated with SARS-CoV-2 infection since no lesions attributable to the viral infection were found. However, infections caused by the Alpha (B.1.1.7) variant in dogs and cats have been tentatively associated with myocarditis [227]. In any case, it is not clear whether SARS-CoV-2 infection in pets may worsen a previous disease, or it is just a subclinical infection concomitant to pre-existing condition.

Due to abovementioned reasons, serum sample collection was essential in this study for assessing SARS-CoV-2 past infections in pets. Globally, we detected evidence of seroconversion in 7.65% of pet cats, 4.95% of dogs, and 4.26% of ferrets, similar to other authors [140,228,237]. We observed that seroprevalences were higher in pets living in households with COVID-19 affected owners compared to those of COVID-19 negative households, which confirmed their major risk of virus exposure, as other authors have found [134,135,140,226,228]. Since groups of the study were classified from data provided by the owners, seroconversion observed in cats (2.04%) and dogs (1.89%) from COVID-19 negative households could be attributed to the pet exposure to SARS-CoV-2 infected asymptomatic or non-diagnosed owners. Besides, a total of 2.35% of stray cats were also exposed to SARS-CoV-2 in our study, consistent with previous investigations [143,144]. These cats could have been in contact with SARS-CoV-2 contaminated environment or with infected humans who take care of them. Furthermore, the exposure or infection of stray cats by transmission of the virus from other susceptible species can also occur [190]. In the Netherlands, stray cats were likely infected

by farmed minks as demonstrated by sequencing analysis [190]. In addition, experimental infections in cats have shown their capacity for viral transmission to other animals [64,66,218]. This finding raises concern about the potential spread and persistence of SARS-CoV-2, particularly within population of stray cats. They usually live forming colonies composed of hundreds of individuals, therefore, the likelihood of them serving as a reservoir for SARS-CoV-2 may not be completely ruled out. However, further investigations are required to validate this possibility. On the other hand, we did not observed correlation between the risk of infection and the sex of pets, similar to other authors [238]. In humans, there are gender-based differences in the risk of experiencing severe COVID-19 and hospitalization, although not necessarily in the risk of exposure to the virus [239,240].

As a novel insight of the present study, we demonstrated in a large collection of sera that nAbs found in cats, dogs, and ferrets can neutralize different VOCs of SARS-CoV-2. Our results indicated lower titers of nAbs against the Omicron (BA.1) variant, while similar titers were observed against the ancestral, Alpha, Beta, and Delta variants within cats and dogs. The pVNT specifically identified nAbs targeting the whole S protein [240]. Previous phylogenetic analysis based on S genomic sequences evidenced that Omicron variants are the most distantly in relation to other variants [241]. Furthermore, Omicron variants exhibit different mutations which are associated with reduced recognition of nAbs, such as E484A located in the S-glycoprotein [242]. This would help to explain the obtained results in animals as well. In addition, we must consider that most of the samples (92.47%) were collected before the Omicron wave started in Catalonia (end of December 2021), thus it is highly likely that animals were not infected by this variant. Apparently, we observed a tendency of the positive sera to exhibit higher titers against the variant that predominated in Catalonia [225] at sampling,

compared to other variants. In the first period, titers of nAbs against the Delta (B.1.617.2) variant were slightly lower than those for previous variants including the ancestral (B.1.), which was the predominant one, the Alpha (B.1.1.7) and the Beta (B.1.351) variants. However, during the second and third periods (from January 2021 to January 2022), titers of nAbs against the Delta (B.1.617.2) variant were similar to those against the ancestral (B.1), Alpha (B.1.1.7), and Beta (B.1.351) variants. Notably, within the second period, the Delta (B.1.617.2) variant was already in circulation, and by the third period, it was considered predominant in the human population. Interestingly, both positive ferrets demonstrated significantly higher titers of nAbs against the Delta (B.1.617.2) variant (ID_{50}>43,740) compared to the other variants, which was the prevailing variant in Spain at the time of sampling in July 2021. These two ferrets were living at the same COVID-19 positive household, which could explain their similar capabilities of neutralizing responses against the different SARS-CoV-2 variants. Since ferret-to-ferret transmission has been demonstrated experimentally [73], we cannot confirm whether the viral transmission took place between the animals or from the owners to each ferret. One of these ferrets exhibited mild bronchitis which could be associated with the potential SARS-CoV-2 infection. Among all studied animals, these ferrets exhibited the highest nAb titers against the Omicron variant, followed by a cat that had direct contact with a positive owner on December 2021 and was sampled on January 2022, according to the period dominated by the Omicron variant among humans in Spain [225]. On the other hand, those dogs living together at the same COVID-19 positive household (No. 14 and No. 15 from the **Supplementary Table 3.1**) exhibited different humoral responses against SARS-CoV-2, although they were sampled at the same time (June 2021). One of them showed high levels of nAbs and its dog mate had low levels of nAbs against all variants. This variability has also been observed in human population; while some

individuals develop strong neutralizing responses against SARS-CoV-2, others develop weak immune responses [240]. Besides the previous comments, we cannot exactly confirm to which variant the seropositive animals were exposed since they tested negative in RT-qPCR, except for C1, D1 and D2. Importantly, the possibility that nAb titers have been reduced, or even lost, in some animals cannot be discarded due to the time interval between the potential infection date and sample collection. At least in cats, the peak of nAbs titers is detected at 10 days after the infection and decreased to the limit of detection of the technique within 110 days [140,235].

Our study showed an overall higher capacity of SARS-CoV-2 neutralization in cats compared to dogs. In humans, higher levels of nAbs have been related to the severity of COVID-19 [240]. Although significant clinical disease seems to be sporadic in pets, different SARS-CoV-2 susceptibility at species level [64,66] may explain differences in humoral responses between species. Viral shedding and tissue tropism have been experimentally demonstrated in both cats and ferrets, whereas no-viral shedding and non-viral replication has been shown in dogs, at least against the ancestral variant [64,66]. Anyways, the differences in susceptibility to SARS-CoV-2 among animal species are not fully understood. Another related factor may be the presence and/or distribution of the ACE2 host cell receptor in these species, as well as the binding affinity between the spike of SARS-CoV-2 and their ACE2 receptors [60]. Low ACE2 levels in the respiratory tract (lung, trachea, and turbinate) from dogs could prevent efficient SARS-CoV-2 replication, whereas high levels of ACE2 in the respiratory tract from cats and ferrets [60,243] may account for a more efficient viral replication in these species.

This study also included the quantitative correlation between the RBD Inhibition ELISA (ELISA-1), used for initial screening, and the pVNT,

considered the gold standard technique. As mentioned, the pVNT used in this study was based on pseudoviruses expressing the S glycoprotein of SARS-CoV-2, incorporating mutations associated with each VOC variant. The RBD, situated in the S glycoprotein, is responsible for recognizing and binding the ACE2 receptor [24]. The ELISA kit used here is based on the RBD sequence of the ancestral variant firstly detected in Wuhan (China). This may explain the higher correlation of this ELISA with the results obtained by the pseudotype expressing the S glycoprotein from the B.1 ancestral variant ($R^2 = 0.77$) in comparison to the other variants. In parallel, this may explain the lower correlation observed between this ELISA and the Omicron pseudovirus assay ($R^2 = 0.62$). Besides, a high specificity of the ELISA was observed, as all ELISA-positive samples were also positive for the pVNT, at least when using the pseudovirus of the B.1 ancestral variant. Further details about the sensitivity and specificity of the ELISA-1 are described and discussed in the *Chapter 7* of this *PhD Thesis*.

From the time that this study was completed (January 2022) until May 2024, there have been advances within the knowledge of the impact of SARS-CoV-2 variants in companion animals. As in humans, differences in susceptibility across different variants in cats, dogs and ferrets have been demonstrated experimentally and naturally [71,72,77]. These species demonstrated to be less susceptible, or even non-susceptible (ferrets), to infection with Omicron and its sub-lineages upon natural and/or experimental conditions [244]. Similar to what is observed in humans, Omicron and its sub-lineages are less virulent and less immunogenic in this group of animal species [72,75,244–246].

In summary, we confirmed evidence of exposure and acute infection by SARS-CoV-2 and its variants in pets from North-Eastern Spain. Although the prevalence of active infection was low, the presence of nAbs in higher at-

risk pets (from COVID-19 households) was relatively high (close to 25% in cats, 10% in dogs, and 40% in ferrets). Thus, taking preventive measures, such as maintaining physical distance when symptoms resembling those of SARS-CoV-2 are noticed or upon receiving a positive COVID-19 diagnosis, and cleaning and disinfecting contaminated surfaces, may contribute prevent viral spill over events and new infections. In addition, considering that SARS-CoV-2 continues to evolve genetically and is still circulating in human population, monitoring and studying the susceptibility of companion animals remains important.

Supplementary Table 3.1 List of animals that tested positive by RT-qPCR (in bold) and/or by ELISA-1. NA*: Not-analyzed.

Animal ID	Specie	Collection Date	COVID-19 (+) household	Gender	RT-qPCR (Cq value nasal swab)	RT-qPCR (Cq value rectal swab)	RT-qPCR (Cq value lung tissue)	Variant of SARS-CoV-2	RBD Inhibition ELISA (% Inhibition)
1	**Cat (C1)**	**April 2020**	**Yes**	**Male**	**UpE: 33.69; RdRp: 34.01; N: 35.1**	**Negative**	**Negative**	**Ancestral variant (B.1 lineage; D614G)**	**96.25**
2	Cat	April 2020	Yes	Male	Negative	Negative	Negative	-	96.59
3	Cat	May 2020	Yes	Male	Negative	Negative	NA*	-	96.87
4	Dog	May 2020	Yes	Male	Negative	NA	NA	-	52.89
5	Dog	June 2020	Yes	Male	NA	NA	NA	-	41.50
6	Cat	June 2020	Yes	Female	Negative	Negative	NA	-	96.87
7	Cat	June 2020	Yes	Female	Negative	NA	NA	-	96.67
8	Dog	July 2020	Yes	Female	Negative	Negative	NA	-	51.07
9	Dog	Dec 2020	Yes	Male	NA	NA	NA	-	52.87
10	Cat	Jan 2021	Yes	Female	NA	NA	NA	-	97.00
11	**Dog (D1)**	**Feb 2021**	**Yes**	**Male**	**UpE: 13.21;**	**UpE: 24.68; RdRP:**	**Negative**	**Alpha (B.1.1.7)**	**NA**

					RdRP: 19.39; N: 19.83	**29.73; N: 30.92**			
12	Stray cat	April 2021	Stray cat	Unknown	Negative	Negative	NA	-	96.06
13	Stray cat	May 2021	Stray cat	Unknown	Negative	Negative	NA	-	96.83
14	Dog	June 2021	Yes	Male	Negative	Negative	NA	-	30.62
15	Dog	June 2021	Yes	Female	Negative	Negative	NA	-	85.51
16	Cat	June 2021	No	Male	Negative	Negative	NA	-	35.16
17	Dog	July 2021	No	Female	Negative	Negative	NA	-	36.06
18	Cat	June 2021	No	Male	Negative	Negative	NA	-	89.93
19	**Dog (D2)**	**July 2021**	**Yes**	**Female**	**UpE: 34.35; RdRP: 35.76; N: >40**	**Negative**	**NA**	**Delta (B.1.617.2)**	**67.93**
20	Ferret	July 2021	Yes	Male	Negative	Negative	NA	-	89.50
21	Ferret	July 2021	Yes	Male	Negative	Negative	NA	-	74.38
22	Stray cat	Aug 2021	Stray cat	Female	Negative	Negative	NA	-	34.20
23	Dog	Sept 2021	Yes	Male	Negative	Negative	NA	-	34.35
24	Dog	Sept 2021	No	Male	Negative	Negative	NA	-	37.22
25	Dog	Oct 2021	Unknown	Unknown	Negative	Negative	Negative	-	59.31
26	Cat	Dec 2021	Unknown	Unknown	Negative	Negative	Negative	-	92.15

27	Dog	Nov 2021	No	Male	Negative	Negative	NA	-	40.62
28	Dog	Nov 2021	No	Male	Negative	Negative	NA	-	36.54
29	Cat	Jan 2022	Yes	Female	Negative	Negative	NA	-	89.93
30	Cat	June 2021	Yes	Male	Negative	Negative	NA	-	96.45
31	Dog	July 2021	Yes	Male	Negative	Negative	NA	-	60.21
32	Dog	July 2021	Yes	Male	Negative	Negative	NA	-	62.21
33	Dog	July 2021	Yes	Female	Negative	Negative	NA	-	54.12
34	Dog	Aug 2021	Yes	Male	Negative	Negative	NA	-	45.60
35	Dog	Oct 2021	Yes	Female	Negative	Negative	NA	-	96.14
36	Cat	Oct 2021	Yes	Male	Negative	Negative	NA	-	96.26
37	Dog	Oct 2021	Yes	Male	Negative	Negative	NA	-	40.48
38	Dog	Oct 2021	Yes	Male	Negative	Negative	NA	-	45.40
39	Dog	Dec 2021	Yes	Female	Negative	Negative	NA	-	41.28
40	Dog	Dec 2021	Yes	Female	Negative	Negative	NA	-	41.41
41	Cat	Jan 2022	Yes	Male	Negative	Negative	NA	-	95.6

Appendix 3.1

Case Report

First Detection of SARS-CoV-2 Delta (B.1.617.2) Variant of Concern in a Dog with Clinical Signs in Spain

Leira Fernández-Bastit [1], Jordi Rodon [1], Edwards Pradenas [2], Silvia Marfil [2], Benjamin Trinité [2], Mariona Parera [2], Núria Roca [1], Anna Pou [1], Guillermo Cantero [1], Cristina Lorca-Oró [1], Jorge Carrillo [2,3], Nuria Izquierdo-Useros [2,3], Bonaventura Clotet [2,4], Marc Noguera-Julián [2,4], Julià Blanco [2,3,4], Júlia Vergara-Alert [1] and Joaquim Segalés [5,6,*]

1 Institut de Recerca i Tecnologia Agroalimentàries (IRTA), Centre de Recerca en Sanitat Animal (CReSA, IRTA-UAB), Campus de la UAB, 08193 Cerdanyola del Vallès, Spain; leirapaula.fernandez@irta.cat (L.F.-B.); jordi.rodon@irta.cat (J.R.); nuria.roca@irta.cat (N.R.); anna.pou@irta.cat (A.P.); guillermo.cantero@irta.cat (G.C.); cristina.lorca@irta.cat (C.L.-O.); julia.vergara@irta.cat (J.V.-A.)
2 IrsiCaixa AIDS Research Institute, 08916 Badalona, Spain; epradenas@irsicaixa.es (E.P.); SMarfil@irsicaixa.es (S.M.); btrinite@irsicaixa.es (B.T.); MParera@irsicaixa.es (M.P.); jcarrillo@irsicaixa.es (J.C.); nizquierdo@irsicaixa.es (N.I.-U.); bclotet@irsicaixa.es (B.C.); mnoguera@irsicaixa.es (M.N.-J.); jblanco@irsicaixa.es (J.B.)
3 IrsiCaixa AIDS Research Institute, Germans Trias i Pujol Research Institute (IGTP), Can Ruti Campus, 08916 Badalona, Spain
4 Infectious Diseases and Immunity, Faculty of Medicine, University of Vic-Central University of Catalonia (UVic-UCC), 08500 Barcelona, Spain
5 Centre de Recerca en Sanitat Animal (CReSA), Institut de Recerca en Tecnologies Agroalimentaries (IRTA), Campus de la UAB, 08193 Barcelona, Spain
6 Departament de Sanitat i Anatomia Animals, Facultat de Veterinaria, Universitat Autònoma de Barcelona, 08193 Cerdanyola del Vallès, Spain
* Correspondence: joaquim.segales@irta.cat

Abstract: Several cases of naturally infected dogs with severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) have been reported despite the apparently low susceptibility of this species. Here, we document the first reported case of infection caused by the Delta (B.1.617.2) variant of concern (VOC) in a dog in Spain that lived with several household members suffering from Coronavirus Infectious Disease 2019 (COVID-19). The animal displayed mild digestive and respiratory clinical signs and had a low viral load in the oropharyngeal swab collected at the first sampling. Whole-genome sequencing indicated infection with the Delta variant, coinciding with the predominant variant during the fifth pandemic wave in Spain. The dog seroconverted, as detected 21 days after the first sampling, and developed neutralizing antibodies that cross-neutralized different SARS-CoV-2 variants. This study further emphasizes the importance of studying the susceptibility of animal species to different VOCs and their potential role as reservoirs in the context of COVID-19.

Keywords: SARS-CoV-2; B.1.617.2; Delta variant; variants of concern; COVID-19; dog; pets; transmission; reverse zoonosis

Citation: Fernández-Bastit, L.; Rodon, J.; Pradenas, E.; Marfil, S.; Trinité, B.; Parera, M.; Roca, N.; Pou, A.; Cantero, G.; Lorca-Oró, C.; et al. First Detection of SARS-CoV-2 Delta (B.1.617.2) Variant of Concern in a Dog with Clinical Signs in Spain. *Viruses* **2021**, *13*, 2526. https://doi.org/10.3390/v13122526

Academic Editor: F. Javier Salguero

Received: 19 November 2021
Accepted: 14 December 2021
Published: 16 December 2021

Publisher's Note: MDPI stays neutral with regard to jurisdictional claims in published maps and institutional affiliations.

Copyright: © 2021 by the authors. Licensee MDPI, Basel, Switzerland. This article is an open access article distributed under the terms and conditions of the Creative Commons Attribution (CC BY) license (https://creativecommons.org/licenses/by/4.0/).

1. Introduction

SARS-CoV-2 is responsible for the ongoing Coronavirus Infectious Disease 2019 (COVID-19). Since the initial outbreak in Wuhan (China) at the end of 2019, the World Health Organization (WHO) has reported more than 270 million cases of COVID-19, causing approximately 5.3 million deaths worldwide (WHO, accessed on 15 December 2021, https://covid19.who.int/) [1]. The massive and rapid transmission of SARS-CoV-2 has led to the emergence of several viral variants, some of which have raised high concern due to their impact on transmissibility, mortality and their putative capacity to escape from immune responses generated after infection or vaccination [2].

To date, there have been four globally recognized variants of concern (VOC), including Alpha or lineage B.1.1.7 (first described in the UK) [3], Beta or lineage B.1.351 (initially identified in South Africa) [4], Gamma or lineage P.1 (first described in Brazil) [5], and Delta or lineage B.1.617.2 (initially detected in India) [6]. The appearance of these VOCs resulted from the accumulation of mutations along the whole SARS-CoV-2 genome; however, those located in the gene that codes for the spike (S) protein have been emphasized because the S protein mediates viral entry into target cells [7–9]. The binding of the S protein to the angiotensin-converting enzyme 2 (ACE2), identified as the main cellular receptor for SARS-CoV-2 entry, determines infectivity, tropism and host range [10].

Bats are believed to be the original host of SARS-CoV-2; however, it is still unclear whether an intermediate host eventually transferred the virus to humans [10,11]. Since the beginning of the pandemic, several domestic and wild animals have shown to be susceptible to SARS-CoV-2 infection [12,13]. Moreover, reverse zoonosis episodes have been documented on farms, in zoos and in familiar households [14–17]. Recently, by infecting wild-type mice, it has been shown that some SARS-CoV-2 variants display an increased virulence in humanized ACE2 transgenic mice and a broadened host range [18,19]. However, little is known about the capabilities of VOC in terms of causing differential virulence or expanded infection tropism in animal species undergoing natural SARS-CoV-2 infection.

Since pet species are at high risk of SARS-CoV-2 exposure due to close contact with their owners, it is crucial to monitor VOC transmission events and understand whether they could pose a higher risk for these animal populations. SARS-CoV-2 transmission from humans to dogs has been described in several parts of the world with early pandemic variants [16,20,21], and recently with both the Alpha (B.1.1.7) [22,23] and the Delta (B.1.617.2) VOCs [24]. Here, we document the first reported case of a symptomatic dog infected with the Delta VOC (B.1.617.2) in Spain, which occurred during the fifth wave of SARS-CoV-2 infection. This dog was living with owners who had been diagnosed with COVID-19 one week prior to the dog developing clinical signs, confirming that transmission of the Delta variant from human to dogs is possible and that animals may develop mild clinical signs similar to those in humans.

2. Materials and Methods

2.1. Clinical Evaluation and Sample Collection

By mid-July 2021, a 13-year-old female Breton dog developed respiratory and digestive signs coinciding with the timing of their owners suffering from COVID-19 (Figure 1). Since mild respiratory signs were still present approximately two weeks later, an oropharyngeal swab was collected on 27 July 2021. On 3 August 2021, an oropharyngeal and a rectal swab were collected from the dog again despite it no longer displaying any clinical signs. Blood extraction for serological analysis was performed at two different time points: on 5 August 2021 (serum 1; Se1) and on 28 September 2021 (serum 2; Se2) (Figure 1). Blood samples were centrifuged at $1800\times g$ for 10 min at 4 °C. The obtained sera were inactivated for 1 h at 56 °C and then stored at −20 °C until further use. All dog samples were collected at the Hospital Clínic Veterinari of the Universitat Autònoma de Barcelona (UAB, Bellaterra, Barcelona, Spain).

2.2. RNA Extraction and Detection by RT-qPCR

Oropharyngeal and rectal swabs were transferred into cryotubes containing 500 μL DMEM (Lonza, Basel, Switzerland) supplemented with 100 U/mL penicillin, 100 μg/mL streptomycin, and 2 mM glutamine (all from Gibco Life Technologies, Madrid, Spain) and finally vortexed. Viral RNA was extracted using the Indimag Pathogen kit (Indical Biosciences, Leipzig, Germany) on a Biosprint 96 workstation (Qiagen, Hilden, Germany), according to the manufacturer's instructions. Detection of SARS-CoV-2 RNA was achieved following a previously described protocol targeting the envelope protein (E)-encoding gene [25] by an RT-qPCR method, applying minor modifications [26]. RT-qPCR was carried out using AgPath-ID™ One-Step RT-PCR Reagents (Applied Biosystems, Life Technologies,

Waltham, MA, USA). Amplification was achieved by using a 7500 Fast Real-Time PCR System (Applied Biosystems, Life Technologies, Waltham, MA, USA) (10 min at 50 °C; 10 s at 95 °C; 45 cycles of 15 s at 94 °C; and 30 s at 58 °C). Samples with a Cq value $\leq$ 40 were considered positive for SARS-CoV-2. To confirm the result, positive samples were also tested by RT-qPCR targeting the RNA-dependent RNA polymerase gene (RdRp) specific to the SARS-CoV-2 [25].

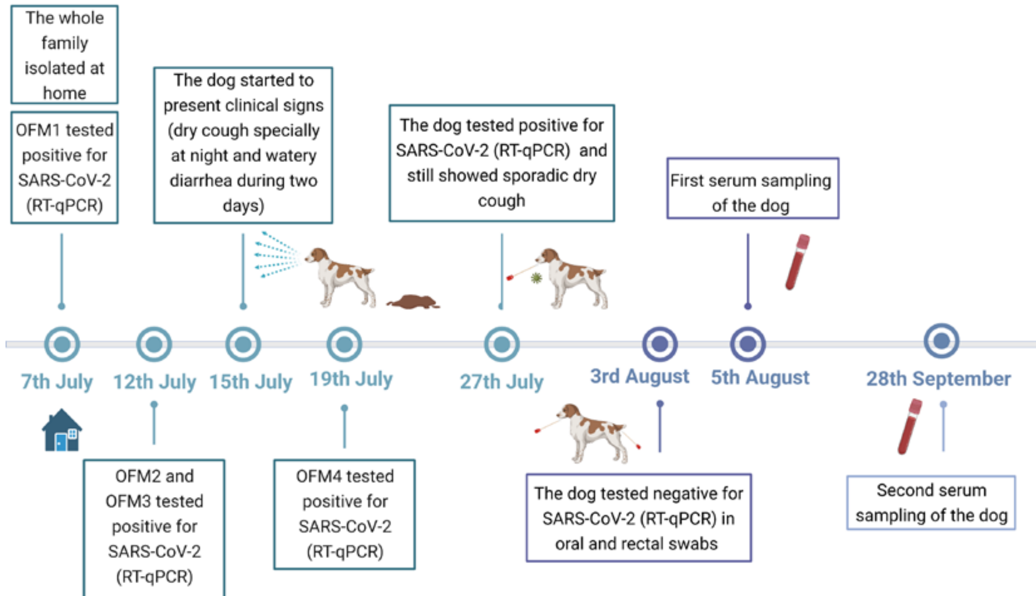

Figure 1. Chronological events relating to the SARS-CoV-2 Delta variant infection of the dog. The timeline shows when the owner's family members and the dog tested positive, as well as the dates that samples were collected. Abbreviations: Owner Family Members (OFM). Reverse transcription quantitative-polymerase chain reaction (RT-qPCR).

2.3. SARS-CoV-2 Genome Sequencing

For the positive samples, viral RNA was extracted and sequenced as previously described [27]. RNA was converted to cDNA with the PrimeScript^{TM} RT reagent kit (Takara Bio Europe SAS, Saint-Germain-en Laye, France) using a combination of oligo-dT and random hexamer methods, following the manufacturer's protocol. cDNA was used for viral DNA enrichment using the ARTIC-CoV v3 PCR protocol and the Q5 Hot-start HF polymerase. The amplified PCR products were used for sequencing-ready library preparation with the Illumina DNA LibPrep kit (Illumina, San Diego, CA, USA). Next, sequencing-ready libraries were loaded onto the Illumina MiSeq platform and a 150 bp paired-end sequencing kit (300 cycles). Raw data analysis was performed using the viralrecon pipeline (https://nf-co.re/viralrecon/1.0.0 (accessed on: 15 December 2021)). Sequence reads were quality-filtered, and adapter primer sequences were trimmed using Trimmomatic [28]. Sequencing reads were then aligned against the reference Wuhan/Hu-1/20219 variant (NCBI accession number: NC_045512.2) using the Bowtie2 tool [29], while consensus genomic sequence was called from the resulting alignments using iVarsoftware at the 25% threshold. Genomic sequence was classified by the Pangolin lineage classification system (v.3.1.16, lineages version 18 October 2021).

2.4. Neutralizing Antibody Detection by SARS-CoV-2 Receptor-Binding Inhibition ELISA

Seroneutralizing antibodies targeting RBD were measured with the GenScript cPass™ SARS-CoV-2 Neutralization Antibody Detection Kit (Genscript, the Netherlands), following the manufacturer's protocol. Serum samples (1:10 diluted) were mixed with equal volumes of recombinant HRP-conjugated RBD and incubated for 30 min at 37 °C. Next, 100 µL of each diluted sample was transferred to 96-well plates pre-coated with a recombinant hACE2

receptor and incubated for 15 min at 37 °C. After four washing steps, the substrate solution (tetramethylbenzidine substrate, TMB) was incubated for 15 min at room temperature, after which the stop solution was added. Absorbance values were read at 450 nm in an automatic microELISA reader, and the percentage of inhibition of each sample was determined using the following formula: %inhibition = (1 − (OD450 sample/OD450 of negative control)) × 100. Each of the samples and controls was included in duplicate (SD ≤ 10%). Inhibition >30% was considered as a positive neutralization.

2.5. SARS-CoV-2 Pseudoneutralization Assay

A pseudovirus-based neutralization assay of the Se2 sample was performed following a protocol previously described [30]. HIV reporter pseudoviruses expressing the SARS-CoV-2 S protein (from different VOCs) and Luciferase were generated. Control pseudoviruses were obtained by replacing the S protein expression plasmid with a VSV-G protein expression plasmid as reported previously [31]. For neutralization assay, 200 TCID50 of pseudovirus supernatant was preincubated with serial dilutions of the heat-inactivated plasma samples and then added onto ACE2 overexpressing HEK293T cells. After 48 h, cells were lysed with Britelite Plus Luciferase reagent (Perkin Elmer, Waltham, MA, USA). Luminescence was measured for 0.2 s with an EnSight Multimode Plate Reader (Perkin Elmer, Waltham, MA, USA).

The neutralization capacity of the plasma samples was calculated by comparing the experimental RLU calculated from infected cells treated with each plasma to the max RLUs (maximal infectivity calculated from infected untreated cells), background minimal signal (non-infected cells), and expressed as percent neutralization: %Neutralization = (RLUmax − RLUexperimental)/(RLUmax − RLUmin) × 100. The SNT50 was calculated by plotting and fitting neutralization values and the log of plasma dilution to a 4-parameters equation in Prism 9.0.2 (GraphPad Software, San Diego, CA, USA).

2.6. SARS-CoV-2 Neutralization Assay

A replicating-virus neutralization assay of the Se2 sample was performed as previously described [15]. The inactivated serum sample was first diluted at 1:10 and then 2-fold serially diluted in DMEM. Next, the diluted sample was mixed 1:1 with an isolate of SARS-CoV-2 (B.1 lineage) [27] and further incubated for 1 h at 37 °C. Each dilution mixture (in four replicates) was transferred onto Vero E6 (ATCC® repository, Manassas, VA, USA, CRL-1586™) cell monolayers containing 100 TCID50 of SARS-CoV-2 per well were cultured for 3 days at 37 °C and 5% CO_2. Then, the cytopathic effect of the SARS-CoV-2 was measured using the CellTiter-Glo luminescent cell viability assay (Promega, Madison, WI, USA), following the manufacturer's protocol. Luminescence was measured as relative luminescence units (RLU) in a Fluroskan Ascent FL luminometer (ThermoFisher Scientific, Waltham, MA, USA). The 50% serum virus neutralization titer (SNT50) was defined as the reciprocal dilution of the sample at which 50% of cells were protected.

The dose–response curve of the serum sample was adjusted to a non-linear fit regression model calculated with a normalized logistic curve with variable slope. Uninfected cells and untreated virus-infected cells were used as negative and positive controls of infection for data normalization (%Neutralization = (RLUmax − RLUexperimental)/(RLUmax − RLUmin) × 100), respectively. All statistical analyses were performed with GraphPad Prism 8.4.3 (GraphPad Software, Inc, San Diego, CA, USA).

3. Results

3.1. Clinical Follow-Up

On 15 July 2021, a 13-year-old female Breton dog developed respiratory signs, especially a dry cough at night, and digestive disorders (watery diarrhea for two days), at the time their owners suffered from COVID-19 (Figure 1). Some days before, on 5 July 2021, one of the owner's family members (OFM1) was confirmed as a contact of a COVID-19-affected patient. Then, on 7 July 2021, OFM1 tested positive for SARS-CoV-2 by RT-qPCR and

started developing symptoms including fever, dyspnea, and dizziness. He was finally hospitalized and diagnosed with bilateral pneumonia and severe respiratory insufficiency. The whole family was quarantined and also developed COVID-19-like symptomatology (fever, coughing and sneezing). Two of them (OFM2 and OFM3) were finally diagnosed with COVID-19 on 12 July 2021, while the last member (OFM4) of the family tested negative by RT-qPCR. It is noteworthy that OFM2 was vaccinated against SARS-CoV-2 three months before. On 19 July 2021, OFM4 was finally diagnosed with COVID-19 as well.

3.2. RNA Detection and SARS-CoV-2 Sequencing

The oropharyngeal swab from the dog collected on 27 July 2021 tested positive for SARS-CoV-2 UpE (Cq of 34.4) and RdRp (Cq of 35.8) genes by RT-qPCR. Eight days later, on 3 August 2021, the animal tested negative for the detection of SARS-CoV-2 RNA in both oropharyngeal and rectal swabs.

SARS-CoV-2 genomic RNA from the first oropharyngeal swab was successfully obtained (GISAID EPI ISL 6344510). The genomic sequence was classified as AY. 43, a sub-lineage within Delta/B.1.617.2 lineage.

3.3. Immune Response Elicited after SARS-CoV-2 Infection

The dog elicited neutralizing antibodies against the RBD of SARS-CoV-2, as determined from serum samples collected 21 days after the display of clinical signs by the receptor binding inhibition assay. The Se1 sample showed an inhibition titer of 68.9% (SD ± 2.40%), and the Se2 sample (two and a half months after displaying the initial clinical signs) had an inhibition of 67.6% (SD ± 0.07%). We then evaluated the neutralization activity of Se2 using a pseudovirus assay; Se2 was able to neutralize the Alpha (SNT50 = 1/260), the Beta (SNT50 = 1/881), the Gamma (SNT50 = 1/207), the WT (SNT50 = 1/340), and the Delta (SNT50 = 1/460) variants (Figure 2). Moreover, titers of neutralizing antibodies against a replicating SARS-CoV-2 isolate (B.1 Pango lineage) were also confirmed (SNT50 = 1/135.8).

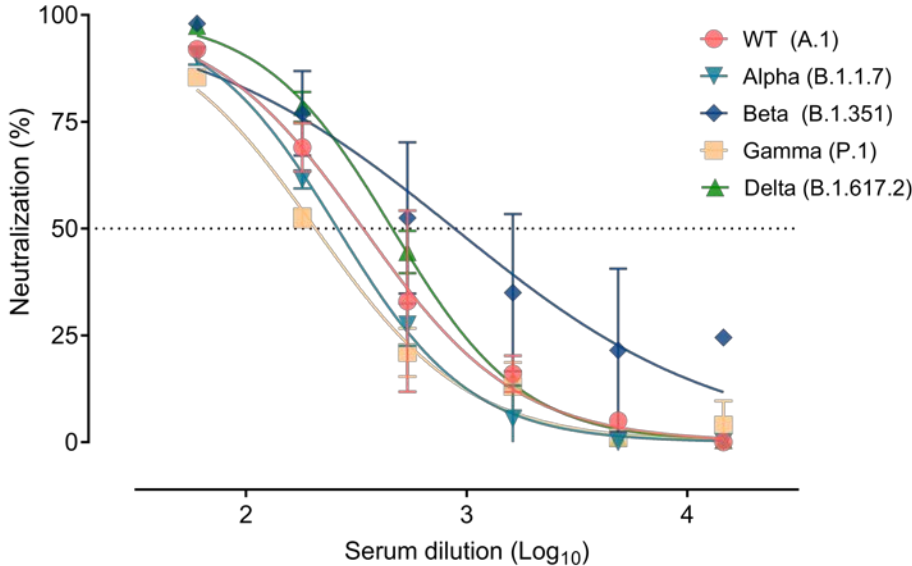

Figure 2. Neutralizing humoral responses developed after SARS-CoV-2 Delta variant infection of the dog. Neutralization assay of the serum sample 2 (Se2) against pseudoviruses expressing the spike proteins of the WT, the Alpha, the Beta, the Gamma and the Delta variants of SARS-CoV-2.

4. Discussion

This is the first case report of SARS-CoV-2 B.1.617.2 (Delta) VOC infecting a dog in Spain; the dog displayed respiratory and digestive clinical signs at the time of infection. It

is speculated that the animal became infected by close contact with its owners since they were diagnosed with COVID-19 a week before the dog displayed clinical signs.

Due to the large number of companion animals infected with SARS-CoV-2 since the beginning of the COVID-19 pandemic, SARS-CoV-2 infection in the dog in this study was suspected [14,16,32,33]. Low viral RNA loads in the oropharyngeal swab confirmed that the animal was infected with SARS-CoV-2; however, subsequent oropharyngeal and rectal swabs collected twenty days after the display of clinical signs were tested and were negative already. Whole-genome sequencing determined infection by the Delta (B.1.617.2) VOC, AY. 43 sub-lineage. Recently, a natural infection by the same variant was also reported in a dog in Kansas (USA) [24]. In addition, natural infections with the B.1.617.2 (Delta) variant have been reported in Asiatic lions, where a human-to-animal transmission was also suspected [34]. In this present study, samples from the owners of the studied dog were not available, but the existing epidemiological information suggests transmission from humans to the dog because the animal did not have other contacts. Furthermore, in agreement with our study, the majority of SARS-CoV-2 natural infections in companion animals, such as dogs and cats, have been reported in animals living in households with at least one SARS-CoV-2-infected owner [14,16,32,35]. In the present case, the infection occurred during the fifth wave of COVID-19 in Spain (July 2021), which was dominated by the Delta VOC variant [36].

The appearance of respiratory disorders in dogs was previously reported upon natural infection with the Alpha variant and was suspected with the Delta variant [23,24]. In fact, Doerksen et al. [24] could not unequivocally attribute observed clinical signs of the dog to the Delta VOC since the animal had other underlying conditions. Importantly, the dog in the present study also displayed digestive clinical signs for two days, which are compatible with SARS-CoV-2 infection. However, it is not possible to rule out the presence of concomitant infections or other conditions affecting the dog at the time of the clinical signs. The low viral load found in the animal in the first oropharyngeal swab sampling suggests that it was already clearing the virus because the clinical signs had started almost two weeks before. In any case, the clinical signs disappeared after the SARS-CoV-2 infection was cleared, supporting the effect of this virus in the clinical condition of the dog. Furthermore, seroconversion to SARS-CoV-2 was confirmed 21 days after the appearance of the clinical signs, and similar levels of antibodies were maintained after two and a half months. Despite the fact that the Delta variant was the variant infecting the dog, the humoral response generated was able to cross-neutralize against the other viral variants in vitro (Alpha, Beta, Gamma, and the first variant reported in Wuhan). Neutralizing responses were developed at similar levels against all the tested SARS-CoV-2 variants. However, the titers of neutralizing antibodies were not high when compared to severely infected human patients [30] and were similar to those that have been described in dogs infected with SARS-CoV-2 [16,22,23].

In summary, the present study confirms that the SARS-CoV-2 Delta VOC (B.1.617.2) can spread to animals exposed to COVID-19 environments and can potentially cause clinical infection. Here we reported the infection of a dog living in contact with COVID-19-positive family members. The dog displayed respiratory and digestive clinical signs during the time of infection, subsequently cleared the virus within twenty days and developed neutralizing responses to different SARS-CoV-2 variants. This case highlights the importance of studying the potential difference of host susceptibility upon transmission of SARS-CoV-2 VOC from humans to animals.

Author Contributions: J.R., G.C., J.V.-A., and J.S. conceived and designed the study. L.F.-B., J.R., E.P., S.M., B.T., M.P., N.R., A.P., M.N.-J., and G.C. performed the sample collection or laboratory experiments. L.F.-B., J.R., E.P., S.M., J.B., M.N.-J., J.V.-A., and J.S. analyzed the data. L.F.-B., J.R., C.L.-O., J.C., N.I.-U., and B.C. interpreted the data. The manuscript was written by L.F.-B., J.R., J.V.-A., and J.S. All authors have read and agreed to the published version of the manuscript.

Funding: This research was partially funded by the BBVA Foundation as part of the project "Investigation on the potential role of pets as animal reservoirs for SARS-CoV-2". M.N.-J. acknowledges funding from Fundació la Marató (202126-30-21). The authors also acknowledge the crowdfunding initiative # Yomecorono, available online at: https://www.yomecorono.com (accessed on 11 November 2021). IRTA is supported by CERCA Programme/Generalitat de Catalunya. E.P. was supported by a doctoral grant from the National Agency for Research and Development of Chile (ANID): 72180406.

Institutional Review Board Statement: Not applicable.

Informed Consent Statement: Not applicable.

Data Availability Statement: All data are available upon request to the authors.

Conflicts of Interest: The authors declare no competing interests.

References

1. World Health Organization (WHO). Official COVID-19 Information. Available online: https://covid19.who.int/ (accessed on 15 December 2021).
2. SARS-CoV-2 Variants of Concern as of 12 November 2021. Available online: https://www.ecdc.europa.eu/en/covid-19/variants-concern (accessed on 12 November 2021).
3. Kirby, T. New variant of SARS-CoV-2 in UK causes surge of COVID-19. *Lancet. Respir. Med.* **2021**, *9*, e20–e21. [CrossRef]
4. Happi, A.N.; Ugwu, C.A.; Happi, C.T. Tracking the emergence of new SARS-CoV-2 variants in South Africa. *Nat. Med.* **2021**, *27*, 372–373. [CrossRef]
5. Faria, N.R.; Claro, I.M.; Candido, D.; Moyses Franco, L.A.; Andrade, P.S.; Coletti, T.M.; Silva, C.A.M.; Sales, F.C.; Manuli, E.R.; Aguiar, R.S.; et al. Genomic characterisation of an emergent SARS-CoV-2 lineage in Manaus: Preliminary findings—SARS-CoV-2 coronavirus/nCoV-2019 Genomic Epidemiology. Available online: https://virological.org/t/genomic-characterisation-of-an-emergent-sars-cov-2-lineage-in-manaus-preliminary-findings/586 (accessed on 18 October 2021).
6. Weekly Epidemiological Update on COVID-19. 2021. Available online: https://www.who.int/publications/m/item/weekly-epidemiological-update-on-covid-19---10-august-2021 (accessed on 15 December 2021).
7. Tegally, H.; Wilkinson, E.; Giovanetti, M.; Iranzadeh, A.; Fonseca, V.; Giandhari, J.; Doolabh, D.; Pillay, S.; San, E.J.; Msomi, N.; et al. Detection of a SARS-CoV-2 variant of concern in South Africa. *Nature* **2021**, *592*, 438–443. [CrossRef]
8. Tang, J.W.; Tambyah, P.A.; Hui, D.S. Emergence of a New SARS-CoV-2 Variant in the UK. *J. Infect.* **2021**, *82*(4), e27–e28. [CrossRef]
9. Sabino, E.C.; Buss, L.F.; Carvalho, M.P.S.; Prete, C.A.; Crispim, M.A.E.; Fraiji, N.A.; Pereira, R.H.M.; Parag, K.V.; da Silva Peixoto, P.; Kraemer, M.U.G.; et al. Resurgence of COVID-19 in Manaus, Brazil, despite high seroprevalence. *Lancet* **2021**, *397*, 452–455. [CrossRef]
10. Zhou, P.; Yang, X.L.; Wang, X.G.; Hu, B.; Zhang, L.; Zhang, W.; Si, H.R.; Zhu, Y.; Li, B.; Huang, C.L.; et al. A pneumonia outbreak associated with a new coronavirus of probable bat origin. *Nature* **2020**, *579*, 270–273. [CrossRef]
11. Deng, J.; Jin, Y.; Liu, Y.; Sun, J.; Hao, L.; Bai, J.; Huang, T.; Lin, D.; Jin, Y.; Tian, K. Serological survey of SARS-CoV-2 for experimental, domestic, companion and wild animals excludes intermediate hosts of 35 different species of animals. *Transbound. Emerg. Dis.* **2020**, *67*, 1745–1749. [CrossRef]
12. Hobbs, E.C.; Reid, T.J. Animals and SARS-CoV-2: Species susceptibility and viral transmission in experimental and natural conditions, and the potential implications for community transmission. *Transbound. Emerg. Dis.* **2021**, *68*, 1850–1867. [CrossRef] [PubMed]
13. Muñoz-Fontela, C.; Dowling, W.E.; Funnell, S.G.P.; Gsell, P.S.; Riveros-Balta, A.X.; Albrecht, R.A.; Andersen, H.; Baric, R.S.; Carroll, M.W.; Cavaleri, M.; et al. Animal models for COVID-19. *Nature* **2020**, *586*, 509–515. [CrossRef] [PubMed]
14. Segalés, J.; Puig, M.; Rodon, J.; Avila-Nieto, C.; Carrillo, J.; Cantero, G.; Terrón, M.T.; Cruz, S.; Parera, M.; Noguera-Julián, M.; et al. Detection of SARS-CoV-2 in a cat owned by a COVID-19-affected patient in Spain. *Proc. Natl. Acad. Sci. USA* **2020**, *117*, 24790–24793. [CrossRef]
15. Fernández-Bellon, H.; Rodon, J.; Fernández-Bastit, L.; Almagro, V.; Padilla-Solé, P.; Lorca-Oró, C.; Valle, R.; Roca, N.; Grazioli, S.; Trogu, T.; et al. Monitoring natural SARS-CoV-2 infection in lions (Panthera leo) at the Barcelona zoo: Viral dynamics and host responses. *Viruses* **2021**, *13*, 1683. [CrossRef]
16. Sit, T.H.C.; Brackman, C.J.; Ip, S.M.; Tam, K.W.S.; Law, P.Y.T.; To, E.M.W.; Yu, V.Y.T.; Sims, L.D.; Tsang, D.N.C.; Chu, D.K.W.; et al. Infection of dogs with SARS-CoV-2. *Nature* **2020**, *586*, 776–778. [CrossRef]
17. Oreshkova, N.; Molenaar, R.-J.; Vreman, S.; Harders, F.; Munnink, B.B.O.; Hakze, R.; Gerhards, N.; Tolsma, P.; Bouwstra, R.; Sikkema, R.; et al. SARS-CoV-2 infection in farmed minks, the Netherlands, April and May 2020. *Euro Surveill.* **2020**, *25*, 2001005. [CrossRef]
18. Montagutelli, X.; Prot, M.; Levillayer, L.; Salazar, E.B.; Jouvion, G.; Conquet, L.; Donati, F.; Albert, M.; Gambaro, F.; Behillil, S.; et al. The B1.351 and P.1 variants extend SARS-CoV-2 host range to mice. *bioRxiv* **2021**. [CrossRef]

19. Tarrés-Freixas, F.; Trinité, B.; Pons-Grífols, A.; Romero-Durana, M.; Riveira-Muñoz, E.; Ávila-Nieto, C.; Pérez, M.; Garcia-Vidal, E.; Pérez-Zsolt, D.; Muñoz-Basagoiti, J.; et al. SARS-CoV-2 B.1.351 (beta) variant shows enhanced infectivity in K18-hACE2 transgenic mice and expanded tropism to wildtype mice compared to B.1 variant. *bioRxiv* **2021**. [CrossRef]
20. Patterson, E.I.; Elia, G.; Grassi, A.; Giordano, A.; Desario, C.; Medardo, M.; Smith, S.L.; Anderson, E.R.; Prince, T.; Patterson, G.T.; et al. Evidence of exposure to SARS-CoV-2 in cats and dogs from households in Italy. *Nat. Commun.* **2020**, *11*, 6231. [CrossRef]
21. Fritz, M.; Rosolen, B.; Krafft, E.; Becquart, P.; Elguero, E.; Vratskikh, O.; Denolly, S.; Boson, B.; Vanhomwegen, J.; Gouilh, M.A.; et al. High prevalence of SARS-CoV-2 antibodies in pets from COVID-19+ households. *One Health* **2021**, *11*, 100192. [CrossRef]
22. Barroso-ar, S.; Dom, L.; Sánchez-Vizcaíno, J.M. First Detection of SARS-CoV-2 B.1.1.7 Variant of Concern in an Asymptomatic Dog in Spain. *Viruses* **2021**, *13*, 1379. [CrossRef] [PubMed]
23. Miró, G.; Regidor-cerrillo, J.; Checa, R.; Diezma-díaz, C. SARS-CoV-2 Infection in One Cat and Three Dogs Living in COVID-19-Positive Households in Madrid, Spain. *Front. Vet. Sci.* **2021**, *8*, 341. [CrossRef]
24. Doerksen, T.; Lu, A.; Noll, L.; Almes, K.; Bai, J.; Upchurch, D.; Palinski, R. Near-Complete Genome of SARS-CoV-2 Delta (AY.3) Variant Identified in a Dog in Kansas, USA. *Viruses* **2021**, *13*, 2104. [CrossRef] [PubMed]
25. Corman, V.M.; Landt, O.; Kaiser, M.; Molenkamp, R.; Meijer, A.; Chu, D.K.W.; Bleicker, T.; Brünink, S.; Schneider, J.; Schmidt, M.L.; et al. Detection of 2019 novel coronavirus (2019-nCoV) by real-time RT-PCR. *Euro Surveil.* **2020**, *25*, 1–8. [CrossRef]
26. Brustolin, M.; Rodon, J.; Rodríguez de la Concepción, M.L.; Ávila-Nieto, C.; Cantero, G.; Pérez, M.; Te, N.; Noguera-Julián, M.; Guallar, V.; Valencia, A.; et al. Protection against reinfection with D614- or G614-SARS-CoV-2 isolates in golden Syrian hamster. *Emerg. Microbes Infect.* **2021**, *10*, 797–809. [CrossRef]
27. Rodon, J.; Muñoz-Basagoiti, J.; Perez-Zsolt, D.; Noguera-Julian, M.; Paredes, R.; Mateu, L.; Quiñones, C.; Perez, C.; Erkizia, I.; Blanco, I.; et al. Identification of Plitidepsin as Potent Inhibitor of SARS-CoV-2-Induced Cytopathic Effect After a Drug Repurposing Screen. *Front. Pharmacol.* **2021**, *12*, 676. [CrossRef]
28. Bolger, A.M.; Lohse, M.; Usadel, B. Trimmomatic: A flexible trimmer for Illumina sequence data. *Bioinformatics* **2014**, *30*, 2114–2120. [CrossRef] [PubMed]
29. Langmead, B.; Salzberg, S.L. Fast gapped-read alignment with Bowtie 2. *Nat. Methods* **2012**, *9*, 357–359. [CrossRef]
30. Trinité, B.; Tarrés-Freixas, F.; Rodon, J.; Pradenas, E.; Urrea, V.; Marfil, S.; Luisa, M.; De La Concepción, R.; Ávila-Nieto, C.; Aguilar-Gurrieri, C.; et al. SARS-CoV-2 infection elicits a rapid neutralizing antibody response that correlates with disease severity. *Sci. Rep.* **2021**, *11*, 2608. [CrossRef]
31. Díez, J.M.; Romero, C.; Cruz, M.; Vandeberg, P.; Merritt, W.K.; Pradenas, E.; Trinité, B.; Blanco, J.; Clotet, B.; Willis, T.; et al. Anti-SARS-CoV-2 hyperimmune globulin demonstrates potent neutralization and antibody-dependent cellular cytotoxicity and phagocytosis through N and S proteins. *J. Infect. Dis.* **2021**. [CrossRef] [PubMed]
32. Newman, A.; Smith, D.; Ghai, R.R.; Wallace, R.M.; Torchetti, M.K.; Loiacono, C.; Murrell, L.S.; Carpenter, A.; Moroff, S.; Rooney, J.A.; et al. First Reported Cases of SARS-CoV-2 Infection in Companion Animals—New York, March–April 2020. *MMWR. Morb. Mortal. Wkly. Rep.* **2020**, *69*, 710–713. [CrossRef]
33. Shi, J.; Wen, Z.; Zhong, G.; Yang, H.; Wang, C.; Huang, B.; Liu, R.; He, X.; Shuai, L.; Sun, Z.; et al. Susceptibility of ferrets, cats, dogs, and other domesticated animals to SARS-coronavirus 2. *Science* **2020**, *368*, 1016–1020. [CrossRef]
34. Mishra, A.; Kumar, N.; Bhatia, S.; Aasdev, A.; Kanniappan, S.; Sekhar, A.T.; Gopinadhan, A.; Silambarasan, R.; Sreekumar, C.; Dubey, C.K.; et al. Sars-cov-2 delta variant among asiatic lions, india. *Emerg. Infect. Dis.* **2021**, *27*, 2723–2725. [CrossRef] [PubMed]
35. Hamer, S.A.; Pauvolid-Corrêa, A.; Zecca, I.B.; Davila, E.; Auckland, L.D.; Roundy, C.M.; Tang, W.; Torchetti, M.K.; Killian, M.L.; Jenkins-Moore, M.; et al. SARS-CoV-2 infections and viral isolations among serially tested cats and dogs in households with infected owners in texas, usa. *Viruses* **2021**, *13*, 938. [CrossRef] [PubMed]
36. SARS-COV-2 Delta Variant Now Dominant in much of the European Region and Efforts must be Reinforced to Prevent TRANSMISSION, Warn WHO/Europe and ECDC. Available online: https://www.ecdc.europa.eu/en/news-events/sars-cov-2-delta-variant-now-dominant-european-region (accessed on 3 November 2021).

CHAPTER 4.

STUDY 2

Survey of SARS-CoV-2 in captive and free-ranging wildlife from Spain.

Manuscript submitted for publication.

4.1 Introduction

Wildlife has been proposed as the source of significant emerging viral diseases in humans (zoonosis), including the COVID-19 caused by the SARS-CoV-2 [3]. The emergence of these zoonosis may, in part, be attributed to human behavior (*e.g.,* hunting practices or consumption of wild meat), population growth and urbanization, and the modification of wildlife habitat structure, which lead to evident human-animal interactions [247]. Although humans are the main host of SARS-CoV-2, from the outset of COVID-19, the SARS-CoV-2 has demonstrated the ability to cross-species barriers in free-ranging and captive scenarios [248].

According to early predictive *in silico* studies, NHP, several carnivore species (mainly felines), and cetaceans were considered at moderate or high risk of infection with SARS-CoV-2 [37,61,249]. These studies were mainly based on comparative and structural analyses of the sequence of the ACE2, the host cell receptor of SARS-CoV-2 [27]. The ACE2 sequence of NHP, carnivores, and cetaceans demonstrated to have high homology with the hACE2, also considering critical amino acid residues for binding with the SARS-CoV-2 RBD [37,249,250]. Additionally, predictive results for the risk of infection based on comparative analysis of the TMPRSS2 sequence of these species, were consistent with those obtained from the ACE2 sequence [249]. The TMPRSS2 is a protease that facilitates the fusion of cell and virus membranes by S protein priming and subsequent viral entry [27]. So far, natural and experimental SARS-CoV-2 infections already confirmed the ability of the virus to infect many NHP and carnivore species [248].

Zoological parks are scenarios in which SARS-CoV-2 animal infections have been documented globally during the pandemic. Most infections were described in Great Apes (*Gorilla gorilla*), tigers (*Panthera*

tigris), lions (*Panthera leo*), and in a variety of large and medium-sized felines [248]. Other mammals, mainly carnivores, have also been infected under captive conditions worldwide including species from the family *Atelidae* (brown-headed spider monkey [*Ateles fusciceps*]), *Canidae* (red fox [*Vulpes vulpes*]), *Hyaenidae* (spotted hyena [*Crocuta crocuta*]), *Hippopotamidae* (hippopotamus [*Hippopotamus amphibius*]), *Mustelidae* (American mink [*Neovison vison*]; Asian small-clawed otter [*Aonyx cinereus*]), *Rhinocerotidae* (white rhinoceros [*Ceratotherium simum*]), and *Viverridae* (South American coati [*Nasua nasua*]) [176,178]. Sequencing analysis and/or epidemiological history supported RZ transmission as the origin of these animal infections [248].

Although animals living in a free-range environment are rarely as close to humans as domesticated or captive animals, the risk of SARS-CoV-2 infection in wildlife has also been proven [249,251,252]. Human household wastes, SARS-CoV-2 contaminated elements (*e.g.,* food and water), or contact with other susceptible animals (*e.g.,* farmed minks) are potential sources for the infection reported in free-ranging wild animals [180,187,188]. In this regard, many spillover events from humans to the WTD (*Odocoileus virginianus*), and even from WTD back to humans, have been described in the United States and Canada, based on sequencing analysis [183,185,253]. WTD are highly abundant in urban and peri-urban areas in North America, being in close contact with humans and human-produced waste. Of concern, SARS-CoV-2 and its variants are able to infect, persist, adapt and can be transmitted within the WTD population, suggesting that this species could serve as a reservoir for SARS-CoV-2 [96,183,253]. Besides, mustelid species have also been exposed and/or infected by SARS-CoV-2 in the wild [187,188,191]. *Mustelidae* species have been involved in one of the most important SARS-CoV-2 animal events to date owing to the number of outbreaks in mink farms in multiple

countries (the Netherlands, Denmark, US, Canada, France, Greece, Italy, Spain, Sweden, Poland, and Lithuania) [248]. Farming has proved to favor the spread of SARS-CoV-2 in other species, as the recently reported outbreak of SARS-CoV-2 Delta (B.1.617.2) variant in farmed beavers (Order Rodentia; *Castor fiber*) in Mongolia [254]. In this sense, several rodent species have shown susceptibility to a variety of SARS-CoV-2 variants (Alpha [B.1.1.7], Beta [B.1.351], Gamma [P.1] and Omicron [B.1.1.529]), although not to the ancestral SARS-CoV-2 lineage (B.1.) [106,107,255]. In northern Spain, two wild species of farming origin, the American mink (*Neovison vison*) and the coypu (Order Rodentia, *Myocastor coypus*) have significantly increased their populations in the past years and are considered exotic invasive. Both species live near aquatic environments, representing a threat to autochthonous biodiversity species [256–258]. However, the SARS-CoV-2 exposure and infection has yet to be evaluated for most exotic and native wild species in Spain.

The present work aimed to investigate SARS-CoV-2 exposure and infection of different free-ranging and captive wildlife species along the COVID-19 pandemic (from 2020 to 2023) in Spain.

4.2 Material and methods

Animals and samples

A total of 420 animals (119 captives and 301 free-ranging) from 40 different species were opportunistically sampled during the 2020-2023 COVID-19 pandemic to detect SARS-CoV-2 RNA and/or specific antibodies.

A total of 137 sera belonging to 33 different species from captive and wild environments from different regions of Spain were collected (**Figure 4.1; Table 4.1**). Serum samples (n = 33) collected before the COVID-19 pandemic (prior to 2019; pre-pandemic period) from 17 different species were included and considered as negative controls (**Supplementary Table 4.1**). Besides, oropharyngeal swabs, rectal swabs, and lung tissue samples were collected from 283 free-ranging animals (141 American minks, 2 Beech marten [*Martes foina*], 3 common genets [*Genetta genetta*], 48 coypus, 48 Eurasian badgers [*Meles meles*], 25 Eurasian otters [*Lutra lutra*], 1 European wildcat [*Felis silvestris silvestris*], and 15 red foxes) located in different regions of Catalonia (**Figure 4.1; Table 4.1).** Each type of sample was collected from almost all the animals depending on availability (282 of each type of sample). Oral and rectal swabs were collected using sterile dry swabs or flocked swabs in 2 mL VTM (Deltalab, S.L. Catalunya, Spain). Lung tissue samples were placed into cryotubes with 500µL DMEM (Lonza, Basel, Switzerland) supplemented with 100 U/mL penicillin, 100µg/mL streptomycin, and 2 mM glutamine (all from Gibo Life Technologies, Madrid, Spain) and containing single zinc-plated, steel 4.5-mm beads. All samples were kept at -20ºC until they were transported properly to the lab for further analysis.

Zoological animals were sampled by Zoo Management veterinarian specialists during routine health assessments or surgical interventions. Sera from free-ranging wildlife were collected by veterinarians from wildlife rehabilitation centers during routine health assessments. Oral swabs, rectal swabs, and lung tissues were sampled from free-ranging animals from Catalonia (Northeastern Spain) during necropsies at the Torreferrusa Wildlife Rehabilitation Centre (license number B2300083). All procedures followed Ethical Principles in Animal Research. Sera from free-ranging cetaceans were

obtained from individuals stranded in the Atlantic and Mediterranean coasts of Spain. Ethical approval by an Institutional Animal Care and Use Committee was not, therefore, deemed necessary. American minks and coypus, subjected to population control programs of the Government of the *Generalitat de Catalunya*, were sampled during necropsies.

Table 4.1 Wild Animals tested for SARS-CoV-2 RNA and/or antibodies. The sample size, animal source (captive or free-ranging), and animals tested for detection of SARS-CoV-2 RNA or antibodies are indicated. NA: not available.

Animal species	Family	Sample size	Animal source	SARS-CoV-2 RNA detection	SARS-CoV-2 antibody detection
Red panda *(Ailurus fulgens)*	*Ailuridae*	3	Captive zoo	NA	3
Fennec fox *(Vulpes zerda)*	*Canidae*	2	Captive zoo	NA	2
Grey wolf *(Canis lupus)*	*Canidae*	1	Captive zoo	NA	1
Iberian wolf *(Canis lupus signatus)*	*Canidae*	1	Captive zoo	NA	1
Red fox *(Vulpes vulpes)*	*Canidae*	15	Free-ranging	15	NA
Atlantic spotted dolphin *(Stenella frontalis)*	*Delphinidae*	1	Free-ranging	NA	1
Bottlenose dolphin (*Turisops truncatus*)	*Delphinidae*	46	Captive zoo	NA	48
		2	Free-ranging		
Killer whale *(Orcinus orca)*	*Delphinidae*	8	Captive zoo	NA	8
Risso's dolphin *(Grampus griseus)*	*Delphinidae*	1	Free-ranging	NA	1
Striped dolphin *(Stenella coeruleoalba)*	*Delphinidae*	14	Free-ranging	NA	14
African lion *(Panthera leo)*	*Felidae*	7	Captive zoo	NA	7
Asian tiger *(Pantera tigris tigris)*	*Felidae*	1	Captive zoo	NA	1
Asiatic lion *(Panthera leo persica)*	*Felidae*	2	Captive zoo	NA	2

Chetaah *(Acinonyx jubatus)*	*Felidae*	4	Captive zoo	NA	4
European wildcat *(Felis silvestris silvestris)*	*Felidae*	1	Free-ranging	1	NA
Jaguar *(Panthera onca)*	*Felidae*	3	Captive zoo	NA	3
Ocelot *(Leopardus pardalis)*	*Felidae*	1	Captive zoo	NA	1
Persian leopard *(Panthera pardus saxicolor)*	*Felidae*	2	Captive zoo	NA	2
Sri Lankan leopard *(Panthera pardus kotiya)*	*Felidae*	2	Captive zoo	NA	2
Sumatran tiger *(Panthera tigris sumatrae)*	*Felidae*	2	Captive zoo	NA	2
Spotted hyena *(Crocuta crocuta)*	*Hyaenidae*	2	Captive zoo	NA	2
Striped skunk *(Mephitis mephitis)*	*Mephitidae*	2	Captive zoo	NA	2
Beluga whale *(Delphinapterus leucas)*	*Monodontidae*	1	Captive zoo	NA	1
American mink *(Neovison vison)*	*Mustelidae*	141	Free-ranging	141	NA
Asian small-clawed otter *(Aonyx cinereus)*	*Mustelidae*	1	Captive zoo	NA	1
Beech marten *(Martes foina)*	*Mustelidae*	2	Free-ranging	2	NA
Eurasian badger *(Meles meles)*	*Mustelidae*	48	Free-ranging	48	NA
Eurasian otter *(Lutra lutra)*	*Mustelidae*	25	Free-ranging	25	NA

Coypu *(Myocastor coypus)*	*Myocastoridae*	48	Free-ranging	48	NA
California sea lion *(Zalophus californianus)*	*Otariidae*	4	Captive zoo	NA	4
South American sea lion *(Otaria flavescens)*	*Otariidae*	9	Captive zoo	NA	9
Grey seal *(Halichoerus grypus)*	*Phocidae*	1	Captive zoo	NA	1
Harbor seal *(Phoca vitulina)*	*Phocidae*	1	Captive zoo	NA	1
Asian black bear *(Ursus thibetanus)*	*Ursidae*	1	Captive zoo	NA	1
Black bear *(Ursus americanus)*	*Ursidae*	1	Captive zoo	NA	1
Brown bear *(Ursus arctos)*	*Ursidae*	7	Captive zoo	NA	7
Giant panda *(Ailuropoda melanoleuca)*	*Ursidae*	1	Captive zoo	NA	1
Sun bear *(Helarctos malayanus)*	*Ursidae*	1	Captive zoo	NA	1
Binturong *(Arctictis binturong)*	*Viverridae*	1	Captive zoo	NA	1
Common genet *(Genetta genetta)*	*Viverridae*	1	Captive zoo	NA	1
		3	Free-ranging	3	NA
Total		**420**		**283**	**137**

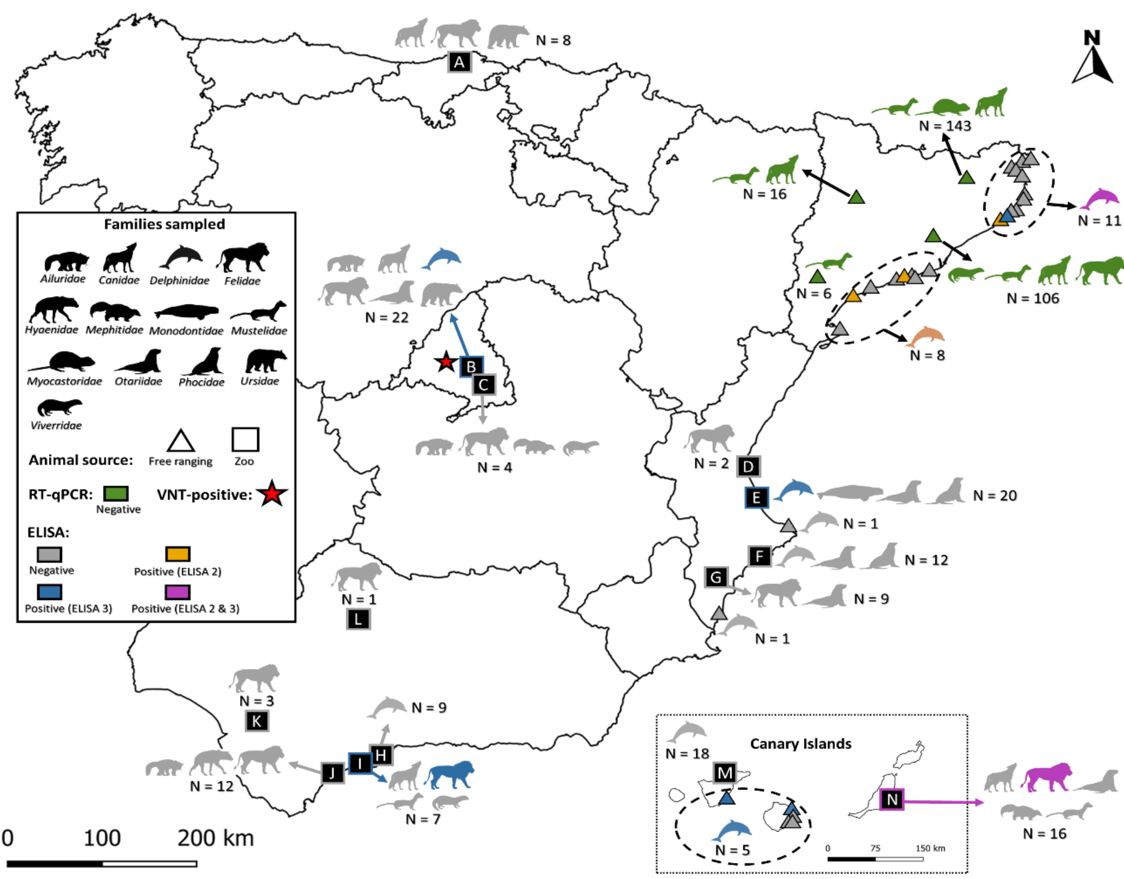

Figure 4.1 Map showing the geographical distribution of the animals sampled in Spain during the COVID-19 pandemic (2020-2023), according to their taxonomy (family category). Zoos and rehabilitation centers are represented by letters (A-N) and the animal source (free-ranging or zoo) is indicated by figures (triangle and square, respectively). Positive results in diagnostic tests are indicated by colors, yellow, blue and violet when positive to ELISA-2, ELISA-3 or both ELISAs, respectively. ELISA-2 corresponds to the SARS-CoV-2 NeutraLISA assay (EUROIMMUNE, Germany), while ELISA-3 refers to the ID Screen® SARS-CoV-2 Double Antigen Multi-species assay (Idvet, France). Positivity to VNT is marked with a red star.

Detection of antibodies against SARS-CoV-2

Available serum samples (137 and 33 from pandemic and pre-pandemic periods, respectively) were tested using two commercially ELISA kits to investigate the presence of specific antibodies against SARS-CoV-2: (i) the SARS-CoV-2 NeutraLISA assay (EUROIMMUNE, Germany) that detects nAbs against the RBD (ELISA-2), and the ID Screen® SARS-CoV-2 Double Antigen Multi-species assay (IDvet, France), which detects antibodies against the viral N protein (ELISA-3). Both tests were performed following the manufacturer's instructions.

Briefly, sera were analyzed by the ELISA-2, which provides S1/RBD coated 96-well plates for capture and soluble biotinylated-ACE2 receptor for detection. In short, each sample was diluted 1:5 with sample dilution buffer containing soluble biotinylated-ACE2, and each mixture was added to the S1/RBD pre-coated wells. Following an incubation period at 37ºC for 60 min, three washing steps were conducted with 300µL of washing solution each. Subsequently, streptavidin-HRP conjugate and the substrate solution were added, and the plate was incubated at room temperature for 15 min. Finally, the stop solution was added to visualize the OD at 450 nm. Results were expressed as an inhibition percentage (% IH) according to the formula provided by the manufacturer's protocol: % IH= 100% - (Sample OD x 100% /mean OD of blank controls). Inhibition (IH) $< 20\%$ was considered negative neutralization, IH= 20-35% doubtful, and IH $\geq 35\%$ was considered positive neutralization. Besides, samples were tested in parallel using the ELISA-3, which provides N-coated plates for capture and HRP-conjugated SARS-CoV-2 N for detection. In summary, 25 µL of each sample were diluted 1:1 with the dilution buffer and added to the 96-well plate. Following incubation at 37ºC for 45 min, five washing steps with 300 µL of washing solution each were performed. Then, 100 µL of N protein recombinant antigen – HRP

conjugate was added to each well and incubated at RT for 20 min. To end, 100 μL of the stop solution was added and the OD were read at 450 nm. Results were analyzed by the following formula provided by the manufacturer's protocol: Sample/Positive control (S/P) % = [(OD sample – OD negative control) / (OD positive control – OD negative control)] x 100. Samples with S/P% ≤50% were considered negative, 50% < S/P % < 60% doubtful, and S/P% ≥ 60 positive. All positive and doubtful samples were tested in duplicates by both ELISAs.

Further, to confirm presence of nAbs against SARS-CoV-2, positive and doubtful samples in at least one of the ELISAs were further tested using a virus neutralization test (VNT) as previously described [78]. Briefly, serum samples were first inactivated at 37ºC for 1 h and diluted 1:10. Then 2-fold serial dilutions were performed in supplemented DMEM. Samples were mixed 1:1 with 100 $TCID_{50}$ of an isolate of SARS-CoV-2 (D614G strain) obtained from a COVID-19 patient (GISAID ID EPI ISL 471472) and incubated at 37 ºC for 1 h. Subsequently, the mixtures were transferred onto Vero E6 (ATCC ® repository, Manassas, VA, USA, CRL- 1586™) cell monolayers and were cultured for 3 days at 37ºC and 5% CO_2. Nine ELISA-negative samples from eight different species were randomly selected and included as negative controls for VNT analyses (**Supplementary Table 4.1**). Experiments were performed in duplicates. The presence of cytopathic effect (CPE) was evaluated at 3 dpi using the CellTiter-Glo luminescent cell viability assay (Promega, Madison, WI, USA) following the manufacturer's protocol. Luminescence was measured as RLU in a Fluroskan Ascent FL luminometer (ThermoFisher Scientific, Waltham, MA, USA). The 50% serum virus neutralization titer (SNT_{50}) was defined as the reciprocal dilution of the sample at which 50% of cells were protected. The dose–response curve of the serum sample was adjusted to a non-linear fit regression model

calculated with a normalized logistic curve with variable slope. Uninfected cells and virus-infected cells were used as negative and positive controls of infection for data normalization (%Neutralization = (RLUmax − RLUexperimental) / (RLUmax − RLUmin) × 100), respectively. All statistical analyses were performed with GraphPad Prism 8.4.3 (GraphPad Software, Inc, San Diego, CA, USA).

RNA extraction and detection of SARS-CoV-2 by RT-qPCR

A total of 283 out of 420 animals were tested for acute SARS-CoV-2 infection by the detection of viral RNA in oropharyngeal swabs, rectal swabs, and lung tissue (**Table 4.1**).

Dry sterile oropharyngeal swabs and rectal swabs were transferred into cryotubes containing 500µL supplemented DMEM and finally vortexed. Those samples obtained by using DeltaSwab Virus with VTM were directly vortexed. Lung tissue was mechanically homogenized at 30 Hz for 1 min using a TissueLyser II (QIAGEN GmbH, Hilden, Germany) and centrifuged for 3 min at 10,000 rpm.

All samples were subjected to viral RNA extraction using the Indimag Pathogen Kit (Indical Biosciences Leipzig, Germany) on BioSprint 96 workstation (Qiagen, Hilden, Germany) following the manufacturer's instructions. Subsequently, detection of SARS-CoV-2 RNA was performed by RT-qPCR using the UpE assay as previously described in *Chapter 3*. Samples with a Cq value <40 were considered positive for SARS-CoV-2 RNA detection.

4.3 Results

Eight out of the 137 sera samples tested positive for antibodies against SARS-CoV-2 by one or the other ELISAs used (**Figure 4.1; Table 4.2).** These samples corresponded to five free-ranging striped dolphins (*Stenella coeruleoalba*), two captive bottlenose dolphins (*Tursiops truncatus*), and one captive Sumatran tiger *(Panthera tigris sumatrae*). From all these ELISA-positive samples, only one captive bottlenose dolphin from zoo B (Madrid province) (**Figure 4.1)** tested positive by VNT although with low titers of nAbs (SNT_{50} 38.15) **(Table 4.2).** Two out of the 33 pre-pandemic samples, one captive Eurasian lynx (*Lynx lynx*) and one free-ranging Risso's dolphin (*Grampus griseus*) also tested positive by both ELISA and ELISA-3, respectively, but negative by VNT (**Table 4.2**).

Table 4.2 Results of the serological analysis including ELISAs detecting RBD (ELISA-2) and N protein (ELISA-3) antibodies against SARS-CoV-2, and VNT. Only ELISA-positive samples are included. A serum sample was considered positive for each test when: IH% ≥ 35 (ELISA-2), S/P% ≥ 60 (ELISA-3) and SNT_{50} >20 (VNT). * NA: Not analyzed due to insufficient volume.

Species	**ELISA-2**		**ELISA-3**		**VNT**	
	IH%	**Results**	**S/P%**	**Results**	**SNT_{50}**	**Results**
Bottlenose dolphin *(Tursiops truncatus)*	<20	Negative	186.5	Positive	38.2	Positive
Bottlenose dolphin *(Tursiops truncatus)*	<20	Negative	712.2	Positive	<20	Negative
Striped dolphin *(Stenella coeruleoalba)*	51.4	Positive	≤50	Negative	<20	Negative
Striped dolphin *(Stenella coeruleoalba)*	<20	Negative	902.2	Positive	<20	Negative
Striped dolphin *(Stenella coeruleoalba)*	<20	Negative	69.8	Positive	NA*	NA
Striped dolphin *(Stenella coeruleoalba)*	35.7	Positive	≤50	Negative	NA	NA
Striped dolphin *(Stenella coeruleoalba)*	49.0	Positive	≤50	Negative	<20	Negative
Sumatran tiger (*Panthera tigris sumatrae*)	<20	Negative	146.6	Positive	<20	Negative
Eurasian lynx *(Lynx lynx)*	81.5	Positive	93.0	Positive	<20	Negative
Risso's dolphin *(Grampus griseus)*	<20	Negative	699.1	Positive	<20	Negative

All tested animals (n = 283) for SARS-CoV-2 RNA detection in oral and rectal swabs, and lung tissue were negative by RT-qPCR (Cq ≥ 40).

4.4 Discussion

Owing to the capacity of SARS-CoV-2 for inter-species transmission, surveillance studies in wildlife are necessary to monitor viral spread and maintenance in wildlife populations, which subsequently may act as reservoirs, promoting genetic evolution and posing a risk for global health. In the present study, we performed an extensive survey of SARS-CoV-2 infection or past exposure to the virus in a variety of captive and free-ranging terrestrial and aquatic species of Spain during the whole pandemic period (2020-2023). We detected the exposure (nAbs) to SARS-CoV-2 in a captive bottlenose dolphin living in a zoological park, whereas all the other animals gave negative results by molecular or serological analyses.

The bottlenose dolphin is commonly housed in zoological collections. Consequently, the close contact between this species and zookeepers or zoo visitors enhances the probability for cross-species transmission of infectious pathogens such as SARS-CoV-2. Importantly, the bottlenose dolphin was predicted to have a high risk of infection with SARS-CoV-2 by *in silico* studies due to the high homology between the ACE2 receptor of this host and the human one [37]. Only five out of the 25 critical SARS-CoV-2 S-binding residues differ between the bottlenose dolphin ACE2 and hACE2, and there is only one non-conserved amino acid substitution between them [37]. Accordingly, cells expressing the ACE2 from bottlenose dolphin allowed cell entry of pseudoviruses expressing the S glycoprotein of an early pandemic isolate of SARS-CoV-2 and Delta (B.1.617.2) and Omicron (B.1.1.529) variants [259]. Additionally, the expression of the ACE2 in the respiratory tract of the bottlenose dolphin also supports the potential susceptibility of this animal under natural conditions [260]. Altogether may explain the putative SARS-CoV-2 exposure of the seropositive bottlenose dolphin in the present study. This animal was

originally from a zoological park of Madrid and was sampled in May 2020, during the first wave of the COVID-19 pandemic. N protein antibodies against SARS-CoV-2 were detected by ELISA-3 in its serum sample and afterwards, presence of nAbs was confirmed by VNT. Considering that the majority of nAbs are known to target RBD and not the N protein of SARS-CoV-2, the ELISA detecting RBD nAbs (ELISA-2) could provide false-negative results. This is consistent with the low sensibility of the commercial kit found in previous comparative analysis of a variety serological assays using VNT as a reference, although testing human samples [261]. ELISAs used in the present study also showed seropositivity against SARS-CoV-2 in other cetacean (*Tursiops truncatus* and *Stenella coeruleoalba*) samples, including one pre-pandemic, but all tested negative by VNT. Considering that VNT is the gold standard technique for detecting specific nAbs, these results suggest a potential cross-reactivity with antibodies against other known or unknown CoV infecting cetaceans [262].

Cetaceans can be infected with CoVs from the genera *Alpha-CoV* and *Gamma-CoV* including bottlenose dolphin CoVs (BdCoVs) [263]. To date, no cases of SARS-CoV-2 infection have been reported in captive or free-range cetacean animal species. This is the first study detecting SARS-CoV-2 exposure in a captive dolphin. Audino et al., described the absence of SARS-CoV-2 infection in a variety of marine mammals from the Italian coastline consistent with negative results obtained by RT-qPCR and/or by immunohistochemistry (IHC) [260]. Nevertheless, past infection or exposure in those tested animals could not be completely discarded since both RT-qPCR and IHC only detect acute infections, contrary to serological analyses [260]. Due to the likely susceptibility of dolphins to SARS-CoV-2, future studies should focus on elucidating the potential impact of this virus on dolphin's individual and population health [260].

SARS-CoV-2 genome has been detected in wastewater and rivers, being used even for epidemiological and predictive studies of incidence of SARS-CoV-2 in human populations [264,265]. This fact suggests the possibility of exposure to SARS-CoV-2 in aquatic and semi-aquatic animals and, thus, supports the relevance of monitoring this group of animals. Indeed, there is a report describing SARS-CoV-2 positivity in water pool samples from a zoological park in Belgium in which two infected hippos (*Hippopotamus amphibious*) were living [178]. However, it should be noted that water treatment procedures (wastewater or pools) and factors of marine water (salinity, pH or dilution effect) may contribute to the SARS-CoV-2 inactivation and reduce the viral load, decreasing the risk of infection [260].

In our study, we also included samples from wild mustelid species that live in aquatic environments. These species were predicted to have a low risk of infection by computational prediction studies due to the low binding affinity between SARS-CoV-2 RBD and the host ACE2 receptor [37]. However, *in vivo* experiments and previous reports describing natural infections already demonstrated their high susceptibility to SARS-CoV-2, probably due to the high levels of ACE2 in the respiratory tract [60,66,84,148]. SARS-CoV-2-seropositivity or infection in mustelids have been reported mainly in livestock industry (American minks), households (ferrets; *Mustela putorius furo*) and zoos (Asian small-clawed otter), but also in free-ranging environments (Eurasian otters, American mink, pine martens [*Martes martes*] and badgers) [248]. So far, most studies have primarily concentrated on monitoring SARS-CoV-2 infection within domestic mustelids than those in the wild, probably due to the difficulties involved in sampling them. Notably, our study prioritized the surveillance of free-ranging mustelid species, with a particular emphasis on the American mink. While none of the sampled animals tested positive for acute infection, it is important to note that viral

exposure cannot be excluded due to the unavailability of serum samples for the detection of SARS-CoV-2 antibodies, as sampling was conducted post-mortem. Experimental infections have demonstrated that American minks usually manifest severe COVID-19, including pronounced lesions in both the nasal mucosa and lung, similarly to those observed in severe cases in humans [84,85]. In natural infections, minks have succumbed to mortality mainly due to interstitial pneumonia associated to the viral infection [147]. Consequently, detecting PCR-positive minks for SARS-CoV-2 infection may pose challenges, given their high susceptibility and mortality rates, unless sampling diseased individuals or during active outbreaks investigations.

The present study did not detect SARS-CoV-2 RNA or SARS-CoV-2 antibodies in any other captive or free-ranging wild animal. Contrarily to our results, many natural infections have been described in wild mammals, mainly carnivore species, and most of them occurred in zoological parks [248]. Zoos are a suitable place for viral cross-species transmission due to the huge diversity of animal species and the frequent human-animal interactions. Especially, medium and large sized wild felids have shown their high susceptibility to SARS-CoV-2 infection even presenting none to mild-moderate clinical signs (respiratory and digestive) [65,165,266,267]. It is worthy to note that the Delta (B.1.612.2) variant has been suggested to cause more severe disease in these group of species, and considered a contributing cause of death in some animals [172]. Additionally, feline species can generate a significant humoral immune response against SARS-CoV-2 after natural infection by presence of RBD nAbs and limited levels of antibodies against the N protein [123,130,165]. RBD nAbs lasted at least up to 4 months and total nAbs may be present at least up to 18 months after natural infection in lions [165,171]. In our study, one Sumatran tiger exhibited positive results for N protein antibodies, and one pre-pandemic Eurasian lynx tested positive for RBD

nAbs and N protein antibodies. Nevertheless, VNT results suggested false ELISA-positive results in both cases and potential cross-reactivity of antibodies from other feline CoVs [268]. A similar study conducted in zoo animals from France reported positive ELISA results for N protein antibodies and RBD nAbs in serum samples from three Springbok (*Antidorcas marsupialis*), three Cameroon sheep (*Ovis aries Cameroon*), and two vicunas (*Vicugna vicugna*) [269]. However, these results were not confirmed by VNT, leading to consider them potential false positives [269]. Regarding free-ranging felid animals, one study described the case of an infected leopard (*Panthera pardus fusca*) by the Delta (B.1.617.2) variant in India [192], and a recent study demonstrated the case of virus exposure in free-ranging Iberian lynx (*Lynx pardinus*) in southern Spain with high titers of nAbs [270].

Noteworthy, animals included in this study for both the detection of acute infection and/or exposure to SARS-CoV-2, were tested opportunistically. Thus, the number of samples for some species was low and sporadic over time, which could have contributed to the failure to detect positive animals. Additionally, the animals could have overcome the infection at the time of sampling, or their immune responses could have decreased below the limit of detection of the techniques. Importantly, ELISAs could not have the same levels of sensitivity or specificity when used in wild species than in domestic species or humans [271]. The difficulty in obtaining species-specific positive and negative controls for serological analyses hinders validation of these diagnostic tests in wildlife. Also, it would be necessary to include other groups of species (*e.g.,* bats and ungulates), so we could acknowledge whether some other species could be infected and missed by our study. Monitoring wildlife species for emerging diseases poses many challenges. Wildlife that runs freely in the wild can be vast and dispersed, making it difficult to access and sampling individuals effectively.

Additionally, monitoring wildlife animals requires of specialized techniques, trained professionals, special equipment or permissions to capture and handle specific species [271]. Altogether, stands out the limitations and challenges to wildlife disease surveillance.

Results from our study give a favorable perspective regarding the absence of SARS-CoV-2 in wildlife animals tested from captive and free-range environments from Spain. However, the promiscuity of SARS-CoV-2 for multiple animal species and its ability to cross-species barriers reinforce the importance to continue monitoring wildlife. Especially, surveillance for SARS-CoV-2 infection should focus on species living in high densities, potential animal reservoirs and those with close animal-human interaction. Our study agrees with previous *in silico* and *in vitro* studies regarding that SARS-CoV-2 infection in marine mammals is feasible. This also supports to take preventive biosecurity measures when interacting with cetaceans and other potentially susceptible species, in case of suspected or confirmed COVID-19 individuals.

Supplementary Table 4.1 Serum samples collected during the pre-pandemic period (prior to 2019) and considered as negative control for ELISA (n=33) and VNT (n=9).

Negative control	Species	Family	Number of animals
ELISA	Red fox (*Vulpes vulpes*)	*Canidae*	1
	Bottlenose dolphin (*Tursiops truncatus*)	*Delphinidae*	4
	Common dolphin (*Delphinus delphis*)	*Delphinidae*	1
	Risso's dolphin (*Grampus griseus*)	*Delphinidae*	3
	Striped dolphin (*Stenella coeruleoalba*)	*Delphinidae*	5
	African lion (*Panthera leo*)	*Felidae*	1
	Asian tiger (*Panthera tigris tigris*)	*Felidae*	2
	Bobcat (*Lynx rufus*)	*Felidae*	1
	Cheetah (*Acinonyx jubatus*)	*Felidae*	2
	Eurasian lynx (Lynx lynx)	*Felidae*	3
	Katanga lion (*Panthera leo bleyenberghi*)	*Felidae*	2
	Ocelot (*Leopardus pardalis*)	*Felidae*	1
	Sri Lankan leopard (*Panthera pardus kotiya*)	*Felidae*	1
	Sumatran tiger (*Panthera tigris sumatrae*)	*Felidae*	1
Total	**Negative control ELISA**		**33**
VNT	Risso's dolphin (*Grampus griseus*)	*Delphinidae*	2
	Striped dolphin (*Stenella coeruleoalba*)	*Delphinidae*	1
	African lion (*Panthera leo)*	*Felidae*	1
	Asian tiger (*Panthera tigris tigris*)	*Felidae*	1
	Cheetah (*Acinonyx jubatus*)	*Felidae*	1
	California sea lion (*Zalophus californianus*)	*Otariidae*	1
	South American sea lion (*Otaria flavescens*)	*Otariidae*	1
	Asian black bear(*Ursus thibetanus*)	*Ursidae*	1
Total	**Negative control VNT**		**9**

CHAPTER 5.
STUDY 3

Monitoring SARS-CoV-2 infection in urban and peri-urban wildlife species from Catalonia (Spain).

Manuscript submitted for publication.

5.1 Introduction

Urban or peri-urban species refer to animals found in urban environments or in the transition area between urban and rural worlds, respectively [272]. These species are heavily influenced by human activities and often thrive in human-altered environments such as parks, gardens, agricultural, industrial areas, and even buildings [212]. They may display adaptive behaviors, such as foraging in garbage bins filled with human-produced waste or nesting in man-made structures. The impact of urban and peri-urban species on humans can vary widely and depends on the specific species and the interaction with the human environment. Regarding health considerations, these species may play a role in the transmission of zoonotic diseases to humans, especially if they act as reservoirs and if there is a close animal-human contact [212].

In Catalonia, Spain, and on a global scale, rodent species including the house mouse (*Mus musculus*; *MM*), black rat (*Rattus rattus*; *RR*), and Norway rat (*Rattus norvegicus; RN*), are recognized as urban pest species [213,273,274]. Generally, *MM* are predominantly found indoors, particularly in buildings and homes, while *RN* are commonly sighted in sewers, garbage areas, and buildings [213,274]. Besides, *RR* are well adapted to naturalized environments, thriving in parks and green areas. Considering that rodents are carriers of at least 60 zoonotic diseases, their proximity to humans may pose a substantial threat to human health [275,276]. Accordingly, *Alpha*-CoVs and *Beta*-CoVs have been identified in these animal species in China and Europe [275,277–279]. Indeed, both HCoV-OC43 and HCoV-KU1, are hCoVs that have a rodent origin, underlining the potential role of these animals in disease transmission [7]. At the outset of the COVID-19 pandemic, rats and mice were considered non-susceptible to SARS-CoV-2 [107]. However, the ongoing

genetic evolution of the virus has triggered the emergence of various viral variants capable of infecting these rodent species [106,107,280,281].

On the other hand, wild boars (*Sus scrofa*) represent a notable example of peri-urban species in Catalonia and most of Europe [214]. Catalonia has a significant population of wild boar, and their presence is influenced by factors including habitat availability, food resources, and human activities. Currently, this animal is predominantly in North-Eastern Catalonia and the province of Barcelona (9-15 individuals/km^2), where expansive urban and agricultural areas, along with abundant vegetation, provide favorable conditions for the population to grow and thrive [214]. Estimates of wild boar density in monitoring programs are determined based on hunting captures in naturalized environments [214]. The invasive population of this species causes significant impacts on local ecosystems, potentially contributing to the spread of diseases that affect both wildlife and domestic animals, and even posing risks to human health [282,283]. Wild boars can transmit diverse zoonotic diseases to humans including Hepatitis E, brucellosis, salmonellosis, tuberculosis, yersinosis, toxoplasmosis and trichinellosis [282]. Moreover, wild boars serve as reservoirs for viruses such as African swine fever or classical swine fever, particularly endangering domestic pigs (*Sus scrofa domesticus*) [283]. Additionally, the Vietnamese Pot-Bellied pig (*Sus scrofa domesticus*) is a small-sized domestic breed that gained popularity as a pet breed in various parts of the world due to its friendly temperament. Importantly, domestic pigs can be infected by six different CoVs [284], but not SARS-CoV-2, at least the ancestral variant upon experimental infection [66,91,92]. However, the susceptibility of pigs to variants that emerged during the pandemic has not been assessed experimentally. Considering that many variants have expanded their host range, assessing the exposure to these variants of SARS-CoV-2 in this animal species should not be ignored.

Therefore, this study aimed to monitor evidence of exposure to and/or acute infection by SARS-CoV-2 in rodent species and wild boars, as well as Vietnamese Pot-bellied pigs, found in Catalonia. This study used samples from the entire COVID-19 pandemic period (from 2020 to 2023) to account for exposure to all the different SARS-CoV-2 variants that emerged in the study area.

5.2 Material and methods

Ethical approval

Permission to carry out the study on rodent species was granted by the Department of Territory and Sustainability of the regional government of Catalonia (reference number: SF/044). Rats were treated according to Directive 2010/63/EU of the European Parliament and Council decision of September 22, 2010 concerning the protection of animals used for scientific purposes (https://faolex.fao.org/docs/pdf/eur98296.pdf).

Wild boars were captured in 14 different municipalities according to the requirements and permissions issued by the Department of Climate Action, Food and Rural Agenda of the Autonomous Government of Catalonia (EPI-53/2019, EPI-29/2021, AC/259-20 and AC/292-21).

Samples

This study included a total number of 582 animals of which 232 were rodents (precisely 57 MM, 26 RR, and 149 RN), 313 wild boars, and 37 Vietnamese Pot-bellied pigs. Samples were collected opportunistically between July 2021 and June 2023 for rodent species, and between March

2020 and May 2023 for wild boar and Vietnamese Pot-bellied pigs (**Figure 5.1**).

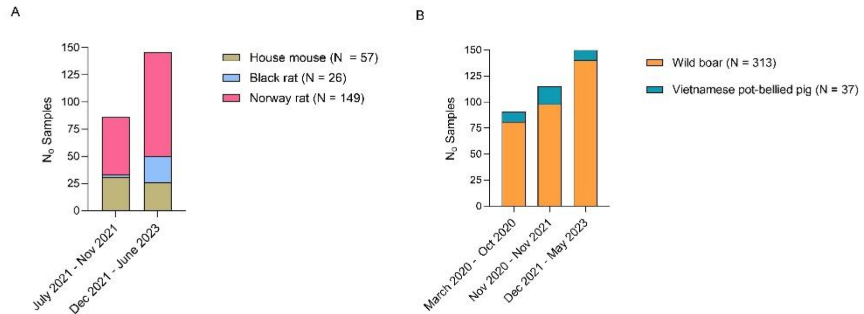

Figure 5.1 Animal sampling in Catalonia during the COVID-19 outbreak with respect to the SARS-CoV-2 variants. **A)** Rodent species sampling distribution in two phases: from July 2021 to November 2021 predominantly for Alpha (B.1.1.7) and Delta (B.1.617.2) variants of SARS-CoV-2; from December 2021 to June 2023 predominantly for the Omicron variant and its subvariants. **B)** Wild boars and Vietnamese Pot-bellied pigs sampling distribution across three phases: from March 2020 to October 2021 predominantly for the ancestral variant (B.1); from November 2020 to November 2021 predominantly for the Alpha (B.1.1.7) and Delta (B.1.617.2) variants, and lastly, between December 2021 and May 2023 predominantly for Omicron and its subvariants.

Oral swabs (57 *MM,* 24 *RR,* and 148 *RN*), lung tissues (57 *MM*, 26 *RR,* and 136 *RN*), and sera (21 *RR* and 11 *RN*) were collected from rodents (n = 232). At least a sample type was collected from each rodent. On the other hand, oral swabs were collected from 29 out of 313 wild boars, and sera samples were collected from all wild boar (n=313) and all Vietnamese Pot-bellied pigs (n=37). The types of samples collected from each animal were determined by both availability and challenges of obtaining samples.

Oral swabs were collected using sterile dry swabs or DeltaSwab Virus 2mL contained in VTM (Delta-lab, S.L., Catalunya, Spain). For lung tissue samples, approximately 0.2 mg was placed into cryotubes containing 500µL of DMEM (Lonza, Basel, Switzerland) supplemented with 100 U/mL penicillin, 100µg/mL streptomycin, and 2 mM glutamine (all from Gibco Life Technologies, Madrid, Spain), and a single 4.5-mm, zinc-plated steel bead.

The sampling of rodent species (*MM*, *RR* and *RN*) was conducted in Barcelona (Catalonia, Spain) by the *Agència de Salut Pública de Barcelona (*ASPB), which is the authority responsible for pest surveillance and control in Barcelona. For *MM*, sampling was carried out in municipal facilities (*e.g.,* libraries, civic centers, retirement homes, and municipal markets). Addressing pest complaints and mice infestation is the major goal of the pest surveillance program. In locations where mice activity was detected, live capture traps were installed. Traps were checked every two days: traps with signs of activity were left in place; traps with no signs of mice activity were removed after a week. Rat samples were obtained from individuals captured during studies in the sewage system (137/149 *RN*) and in public green areas of the city (12/149 *RN* and 26 *RR*). Rats in the sewers were captured with snap traps, while live traps were used in public green areas (**Figure 5.2**).

Figure 5.2 Traps for capturing black rats in public green areas. Image sourced from Tomás Montalvo (ASPB).

On the other hand, a total of 221 out of the 313 (70.60%) wild boars and 10 out of the 37 (27.03%) Vietnamese Pot-bellied pigs were captured with traps located in urban and peri-urban areas of various municipalities of Barcelona. The remaining 92 wild boars (29.39%) were captured at the Vacarisses landfill (Vallès Occidental, Barcelona). Besides, from the 27 Vietnamese Pot-bellied pigs that were not free-ranging, 14 (51.85%) came from sanctuaries in Barcelona, 10 (37.04%) came from Tarragona (Catalonia), and three (11.11%) were from separate households in Barcelona. Sampling of wild boars and free-range Vietnamese Pot-bellied pigs was performed using trap capture and anesthesia. The cage traps were 1.03 m in length, 1 m in width, and 1.48 m in height and were made from welded rods (**Figure 5.3**). These rods, with a diameter of 8 to 10 mm, formed a mesh with squares measuring 5 cm x 5 cm. They featured downward-opening doors that were activated by a trigger mechanism connected to these doors by steel cables. The traps were baited with corn and checked daily. The animals were kept in the traps for an average period of 12 h (range 8–16 h) before blood

collection. In order to minimize stress, animal handling took place during the early morning hours, and anesthesia was administered by a single person approaching the animal. Animals were anesthetized using a combination of tiletamine–zolazepam (6 mg/kg, Zoletil Virbac Salud Animal, Esplugues de Llobregat, Spain) and xylazine (3 mg/kg, Xilagesic 20%, Calier Laboratories, Les Franqueses del Vallès, Spain), delivered via a dart syringe dispatched with a blowpipe (Telinject, Global Veterinaria, Mataró, Spain). Once anesthetized, the animals were placed in lateral recumbency and blood samples were collected from the heart using 18 G 1½″ disposable needles (Sterican; Bbraun, Rubí, Spain) and 10 mL syringes (Omnifix; Bbraun). Euthanasia was then performed by the same administration methods (1 ml/10 kg, Euthasol, Dechra Veterinary Products SLU, Barcelona, Spain). Vietnamese Pot-bellied pigs originated from households were anesthetized using the same method as for free-range wild boar.

Figure 5.3 Representation of the traps for capturing wild boar. Image sourced from Francesc Closa (Vets & Wildlife).

RNA extraction and detection of SARS-CoV-2 RT-qPCR

All rodents (n = 232; 57 *MM*, 26 *RR*, and 149 *RN*) and 29 out of 313 wild boars were tested for acute infection of SARS-CoV-2. The presence of SARS-CoV-2 RNA in oral swabs and/or lung tissue samples was assessed using RT-qPCR as previously described in *Chapter 3*. Cq values < 40 indicated a positive result for SARS-CoV-2 RNA detection.

Detection of SARS-CoV-2 antibodies

Blood samples (21 *RR*, 11 *RN*, 313 wild boars, and 37 Vietnamese Pot-bellied) were used to test exposure to SARS-CoV-2 by detecting nAbs against the RBD. First, blood samples were centrifuged at 1800 x g for 10 min at 4ºC, and the resulting sera were then inactivated at 56ºC for 30 min. The assessment of nAbs against the SARS-CoV-2 RBD was performed using ELISA-1 following the manufacturer's protocol (*Chapter 3*). The percentage of inhibition of the RBD-ACE2 interaction was calculated using the following formula: % Inhibition = (1− (OD_{450} sample/OD_{450} negative control)) × 100. Samples with an inhibition proportion of ≥30% were considered positive for presence of SARS-CoV-2 RBD nAbs.

ELISA-positive samples were further analyzed by the VNT as previously described in *Chapter 4.1.*

Seroprevalence and 95% CI were calculated in each population.

5.3 Results

All animals tested for the presence of SARS-CoV-2 RNA by RT-qPCR (232 rodents and 29 wild boar) were negative (Cq ≥ 40).

As assessed with ELISA-1, three out of the 313 (0.96 %; CI: [0.0%-2.06%]) wild boars tested positive for the presence of nAbs against the RBD with a low percentage of inhibition in each sample: 35.22% (Wild boar 1 – Wb1), 34.87% (Wild boar 2 – Wb2), and 30.20% (Wild boar 3 – Wb3). Wb1 was sampled in April 2020, Wb2 in May 2021, and Wb3 in August 2021. ELISA-positive sera were subsequently tested with the VNT, the gold standard technique, and all tested negative. The remaining 310 wild boars, 21 *RR* and 11 *RN* tested negative by ELISA and were not subjected to further VNT testing.

5.4 Discussion

Wild animals living in urban and peri-urban areas exhibit a high level of adaptability to human-altered environments, where they can find food-resources and suitable opportunities to thrive. This contributes to the establishment of a human-animal interface that importantly creates a high risk for zoonotic spillover of infectious diseases [195]. In Catalonia, the abundance of certain urban and peri-urban species - including common rodent species and wild boars – raises concerns about their impact on the human population, posing risks to public safety and property. Disease transmission between humans and wild urban/peri-urban species can occur bidirectionally. Inadequate waste management, hunting, and wildlife trade can contribute the spread of infectious diseases from humans to wild animal populations [212,273,285,286]. Conversely, these species can also transmit pathogens to humans through direct contact with them or their bodily fluids, as well as through contaminated food, water, or surfaces [212,273,285,286].

Previous events of RZ transmission of SARS-CoV-2, as well as the virus's ability to adapt and spread among certain animal species, have underscored the need for surveillance studies in species at risk of infection.

Initially, murine and wild boars were not deemed susceptible to SARS-CoV-2. However, variants of SARS-CoV that emerged during the pandemic demonstrated their potential to infect a wider host range as well as previously non-susceptible species, such as rats and mice [106,107,114]. As a result, this study aimed to assess the presence of acute infection or exposure to SARS-CoV-2 throughout the entire pandemic period (2020-2023) in rodents and wild boar to better understand the prevalence and distribution of the disease in urban and peri-urban wildlife populations.

Essentially, results revealed that none of the animals included in this study, whether rodents or wild boars, had an acute SARS-CoV-2 infection at the time of sampling, as negative results were observed by RT-qPCR. Additionally, serological analyses indicated that none of the animals had been exposed to the virus, as no specific nAbs were detected in blood samples. Initial serological screening using the ELISA-1 revealed that three wild boars out of 313 had nAbs against SARS-CoV-2. Nevertheless, these animals tested negative by VNT, a more specific and reliable technique, suggesting potential false positives in the ELISA results due to possible cross-reactivity with other CoVs [287]. Indeed, six different CoVs (four *Alpha*-CoVs, one *Beta*-CoV, and one *Delta*-CoV) are known to infect pigs [284,288], and a certain degree of cross-reactivity between antibodies for these and SARS-CoV-2 has already been proposed [287].

At the beginning of the COVID-19 pandemic, experimental infections demonstrated that domestic pigs were not susceptible to the ancestral variant of SARS-CoV-2 by IN, IT, IM, and IV routes of inoculation [66,91,92,289]. However, when piglets were parenterally inoculated (IM and IV), antibodies against the S glycoprotein were observed at least 14 dpi and nAbs were detected at 22 dpi [92]. Notably, the inoculation doses ($\approx 10^5$ - 10^6 $TCID_{50}$/mL) used in most studies on pig susceptibility were likely higher than

what a host might encounters naturally [66,91,92]. Besides, *in vitro* studies demonstrated that SARS-CoV-2 can replicate and cause CPE in porcine cell lines, including swine testicle and porcine kidney cells (PK-15) [290,291]. Accordingly, the expression of the ACE2, the primary cell receptor for SARS-CoV-2, has been verified in the pig intestine and kidneys, contrasting with its absence in the respiratory tract [60]. Since SARS-CoV-2 mainly utilizes the respiratory tract as entry point, the risk of infection in pigs and wild boar under natural conditions might be considered low. Nonetheless, wild boar's urban behavior, proximity to human populations, and interaction with human-produced waste justify their inclusion in monitoring studies to assess viral exposure. Additionally, the possibility of alternative virus receptors enabling infection in specific species cannot be ruled out.

On the other hand, we examined the exposure to SARS-CoV-2 in *21* RR and *11* RN, with all individuals testing negative for SARS-CoV-2 RBD nAbs. However, the limited number of serum samples from this group of animals may restrict the generalizability of our findings to the entire population. Consequently, our results do not conclusively rule out the possibility of SARS-CoV-2 exposure in rodents in Barcelona during the pandemic. *RN* are likely thriving in the sewer environment where they have access to food and water. Notably, SARS-CoV-2 has been detected in wastewater from the sewer system in various countries due to virus particles in feces and urine of infected humans [264,265]. Indeed, detection of SARS-CoV-2 RNA in wastewater has been utilized in epidemiological studies to determine SARS-CoV-2 incidence and predict the emergence of novel variants in the human population in Catalonia [265]. However, the absence of evidence of infectious virus in wastewater or fecal waste significantly reduces the risk of infection among animals [292]. Consistent with our findings, a study on SARS-CoV-2 surveillance in *RN* within the Antwerp, Belgium, sewage

system also reported a lack of exposure to the virus, as they did not detect SARS-CoV-2 nAbs [293]. In contrast, Wang et al. proposed that this species may have been exposed to SARS-CoV-2 in the sewage system of New York City, based on ELISA testing [255]. However, conflicting negative results from the microneutralization assay cast doubt on this assertion [255]. The same authors also found partial genomic sequences of SARS-CoV-2 (coverage from 1.6% to 21.3%) associated with the B.1 lineage in four *RN*, with two of them being from rats that tested positive in ELISA testing [255]. Another study performed in Liverpool, UK, also supported the possibility of SARS-CoV-2 exposure in this species in the sewer system, as antibodies in lung and heart tissue fluid partially neutralized pseudovirus particle infection [285]. Low titers of nAbs against SARS-CoV-2 were also found by VNT in one *RN* in Hong Kong, China, on May 2021, as part of a surveillance study in rodent species[294].

The need to monitor murine species arose as variants of SARS-CoV-2 gained the ability to infect them, contrary to the ancestral variant (B.1) [106,107]. Due to specific amino acid substitutions within the ACE2-RBD interacting surface on murine ACE2 compared with hACE2, the SARS-CoV-2 ancestral variant was not able to use murine ACE2 for cell entry [107]. However, viral variants carrying the N501Y mutation in the RBD of SARS-CoV-2, including Alpha (B.1.1.7), Beta (B.1.351), Gamma (P.1), and Omicron (B.1.1.529), increased the ability of SARS-CoV-2 to bind murine ACE2 and thus infect murine species [107,295]. Rodents are known to be suitable reservoirs of zoonotic diseases due to several factors that facilitate the transmission of pathogens to humans [276,286]. These factors include rapid reproduction rates and their adaptability to diverse environments [276]. Rodents nesting close to human dwellings and feeding on stored food in homes or urban areas can also contribute to the transmission of diseases [276]. Thereby,

mitigating the risk of SARS-CoV-2 variants spreading to these species and understanding the potential role these species may play in transmission is crucial. Additionally, since rodent species host a range of CoVs, there is a possibility of viral recombination, including the recombination of SARS-CoV-2 with other CoVs [277]. This could lead to the emergence of viral variants that pose a major risk for both human and animal well-being [277,285].

Findings from our study indicated that urban and peri-urban populations of wild boars and rodents in Catalonia reported no signs of exposure to or acute infection with SARS-CoV-2. This suggests that these species were unlikely to have played a role in spreading or transmitting the virus during the COVID-19 pandemic. However, the potential for new variants of SARS-CoV-2 to expand their host range underlines the importance of ongoing surveillance of these animal populations, especially rodent species. This is crucial due to their close contact with human communities, which could pose future risks of zoonotic transmission.

CHAPTER 6.
STUDY 4

Susceptibility of domestic goat (*Capra aegagrus hircus*) to experimental infection with SARS-CoV-2 B.1.351/Beta variant.

Chapter adapted from the article:

Fernández-Bastit, L. *et al.* Susceptibility of Domestic Goat (*Capra aegagrus hircus*) to Experimental Infection with Severe Acute Respiratory Syndrome Coronavirus 2 (SARS-CoV-2) B.1.351/Beta Variant. *Viruses* **14**, 2002 (2022).

6.1 Introduction

In an increasingly connected world, the emergence of multiple viral variants of SARS-CoV-2 since the start of the COVID-19 pandemic has urged the implementation of rapid measures to control the spreading of the virus. Several VOCs have been identified with different infectivity or transmissibility properties in humans and animal species. Natural infections by SARS-CoV-2 and its variants have been reported in zoo animals (*i.e.,* felids and NHP, among others), farmed animals (mainly minks), and pets (*i.e.,* cats, dogs, ferrets, and hamsters) [57,165,167]. The susceptibility of many animal species (*i.e.,* NHP, hamsters, ferrets, cats, deer, rabbits, raccoon dogs, fruit bats, and skunks) has also been demonstrated by experimental infections with SARS-CoV-2 and different variants [64,87,91,97,100,103,296]. Importantly, WT mice are not susceptible to early pandemic variants of SARS-CoV-2 under experimental conditions, but susceptible to certain variants such as Alpha (B.1.1.7), Omicron (BA.1.1), and especially Beta (B.1.351) [80,106,107,297].

The Beta variant was first reported in South Africa in late 2020, and showed nine mutations in the S glycoprotein compared to the ancestral variant [298]. Especially three mutations (K417N, E484K, and N501Y) have been reported to had an important role for enhancing viral infectivity, leading to escape from previously acquired SARS-CoV-2 nAbs, and increasing virus transmissibility [299–302]. SARS-CoV-2 variants acquired mutations along the genome, but most importantly in the RBD of the SARS-CoV-2 S glycoprotein, which is responsible for the recognition of the ACE2 host receptor [27]. The affinity between the RBD and the ACE2 receptor as well as the distribution in different tissues of the ACE2 in animal species is a putative indicator of host susceptibility to infection [243]. These differences between variants raise concerns regarding how the risk of new mutations in SARS-

CoV-2 could increase susceptibility in novel species, with the danger of becoming zoonotic reservoirs.

The interactions between livestock animals and wildlife increase cross-transmission of infectious diseases which can reduce food safety and also result in economic impact [303]. Among ruminants, WTD has been found to be the most susceptible species to ancestral and Alpha (B.1.1.7) SARS-CoV-2 variants, showing high seroprevalence in free-ranging animals in the USA [180]. Moreover, subclinical infection has been demonstrated following experimental challenge, as well as viral transmissibility between in-contact WTD and vertically from doe to fetus [95,97]. Regarding other ruminant species, sheep, cattle, and goat have shown low susceptibility to SARS-CoV-2 ancestral variant [87,89] or the combination of ancestral plus Alpha (B.1.1.7) variants [88]. The domestic goat (*Capra aegagrus hircus*) is a small ruminant species raised worldwide in a broad range of production systems for its meat, milk, hide, and hair [304]. Since goats are in constant contact with humans and other SARS-CoV-2 susceptible animal species, it is of high interest to investigate their susceptibility to this viral infection. At the moment of the present study the potential reservoirs and intermediate hosts of SARS-CoV-2 were still being investigated and little information was available regarding the susceptibility of livestock to different variants. Therefore, the aim of the present study was to evaluate the susceptibility of the domestic goat to the Beta (B.1.351) variant of SARS-CoV-2, one of the most important VOCs that already showed expanded species tropism [106,107,297].

6.2 Material and methods

In silico studies

We ran MODELLER v10.1 [305] to generate ten different homology models of goat ACE2 in complex with the spike RBD of the SARS-CoV-2 ancestral variant. The crystallographic structure of hACE2 in complex with the spike RBD of the ancestral variant (PDB ID 6M0J) was used as a template. Then, using the MODELLER models as input, we used FoldX v5 [306] to predict the changes in binding affinity induced by the mutations of the variants. We considered the following mutations located at the RBD: K417N, E484K, N501Y for B.1.351/Beta, L452R, T478K for B.1.617.2/Delta, K417T, E484K, N501Y for P.1/Gamma, and G339D, S371L, S373P, S375F, K417N, N440K, G446S, S477N, T478K, E484A, Q493R, G496S, Q498R, N501Y, Y505H for B.1.1.529/Omicron (BA.1) variants. We ran FoldX modules repairPDB followed by BuildModel. We fixed all FoldX parameters to their default values except parameter vdwDesign, which was set to zero. As a positive control, we performed the same steps using the hACE2 sequence to predict the changes in binding affinity caused by the mutations of the SARS-CoV-2 VOC in humans.

Cell and virus

Vero E6 cells (ATCC® repository, CRL-1586™) and SARS-CoV-2 Beta (B.1.351) variant isolate (passage 3; hCoV-19/Spain/CT-IrsiCaixaR008CC8B3/2021; GISAID ID EPI_ISL_3164134) were prepared as previously reported [78] in DMEM (Lonza, Basel, Switzerland) supplemented with 5% fetal calf serum (FCS; EuroClone, Milan, Italy), 100 U/mL penicillin, 100 µg/mL streptomycin, and 2 mM glutamine (all from Gibco Life Technologies, Madrid, Spain).

The infectious titer of the SARS-CoV-2 stock was calculated by determining the dilution that caused CPE in 50% of the inoculated Vero E6 cells ($TCID_{50}$).

Experimental study design

This experimental study was approved by the Institutional Animal Welfare Committee of the Institute of Agrifood Research and Technology (CEEA-IRTA) and by the Ethical Commission of Animal Experimentation of the Autonomous Government of Catalonia (CEA-OH/11586/3) and conducted by certified staff. Experiments involving SARS-CoV-2 were performed at the Biosafety Level-3 (BSL-3) facilities of the Biocontainment Unit of IRTA-CReSA (Barcelona, Spain).

A total of 18 goats of 2–3 months of age were acquired from a Spanish commercial farm (La botiga d'Ullastrell S.L, Barcelona, Spain) and included in the study. Three animals were used as non-inoculated controls and were necropsied on the day of animal arrival. The remaining 15 animals were allocated inside an experimental box of the animal BSL-3 facilities, and after 7 days of acclimatization, they were IN inoculated with 2 mL of 10^6 $TCID_{50}$/mL of SARS-CoV-2 Beta (B.1.351) variant (1 mL in each nostril) using a diffusor device (LMA™ MAD® Nasal, Teleflex LLC). At 2, 4, 7, 10, and 18 dpi, three goats/day were euthanized and complete necropsies were performed (**Figure 6.1).** At necropsy, nasal and rectal swabs, blood, and the following tissues were collected: nasal turbinate (NT; caudal and cranial portions), olfactory bulb, parotid gland, trachea, pharynx, tonsil, lung (apical, medial, and caudal portions), lymph nodes (LN; cervical, mesenteric, and mediastinal ones), kidney, spleen, jejunum, colon, and heart. Clinical signs and rectal temperatures were recorded daily during the whole study.

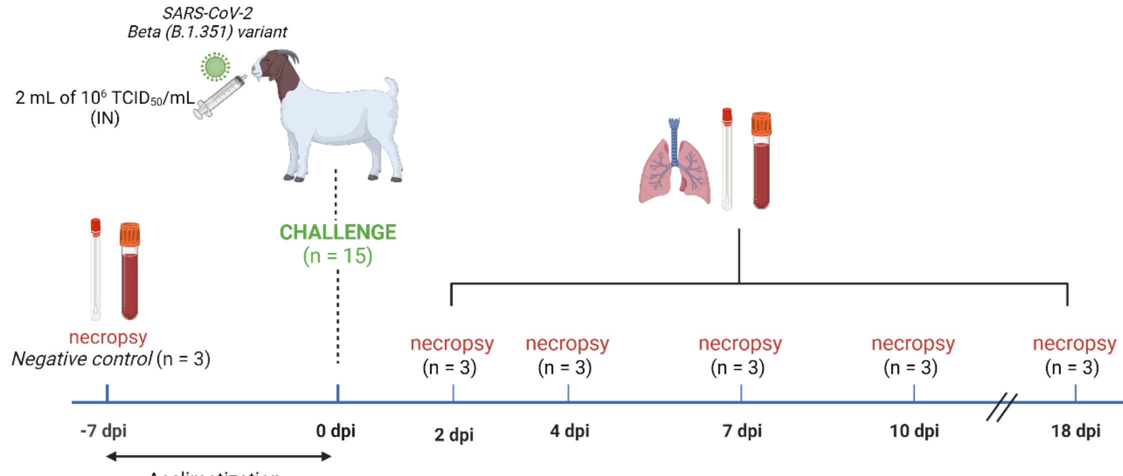

Figure 6.1 Schematic representation of the study design. Three out of eighteen goats were necropsied on the day of arrival and served as non-inoculated controls. Nasal and rectal swabs, blood, and tissue samples were collected. After acclimatization, the remaining fifteen goats were intranasally inoculated with the SARS-CoV-2 Beta (B.1.351) variant (2 mL of 1 × 10^6 $TCID_{50}$/animal). At 2, 4, 7,10, and 18 days post-infection, three goats were necropsied each day and nasal and rectal swabs, blood, and tissues were collected. Clinical signs and rectal temperature were recorded daily.

Virus detection and isolation

Nasal and rectal swabs were collected during necropsy and were transferred into cryotubes containing 500µL DMEM supplemented with 100 U/mL penicillin, 100 µg/mL streptomycin, and 2 mM glutamine. The samples were then vortexed. Tissue samples were placed into cryotubes with 500µL supplemented DMEM and containing single zinc-plated, steel 4.5-mm beads. Blood was centrifuged (1800× *g*, 10 min at 4 ºC) and sera were further collected. All samples were kept at −20 ºC until use.

Viral RNA was extracted from all samples using the Indimag Pathogen Kit (Indical Biosciences, Leipzig, Germany) on a BioSprint 96 workstation (Qiagen, Hilden, Germany) in accordance with the manufacturer's instructions. gRNA was detected by the UpE RT-qPCR assay as previously described in *Chapter 3*. Additionally, to evaluate viral replication in all samples, sgRNA were detected using the sgRNA RT-qPCR assay following a previously published protocol [307]. Samples with a Cq value <40 were considered positive for SARS-CoV-2 RNA.

RT-qPCR positive samples for gRNA with Cq <30 were evaluated for the presence of infectious virus by titration in Vero E6 cells, as previously reported [78]. Briefly, ten-fold dilutions from each sample were transferred to Vero E6 monolayers and incubated at 37ºC and 5% CO_2. Plates were monitored under a light microscope and wells were assessed for the presence of CPE for 6 days. The amount of infectious virus was calculated by determining the $TCID_{50}$ using the Reed and Muench method [308].

Neutralizing antibodies

Initially, serum samples from the 18 experimental goats were inactivated at 56 ºC for 1 h. Subsequently, nAbs targeting SARS-CoV-2 RBD were measured using ELISA-1, following the manufacturer's protocol and as previously described in *Chapter 3*. The percentage of inhibition of each sample was calculated by the following formula: % Inhibition = (1 – [OD_{450} sample/OD_{450} negative control]) × 100. Samples and controls were included in duplicate (SD ≤ 10%). Inhibition of ≥ 30% was considered a positive neutralization.

In addition, VNT was performed as previously described [78]. Briefly, pre-inactivated serum samples were initially diluted 1:20 and then 2-fold

serially diluted in supplemented DMEM, mixed 1:1 with the SARS-CoV-2 isolate (Beta variant) (EPI_ISL_3164134), and incubated for 1 h at 37 ºC. Then, each dilution mixture (in duplicates) was transferred to Vero E6 monolayers containing 100 $TCID_{50}$ of SARS-CoV-2 per well and cultured for 3 days at 37 ºC and 5% CO2. The CPE was measured as explained in *Chapter 4*. SNT_{50} was defined as the reciprocal value of the sample dilution that showed 50% of the SARS-CoV-2-induced CPE in Vero E6 cells.

Histopathology

NT and tonsil were harvested and fixed by immersion in 10% buffered formalin solution. Fixed tissues were embedded in paraffin and cut sections (3 µm thick) were stained with hematoxylin and eosin (H/E) for histopathology studies using an optical microscope.

A previously described IHC technique to detect SARS-CoV-2 NP antigen [101] [101] using a Rabbit monoclonal antibody (40143-R019, SinoBiological, Beijing, China) at dilution 1:10,000 was applied on NT and tonsil. The amount of viral antigen in tissue samples was semi-quantitatively scored: lack of antigen detection, low, moderate, and high amount following a previously published scoring system [78].

Statistical analyses

The Whitney–Wilcoxon test was applied to study differences in binding affinities changes of the RBD protein of SARS-CoV-2 variants compared to the ancestral variant ($\Delta\Delta G = \Delta G_{variant} - \Delta G_{ancestral}$), with both the goat and the human ACE2 receptors, respectively. Additionally, the results between the two receptors were also compared using the same test. *P*-values < 0.05 were considered statistically significant.

Dose–response curves of neutralization assay in serum samples were adjusted to a non-linear fit regression model calculated with a normalized logistic curve with variable slope. For data normalization, uninfected cells and untreated virus-infected cells were used as negative and positive control of infection (% Neutralization = (RLUmax − RLUexperimental)/ (RLUmax − RLUmin) × 100), respectively. This was performed using GraphPad Prism 8.4.3 (GraphPad Software, Inc., San Diego, CA, USA).

6.3 Results

In silico predictions of binding affinity changes in Beta (B.1.351), Delta (B.1.617.2), Gamma (P.1), and Omicron (B.1.1.529) variants

We ran FoldX to predict the changes in binding affinity (ΔΔG = ΔGvariant − ΔGancestral) for the spike RBD in complex with goat ACE2, induced by the mutations of the variants we studied (**Figure 6.2**). As a control, we also computed the binding affinity changes for the spike RBD in complex with hACE2. Results for goat ACE2 indicated that Beta ΔΔG was significantly lower (higher binding affinity changes) than Delta, Gamma, and Omicron ΔΔGs (Mann–Whitney–Wilcoxon test *P*-values equal to $1.06x10^{-2}$, $3.64x10^{-3}$, and $3.20x10^{-2}$, respectively). We found a similar trend for hACE2 (Mann–Whitney– Wilcoxon test *P*-values equal to $6.58x10^{-4}$, $2.91x10^{-4}$, and $1.06x10^{-2}$ for Delta, Gamma, and Omicron, respectively). Positive/negative ΔΔGs values indicated if the binding affinity was lower/higher for the evaluated variant in comparison with the ancestral spike RBD in complex with goat ACE2. The average ΔΔGs for the Beta variant was −2.18, with a 95% CI of (−3.67, −0.69), for the Delta variant −0.06, with a 95% CI of (−0.58, 0.46), for the Gamma variant 0.25 with a 95% CI of (0.97, 1.48), and

for the Omicron variant 0.20, with a 95% CI of (−1.86, 2.26). We did not find significant differences between the predicted ΔΔGs for goat and human for any of the variants (Mann–Whitney–Wilcoxon test *P*-values of 4.85×10^{-1}, 5.15×10^{-1}, 7.64×10^{-1}, and 6.61×10^{-1} for Delta, Beta, Gamma, and Omicron, respectively).

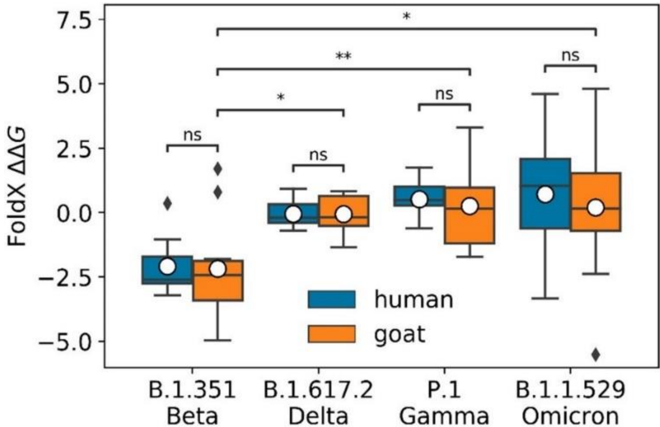

Figure 6.2 FoldX predicted ΔΔG for Beta(B.1.351), Delta(B.1.617.2), Gamma (P.1), and Omicron (B.1.1.529) variants for human and goat ACE2 (blue and orange boxes, respectively). For goat, the computed ΔΔG was significantly lower for the Beta variant than for the Delta, Gamma, and Omicron variants, with Mann–Whitney–Wilcoxon test *P*-values of 1.06×10^{-2}, 3.64×10^{-3}, and 3.20×10^{-2}, respectively. We found no significant differences between the predicted ΔΔG values for human and goat of the different variants (Mann–Whitney–Wilcoxon test p-values of 4.85×10^{-1}, 5.15×10^{-1}, 7.64×10^{-1}, 6.61×10^{-1} for Delta, Beta, Gamma, and Omicron, respectively). In this boxplot representation, the lower and upper ends of each box represent the first (Q1) and third (Q3) quartiles of the ΔΔG predicted values, respectively. The horizontal line, inside each box, represents the median, or second quartile (Q2), and the mean is plotted as a white dot for each variant. The box "whiskers" extend to values that are 1.5 times the size of the interquartile range (IQR = Q3 − Q1). Values that fall outside this range are displayed independently as black diamonds. Mann-Whitney-Wilcoxon test p-values are annotated according to the following criteria: ns ($0.05 < P\text{-value} \leq 1$), * ($0.02 < P\text{-value} \leq 0.05$), ** ($0.001 < P\text{-value} \leq 0.02$).

Clinical signs and pathological findings

No clinical signs or increase in rectal temperature from baseline were observed after challenge in any of the goats along the study. Moreover, no

gross or microscopic lesions attributable to SARS-CoV-2 infection were recorded in any of the studied animals.

Virus detection and replicative viral isolation in goat samples

Fourteen out of the fifteen goats tested positive for SARS-CoV-2 by genomic RT-qPCR in nasal swabs at 2 dpi, and ten out of twelve at 4 dpi with low viral loads (Cq $\geq$ 27). Only two out of the nine remaining goats were positive with low viral loads (Cq > 30) at 7 dpi (**Figure 6.3A**). Moreover, low levels of viral RNA were observed in caudal and cranial NT (Cq $\geq$25) **(Figure 6.3B)**, lymphoid tissues including tonsil (Cq $\geq$23) and cervical and/or mediastinal LN (Cq >30) **(Figure 6.3C, D)**, and respiratory tract including trachea and lung (Cq >30) **(Figure 6.3E, F)**. SARS-CoV-2 RNA from tonsil and LN samples were detected until 18 dpi in goats **(Figure 6.3C, D)**. Blood and all other samples (rectal swabs, olfactory bulb, parotid gland, trachea, spleen, kidney, jejunum, and heart) tested negative by genomic RT-qPCR (Cq $\geq$ 40).

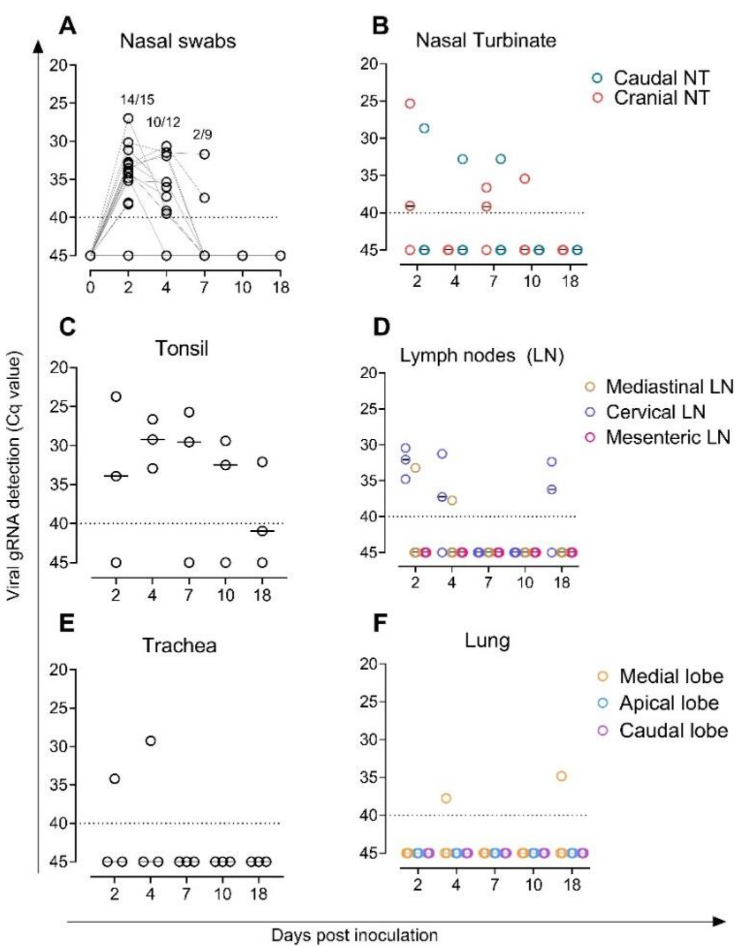

Figure 6.3 Detection of SARS-CoV-2 genomic RNA (gRNA) by RT-qPCR. SARS-CoV-2 loads in **(A)** nasal swabs; **(B)** cranial and caudal nasal turbinate; **(C)** tonsil; **(D)** mediastinal, cervical, and mesenteric lymph nodes; **(E)** trachea; and **(F)** lung. Horizontal bars indicate median viral loads. Dotted lines reflect the limit of detection (Cq = 40).

Inoculated goats were positive for viral sgRNA in tonsil samples at 2, 4, and 7 dpi (Cq > 28), and in cranial NT at 2 dpi (Cq >29) **(Figure 6.4; Supplementary Table 6.1**). Blood and all other samples (rectal swab, olfactory bulb, parotid gland, trachea, spleen, kidney, jejunum, and heart) tested negative (Cq ≥ 40) via subgenomic RT-qPCR.

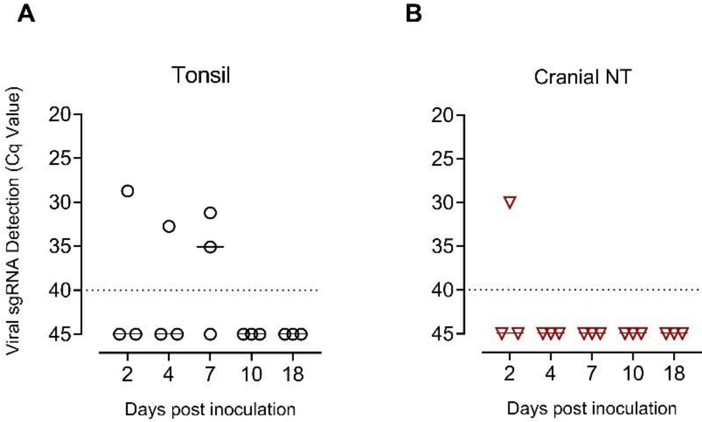

Figure 6.4 Detection of SARS-CoV-2 subgenomic RNA (sgRNA) by RT-qPCR. SARS-CoV-2 loads in **(A)** tonsil and **(B)** cranial nasal turbinate (NT). Horizontal bars indicate median viral loads. Dotted lines reflect the limit of detection (Cq = 40).

Only samples with a Cq< 30 by genomic RT-qPCR were tested for virus titration on cell culture (**Supplementary Table 6.1**). We isolated infectious virus on Vero E6 cells at 2 dpi from one tonsil (3.1 $TCID_{50}$/mL) and one cranial NT (1.9 $TCID_{50}$/mL) from two different goats. SARS-CoV-2 could not be isolated from all other tested samples.

Detection of SARS-CoV-2 nucleoprotein in tissues by immunohistochemistry

Only samples with a Cq <30 by genomic RT-qPCR were tested by IHC (**Supplementary Table 6.1**). Tonsils from inoculated goats collected at 2, 4, and 7 dpi (one goat per day) were positive for IHC staining (**Figure 6.5);** labelling was mainly found in dendritic-like cells mostly located around tonsillar crypts. The amount of viral antigen was low in all animals. All other samples were negative.

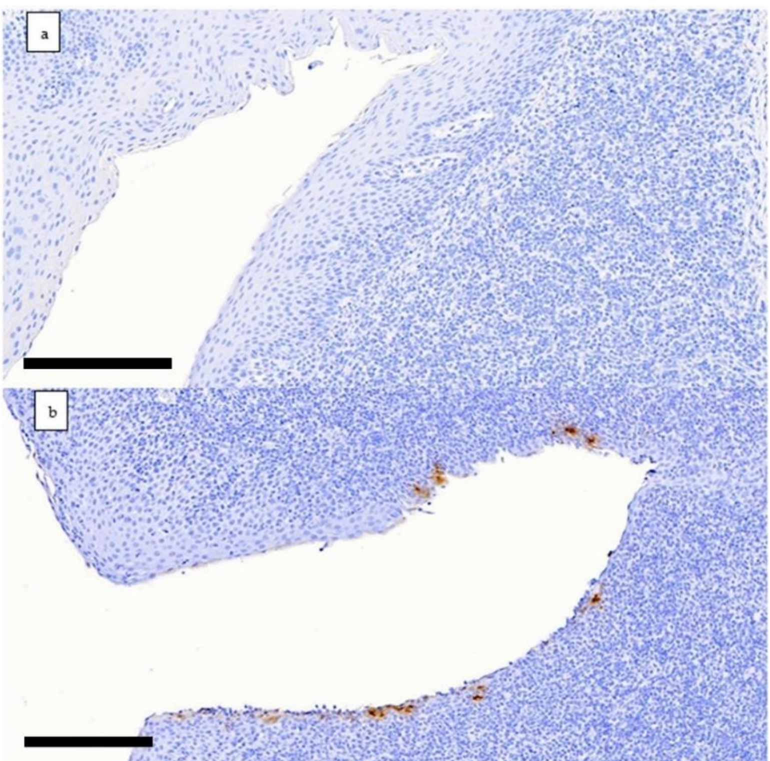

Figure 6.5 Immunohistochemistry staining to detect the nucleocapsid protein of SARS-CoV-2 in goat tonsils (scale bar: 200 μm). **(a)** Negative control animal with no antigen labelling. **(b)** Positive result in the tonsil of a goat euthanized at 2 dpi (Goat 1); immunolabelling is seen as brownish staining in dendritic-like cells around a tonsillar crypt.

Inoculated goats developed humoral responses against SARS-CoV-2

nAbs targeting the RBD of SARS-CoV-2 were detected in goats at 10 and 18 dpi by the ELISA-1 **(Figure 6.6A).** Seroneutralisation capacity against SARS-CoV-2 Beta (B.1.351) variant was observed at 7 dpi (SNT_{50} 105.0 ± 94.98), 10 dpi (SNT_{50} 357.90 ± 74.90), and 18 dpi (SNT_{50} 158.5 ± 99.24) by VNT **(Figure 6.6B).**

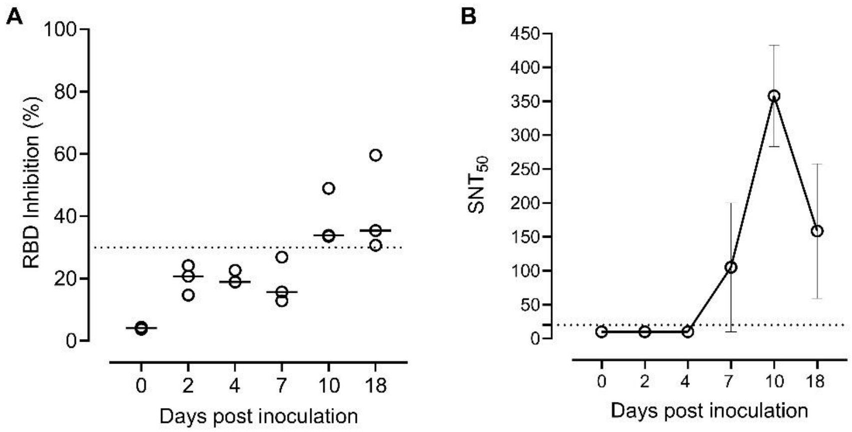

Figure 6.6 (A) Neutralizing antibodies detected by the SARS-CoV-2 RBD Inhibition ELISA (Positive ≥ 30% RBD inhibition). **(B)** Neutralization titers in sera samples from 0, 2, 4, 7, 10, and 18 dpi determined by the live virus neutralization assay (Positive ≥ 20 SNT_{50}). Data reported as values of reciprocal dilution of SNT_{50} (mean ± SEM). The horizontal dotted lines indicate the cut-off value of the assay. Abbreviations: RBD, receptor-binding domain; SNT_{50}, serum virus neutralization titer (reciprocal dilution) that showed 50% protection of virus growth.

6.4 Discussion

Goat is an important livestock animal that has already been suggested to be susceptible to SARS-CoV-2 by previous studies, both *in vitro* and *in vivo* [87,215]. Similarity between goat and hACE2 receptors has been demonstrated via comparative genomic analysis, as well as binding affinity between goat ACE2 and SARS-CoV-2 S protein [37]. Other previous *in vitro* studies also reported that goat ACE2 supports cell entry of SARS-CoV-2 [215,309] and its replication has been demonstrated in non-susceptible avian fibroblast cells (DF1) expressing both goat ACE2 and TMPRSS2 [215]. Since the susceptibility of animal species could change after the appearance of SARS-CoV-2 variants, and our *in silico* study predicted potential affinity between the Beta (B.1.351) variant and ACE2 goat receptor, the aim of the present study was to investigate the susceptibility of goat to this SARS-CoV-2 variant by means of an *in vivo* experimental inoculation.

Previously, Bosco-Lauth et al., described SARS-CoV-2 RT-qPCR positive results in two out of three inoculated goats at 3 days after experimental challenge with the ancestral variant (dose of $10^{5.4}$ pfu) [87]. However, none of the animals shed detectable infectious virus, nor did other ruminants such as sheep (dose of $10^{4.5}$ pfu) and cattle (dose of $10^{5.4}$ pfu) [87]. The aforementioned study together with other previous studies reported that goat and cattle developed limited subclinical infection after SARS-CoV-2 inoculation [87–89]. Sheep co-infected with ancestral and Alpha (B.1.1.7) variants by IN and oral route simultaneously (dose of 10^6 $TCID_{50}$) also developed a subclinical infection [88]. The authors detected viral RNA in respiratory and lymphoid tissues of various animals at 4, 8, and 21 dpi, and achieved virus isolation in the trachea sample from one sheep [88]. Although our study performed in goats was carried out with a higher sample size than the mentioned experimental infections in livestock ruminants, comparable

results regarding overall susceptibility in goats were observed. Low amounts of viral genome and antigen in tissues were found in the inoculated goats. Although the detected nucleic acid may be considered remnants of the inoculum, the detection of sgRNA in NT and tonsil samples at 2 dpi, 4 dpi, and 7 dpi, suggests recent virus replication, at least in those tissues. In addition, the virus isolation from these tissues supports the presence of infectious virus in this species after challenge with the Beta (B.1.351) variant. On the other hand, results from IHC exhibited presence of N protein of SARS-CoV-2 in the tonsil of a single goat at each of the time points (2, 4, and 7 dpi) in cells with morphology compatible with dendritic cells (DCs). It is likely that this immunolabelling represents viral internalization and accumulation in these cells, since at least human DCs have been shown to trap SARS-CoV-2 even though not showing a productive infection [310,311]. Further studies would be needed to understand whether viral replication or DC internalization occur in tonsils in this or other potentially susceptible species.

Differences in viral loads observed in our study compared to those of previous experimental infections in goats, as well as in the other ruminant species, may be due to the higher affinity predicted for Beta (B.1.351) variant in goats than for ancestral variants [87,88]. This may confirm that the emergence of new variants raises concerns regarding the risk of new mutations in SARS-CoV-2 that could increase the range of susceptibility of zoonotic reservoirs. In our study, the dose of challenge may be higher than the one that goats could be exposed to under natural conditions. Thus, it is unknown whether inoculation with a lower dose of virus would have shown evidence of infection or not. In a parallel study, we tested 208 goats that were in contact with COVID-19 positive farmers, yet none of the goats developed humoral immune responses against SARS-CoV-2 (unpublished work). This latter

result further supports the idea that goats were not exposed to the virus in natural conditions albeit the circumstances.

Overall, our results suggest that domestic goat has low susceptibility to SARS-CoV-2 Beta (B.1.351) variant infection with low amounts of viral genome and antigen in tissues and evidence of seroconversion from 7 dpi onwards. Moreover, challenged goats did not show clinical signs or gross and microscopic lesions consistent with SARS-CoV-2 infection. Thus, the domestic goat seems to be a poorly competent host for SARS-CoV-2 (at least for the Beta variant) and probably has a negligible role in virus transmission, in agreement with studies carried out in other ruminant livestock species [87–89]. However, it is important to continue monitoring potential susceptible wild and domestic species to minimize the risk of transmission at the human-animal interface. Moreover, the circulating variants such as Omicron and the emergence of new ones raise concerns regarding the risk of new mutations in SARS-CoV-2 that could expand host range susceptibility and generate new zoonotic reservoirs. In fact, a previous study comparing 27 ACE2 orthologues including goat ACE2, suggests a broader species receptor binding of Omicron compared to other variants such as Delta [312] . Thus, the infectivity of new viral variants in different animal species should be continuously monitored as improved viral replication cannot be ruled out in species currently defined as poorly susceptible.

Supplementary Table 6.1 Goat samples with Cq values < 30 by genomic RT-qPCR and tested for virus isolation and IHC. Results obtained by genomic and subgenomic RT-qPCR (Cq), virus titration (log10 $TCID_{50}$/mL), and IHC (positive [+] / negative [-]) are indicated for each sample. NA: not-analyzed.

DPI	Goat	Tissue	genomic RT-qPCR (Cq < 30)	subgenomic RT-qPCR	Virus isolation (log10 $TCID_{50}$/mL)	IHC
2	1	Tonsil	23.73	28.71	3.1	+ (low)
	2	Caudal NT	28.66	<40	<1.8	-
		Cranial NT	25.32	29.97	1.9	-
	11	Nasal swab	26.96	<40	<1.8	NA
4	4	Tonsil	26.64	<40	<1.8	-
	6	Tonsil	29.22	32.74	<1.8	+ (low)
7	8	Tonsil	25.71	30.46	<1.8	-
	9	Tonsil	29.55	35.07	<1.8	+ (low)
10	10	Tonsil	29.42	<40	<1.8	-

CHAPTER 7.
STUDY 5

Comparison of three commercial ELISA kits for detection of antibodies against SARS-CoV-2 in serum samples from different animal species.

Manuscript in preparation.

7.1 Introduction

The zoonotic origin of SARS-CoV-2, together with ongoing reports of SARS-CoV-2 infections in various animal species, underscore the need for sustained surveillance studies in animal populations [313]. Serosurveillance is the use and analysis of blood or sera specimens for the detection of antibodies against a specific pathogen [314]. Regarding EZDs, including COVID-19, serological tests are useful tools to detect exposure to the pertinent infectious agent, to detect susceptible animal species, and to identify potential animal reservoirs [194].

ELISA is a common serological assay that offers significant utility for conducting high-throughput analyses due to its cost-effectiveness and minimal time consumption [262,315]. More complex but also more specific and sensitive techniques including pVNT, VNT, or plaque reduction neutralization test (PRNT), are essential to confirm the results obtained by initial screenings performed through ELISA [262]. pVNT is a technique that uses viral particles named as pseudoviruses, which are coated with an heterologous E protein responsible for entry to cells [316]. This assay allows to assess the functional capability of antibodies to neutralize the virus by blocking viral entry into cells and preventing further infection. pVNT relies on infectious pseudoviruses and live cells, which clearly better simulates the live virus entry and infection compared to ELISA [262,316]. ELISA does not evaluate the functional capacity of antibodies but the capacity of them to bind purified recombinant proteins of the virus. Accordingly, pVNT highly correlates with VNT and PRNT, which both measure nAb titers using infectious viruses [316]. Importantly, pVNT can be carried out in BSL-2 facilities, which is a clear advantage over VNT or PRNT, which need to be performed under BSL-3 facilities when studying highly pathogenic viruses

like SARS-CoV-2. This is because pseudoviruses lack autonomous replication capability and can infect cells only in a single cycle [262,316].

So far, different serological tests (commercial and in-house assays) have been employed worldwide to assess the presence of SARS-CoV-2 antibodies in animal samples [162,317–319]. Within the present *PhD Thesis*, three different commercial ELISAs kits have been used for initial screenings to detect antibodies against the most immunogenic antigens of SARS-CoV-2: the RBD and the N protein [320,321]. Works presented from *Chapters 3 to 6*, aimed to study humoral responses in different animal species for epidemiological reasons and/or to assess their susceptibility to SARS-CoV-2 infection. Both, the cPass SARS-CoV-2 Neutralization Antibody detection kit (Genscript, The Netherlands; ELISA-1) and the SARS-CoV-2 NeutraLISA kit (EUROIMMUNE, Germany; ELISA-2) are competitive ELISA-based assays that detect nAbs against the RBD. Both kits use purified recombinant RBD and hACE2 proteins. Besides, the ID Screen® SARS-CoV-2 Double Antigen Multi-species assay (IDVET, France; ELISA-3) detects total antibodies against the N protein of SARS-CoV-2. Owing to different reasons (*e.g.,* economic resources or limited volumes of sera samples), all three ELISA kits were not applied in each study from this *PhD Thesis*. The pVNT was also carried out in *Chapter 3* to confirm presence of nAbs in sera samples from pets and stray cats that previously tested positive by ELISA-1. The pVNT was based on pseudoviruses derived from the modified HIV, engineered to express the S glycoprotein of SARS-CoV-2 along with a luciferase reporter gene, as previously documented [240].

In human COVID-19 patients and animals, most of the nAbs are being developed against the RBD, while N protein antibodies are mainly considered binding antibodies (bAbs) [322]. RBD nAbs are associated with protective immunity in humans and some animal species by blocking viral

entry and subsequent infection [64,78,99,323,324]. bAbs have been recognized to mitigate severe COVID-19 in human patients and promote resolution of SARS-CoV-2 infection [320]. This is partially explained by the enhancement of the cell immune response produced by these bAbs, that promotes the uptake of SARS-CoV-2 or elimination of toxins and infected cells [320,325,326]. Importantly, the N encoding-gene is highly conserved among CoVs, leading to the possibility for cross-reactivity of antibodies against this antigen [320,327]. Meanwhile, the RBD genomic sequence is highly variable among CoVs, thereby, serological tests targeting this antigen may be more specific [318,327,328]. This is of particular importance to consider when developing serological assays for detecting SARS-CoV-2 antibodies in animal samples, given that numerous other CoVs have the capability to infect a wide range of animal species [18]. Moreover, most of the commercialized ELISAs used for analyzing animal samples were developed and validated exclusively using human samples but not animal ones [318,329]. This is the case of ELISA-1 [329] and ELISA-2 (Manufacturer's instructions). Hence, it is noteworthy that the interpretation of results may vary between humans and animal samples, and even among samples from different animal species.

The present study sought to compare ELISA-1, ELISA-2, and ELISA-3, to ascertain the most appropriate kit for detecting seropositive animals against SARS-CoV-2. This evaluation aimed to facilitate the initial screening process in epidemiological studies dealing with a large number of animal samples. The diagnostic performance of each ELISA was evaluated and compared with the pVNT, the reference technique to assess neutralizing activity against SARS-CoV-2.

7.2 Material and methods

Experimental design

The *PhD Thesis* comprises four works (*Chapters 3 to 6*) investigating the humoral response to SARS-CoV-2 in various animal species using different ELISA kits (ELISA-1, ELISA-2, and/or ELISA-3), as represented in **Table 7.1**. ELISA-1 was used in all studies except for *Chapter 4*, which utilized ELISA-2 and ELISA-3; limited volumes of samples prevented further testing with ELISA-1. The use of different ELISAs led to perform a comparison and identification of the most suitable kit for animal serosurveillance.

Table 7.1 ELISAs (ELISA-1, ELISA-2, or ELISA-3) used for each *Chapter* of this *PhD Thesis* are indicated. The group of animals sampled in each study is also summarized.

Chapter	ELISA test	Group of animals sampled
Chapter 3	ELISA-1	Domestic pets (cats, dogs and ferrets) and stray cats
Chapter 4	ELISA-2 and ELISA-3	40 wildlife species: terrestrial and aquatic free-range and captive animals
Chapter 5	ELISA-1	Urban and peri-urban species: wild boar and rodents
Chapter 6	ELISA-1	Domestic goats

A total of 101 sera samples were included in the present comparison study. Samples corresponded to different animal species: 36 domestic cats (*Felis catus*), 41 dogs (*Canis familiaris*), 4 ferrets (*Mustela putorius furo*), 10 wild boars (*Sus scrofa*), 6 domestic goats (*Capra aegagrus hircus*) and 4 lions (*Panthera leo*) (**Supplementary Table 7.1**).

Samples belonged to: i) the epidemiological study in pets and stray cats from *Chapter 3* (n = 81), ii) the epidemiological study in wild boar from *Chapter 5* (n = 10), iii) the experimental infection with SARS-CoV-2 Beta variant in goats from *Chapter 6* (n = 6), and iv) our previous study monitoring lions naturally infected with SARS-CoV-2 at the Barcelona Zoo (n = 4) [165].

SARS-CoV-2 acute infection was previously assessed by RT-qPCR in all animals, except for wild boars, in each corresponding study. One dog (Dog 36 in **Supplementary Table 7.1**), all experimentally infected goats, and all lions (**Supplementary Table 7.1**) tested positive. Additional information, including animal species, date of blood sampling, predominant SARS-CoV-2 variant at the sampling period, and results of RT-qPCR, is summarized within the **Supplementary Table 7.1**. From the experimental study in domestic goats (*Chapter 6*), three animals sampled after 2 dpi and three animals sampled after 18 dpi were included in the present study (**Supplementary Table 7.1**).

Herein, serum samples were analyzed by ELISA-1, ELISA-2, ELISA-3, and pVNT (used as a reference) to assess sensitivity and specificity of each ELISA kit. The diagnostic performance of each assay was also evaluated according to the cut-off established for the pVNT. Due to limited volumes, not all the samples were tested by each serological test (**Supplementary Table 7.1**).

Reference assay: Pseudovirus neutralization assay (pVNT)

All samples (N = 101) were tested by pVNT (**Supplementary Table 7.1**), following the protocol described within *Chapter 3*, with some modifications. Briefly, HIV reporter pseudoviruses expressing SARS-CoV-2 S protein from the ancestral virus (B.1 lineage) and luciferase were generated.

Pseudoviruses expressing a VSV-G protein instead of the S protein were generated and used as control of specificity as previously described [240].

For the neutralization assay, 200 $TCID_{50}$ of pseudovirus were pre-incubated with three-fold serial dilutions (from 1/60 to 1/14,580) of heat-inactivated sera samples for 30 min at 37ºC. Next, human ACE2-overexpressing HEK293T cells were added onto mixed samples. After 48 h, cells were lysed with Britelite Plus Luciferase reagent (Perkin Elmer, Waltham, MA, USA) and luminescence was measured for 0.2 s with EnSight multimode late reader (Perkin Elmer).

The neutralization capacity of the sera samples was calculated by comparing the experimental RLU from infected cells treated with each serum to the maximum RLUs (maximal infectivity calculated from infected untreated cells) and minimum RLUs (minimal infectivity calculated from uninfected cells), and expressed as percentage of neutralization: % Neutralization = $(RLU_{max} - RLU_{experimental}) / (RLU_{max} - RLU_{min}) \times 100$. ID_{50} values were calculated by plotting and fitting neutralization values and the log of plasma dilution to a four-parameter equation in Prism 10.0.2 (GraphPad Software, San Diego, CA, USA). All ID_{50} values are reported as reciprocal dilution. Titers of nAbs equal or higher than 60 were considered positive, while those samples with lower titer ($ID_{50} < 60$) were considered negative.

ELISA assays

ELISA-1: cPass SARS-CoV-2 Neutralization Antibody detection kit (Genscript, USA)

All samples (N=101) were previously evaluated by ELISA-1 in each corresponding study (**Supplementary Table 7.1**). The protocol was performed following the manufacturer's instructions and as previously described in *Chapter 3*. Results were expressed by the formula provided by the manufacturer's protocol: % Signal Inhibition (%IH) = (1 – (OD value of sample/OD value of negative control)) x 100%. Samples with %IH ≥ 30% were considered positive for RBD nAbs.

This test is based on the RBD of the ancestral variant (B.1) of SARS-CoV-2 firstly detected in Wuhan (China). ELISA-1 was validated using human samples from the United States from early stages of the pandemic (from March 2020 to November 2020), when other circulating variants still not emerged [30].

ELISA-2: SARS-CoV-2 NeutraLisa kit (Euroimmune, Germany)

A total of 87 out of 101 samples were tested by ELISA-2 (**Supplementary Table 7.1**), following the manufacturer's instructions, and as previously described in *Chapter 4*. Results were expressed as an inhibition percentage (%IH) according to the formula provided by the manufacturer's instructions: %IH = 100% - (Sample OD x 100% /mean OD of black controls). A %IH ≥35% was considered positive neutralization, while %IH ≥ 20 to < 35 was considered doubtful, and %IH < 20 was considered as a negative neutralization.

This test provides S1/RBD from the B.1 ancestral variant of SARS-CoV-2. It was validated using human samples; therefore, it is recommended for serum and plasma samples from humans.

ELISA-3: ID Screen® SARS-CoV-2 Double Antigen Multi-species assay (IDVET, France)

A total of 99 out of 101 samples were tested by the ELISA-3 (**Supplementary Table 7.1**) following the manufacturer's protocol and as described in *Chapter 4*. Results were analyzed by the following formula provided by the manufacturer's protocol: Sample/Positive control (S/P) % = [(OD sample – OD negative control) / (OD positive control – OD negative control)] x 100. Samples with S/P% ≥ 60 were considered positive for N protein antibodies, S/P% from 50 to 60 were considered doubtful, and S/P% ≤ 50 were considered negative.

The use of this test is recommended for serum or plasma samples from cats, dogs, bovines, sheep, goats, horse, and any other susceptible animal species.

Statistical analyses

Sensitivity and specificity, and predictive positive and negative values (PPV and PNV, respectively) were calculated for each ELISA using pVNT as a reference (cut-off = ID_{50} 60). The overall diagnostic performance of each test was determined using receiver operating curve (ROC) analysis and calculating the area under the curve (AUC).

A Spearman correlation analyses were conducted between each ELISA and pVNT, and among ELISAs detecting RBD nAbs (ELISA-1 and

ELISA-2), and among ELISA-1 and ELISA-3, to study the correlation between presence of RBD nAbs and N protein antibodies. Correlation analyses with *P-values* < 0.05 were considered significant.

All statistical analyses were performed using GraphPad Prism (version 10.0.2).

7.3 Results

Detection of SARS-CoV-2 humoral response by pVNT

All samples (N = 101) were evaluated by pVNT using a pseudotype containing the S glycoprotein of the initial Wuhan variant of SARS-CoV-2 (B.1 lineage). A total of 33 out of 101 (32.67%) samples tested positive, with ID_{50} values ranging from 92.73 to 9570, and the remaining 68 (67.33%) samples tested negative (**Supplementary Table 7.1)**

Detection of SARS-CoV-2 humoral response by ELISA-1, ELISA-2, and ELISA-3

All samples (N = 101) that were tested by pVNT, were also evaluated by ELISA-1 (**Supplementary Table 7.1**). From 33 pVNT-positive samples, 32 (96.97%) were positive (%IH ≥ 30) and 1 (3.03%) was negative by ELISA-1 (**Figure 7.1**; **Table 7.1A**). However, from 68 pVNT-negative samples, 2 (2.94%) sera samples tested positive by ELISA-1. These samples corresponded to two wild boars that exhibited %IH values of 34.87% and 30.02%, respectively (**Supplementary Table 7.1**).

Besides, a total of 87 samples out of 101 pVNT-evaluated samples were also tested by ELISA-2 (**Supplementary Table 7.1**). From 29 pVNT-

positive samples, 15 (51.72%) samples were positive (%IH≥35), 4 (13.79%) samples were considered doubtful (%IH ≥ 20 to < 35) and 10 (34.48%) samples were considered negative (%IH < 20) by ELISA-2 (**Figure 7.1B**). This is represented by a wide distribution of the %IH values of positive pVNT samples within the ELISA-2 compared to ELISA-1 (**Figure 7.1A-B**). No false positive results were obtained by ELISA-2 (**Figure 7.1B).**

Finally, a total of 99 out of 101 samples were tested by the ELISA-3 (**Supplementary Table 7.1**). Although false positive results were not observed, 19 (61.29%) samples tested negative (S/P% ≤ 50) from a total of 31 pVNT-positive samples (**Figure 7.1C**). This test has a similar distribution of the pVNT-positive samples as ELISA-2 (**Figure 7.1B-C**).

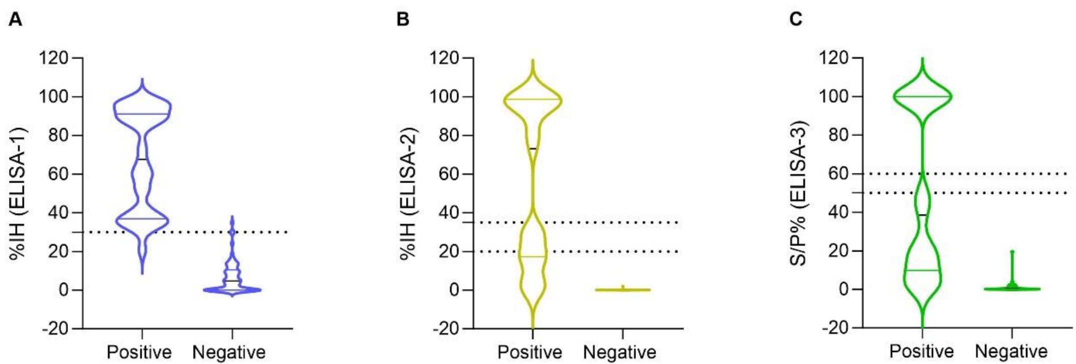

Figure 7.1 Distribution of inhibition (%IH) values of **(A)** ELISA-1 (N = 101), **(B)** ELISA-2 (N = 87) and sample/positive % (S/P%) values of **(C)** ELISA-3 (N = 101) among pVNT-positive and pVNT-negative results (X axes). Dashed lines indicate the cut-off for each serological assay. Negative values were represented as zero, and values exceeding 100 were represented as 100 for illustrative purposes. The values of pVNT-positive samples exhibited a wider distribution within ELISA-2 and ELISA-3, compared to ELISA-1. Two false positive results were observed by ELISA-1, but none by ELISA-2 and ELISA-3.

In all ELISA tests, the higher the pVNT-titer, the less likely occurrence of false negative results (**Figure 7.2**). Notably, pVNT-positive samples with titers <1,000 were detected as doubtful or negative by ELISA-2. In parallel, all samples with pVNT titers ≥1,000 were detected positive with IH% values ≥79% by ELISA-2 **(Figure 7.2B).** Meanwhile, ELISA-1 was able to detect positive samples at least from pVNT titers ≥60 **(Figure 7.2A)**. Besides, all positive ELISA-3 samples that were also positive by pVNT gave values from S/P% > 100. In contrast, around two thirds of positive pVNT samples yielded negative results by ELISA-3 **(Figure 7.2C).**

A significant correlation (*p-value* < 0.05) was observed between each ELISA and pVNT, using Spearman correlation analysis. ELISA-1 showed a Spearman correlation coefficient (r) value of 0.7985 (%95 CI = [0.7118-0.8613]), ELISA-2 exhibited an r value of 0.7935 (%95 CI = [0.6966 – 0.8620]), and finally, ELISA-3 exhibited an r value of 0.6663 (%95 CI = [0.5357 – 0.77658]).

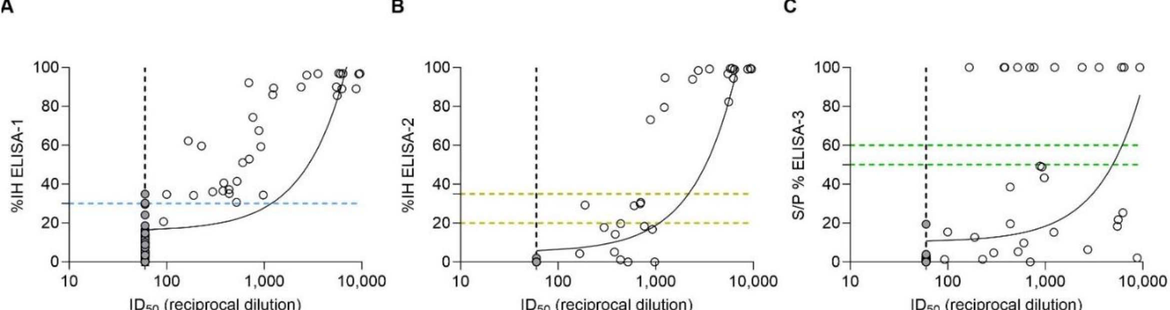

Figure 7.2 Spearman Correlation analysis between pVNT and **(A)** ELISA-1 (r = 0.7985), **(B)** ELISA-2 (r = 0.7935), and **(C)** ELISA-3 (r = 0.6663). Dashed lines indicate the cut-off for each serological assay (blue color for ELISA-1, yellow color for ELISA-2 and green color for ELISA-3). Within ELISA assays, negative values were represented as zero, and values exceeding 100 were represented as 100 for illustrative purposes. ID_{50} (pVNT) is represented as the reciprocal dilution. Filled grey circles indicate samples out of the minimum limit of quantification of the pVNT.

Qualitative comparison of ELISAs with pVNT

To calculate sensitivity and specificity of each ELISA (ELISA-1, ELISA-2, and ELISA-3), the pVNT was used as the gold standard technique with a cut-off = ID_{50} 60. ELISA-1 exhibited the highest sensitivity (96.90%) followed by ELISA-2 (51.72%) and ELISA-3 (38.70%) (**Table 7.2**). When doubtful samples from ELISA-2 (n = 4) were considered positive, the sensitivity increased (65.51%), although this test still showed less sensitivity than ELISA-1, and higher sensitivity than ELISA-3. ELISA-1 showed the lowest specificity (97.05%), compared to ELISA-2 (100%) and ELISA-3 (100%). PPV and NPV are also represented in **Table 7.2**.

Table 7.2 Comparison of sensitivity and specificity of ELISA-1, ELISA-2, and ELISA-3 using the pVNT as a reference (Cut-off = ID_{50} 60). Seropositivity was defined by a cut-off of %IH ≥30% for ELISA-1, % IH ≥35% for ELISA-2, S/P% ≥ 60 for ELISA-3. P: Positive; N: Negative; PPV: positive predictive value; NPV: negative predictive value. The confidence interval (95%) is also represented for each parameter.

		pVNT						
		P	**N**		**Sensitivity (% 95 CI)**	**Specificity (% 95 CI)**	**PPV (% 95 CI)**	**NPV (% 95 CI)**
ELISA 1	**P**	32	2	34	96.90%	97.05%	94.10%	98.50%
	N	1	66	67	(91.1%-102.8%)	(93.0%- 101.1%)	(86.2% - 102.0%)	(50.6%-101.4%)
		33	68	**101**				
ELISA 2	**P**	15	0	15	51.72% *	100.00%	100.0%	80.60% *
	N	14	58	72	(33.50%-69.9%)	(100.0%-100.0%)	(100.0%- 100.0%)	(71.4%- 89.7%)
		29	58	**87**	65.50% (48.2%- 82.8%)			85.30% (76.9%- 93.7%)
ELISA 3	**P**	12	0	12	38.70%	100.00%	100.00%	78.20%
	N	19	68	87	(21.6% - 55.9%)	(100.0% -100.0%)	(100.0%- 100.0%)	(69.5% - 6.8%)
		31	68	**99**				

*The sensitivity and NPV increased from 51.72% to 65.51% and from 80.60% to 85.30%, respectively, when doubtful samples (n=4) from ELISA-2 were considered positive.

Serum samples from cats and dogs were also analyzed independently to assess sensitivity and specificity of each ELISA for each species using pVNT as a reference (**Table 7.3**). Within cats, ELISA-1 exhibited the highest sensitivity (100.00%), followed by the ELISA-2 (72.70%), and ELISA-3 (66.70%). When doubtful samples from ELISA-2 (n = 2) were considered positive, the sensitivity increased to 90.90%, although this test still showed less sensitivity than ELISA-1, and higher sensitivity than ELISA-3. Regarding dogs, ELISA-1 still exhibited the highest sensitivity (100.00%), followed by ELISA-3 (33.30%), and ELISA-2 (16.70%). The sensitivity of

ELISA-2 increased to 33.30% when doubtful samples (n = 2) were considered positive. All tests showed a specificity of 100.00% for both cats and dogs. PPV and NPV are also represented in **Table 7.3.** All other species were not included in these analyses since the number of samples per species was very low and/or samples were not analyzed with all the different assays.

Table 7.3 Comparison of sensitivity and specificity of ELISA-1, ELISA-2, and ELISA-3 using the pVNT as a reference (Cut-off = ID_{50} 60) in cats and dogs, independently. Seropositivity was defined by a cut-off of %IH ≥30% for ELISA-1, % IH ≥35% for ELISA-2, S/P% ≥ 60 for ELISA-3. P: positive; N: negative PPV: positive predictive value; NPV: negative predictive value. The confidence interval (95%) is also represented for each parameter.

			pVNT						
			P	**N**		**Sensitivity** (% 95 CI)	**Specificity** (% 95 CI)	**PPV** (% 95 CI)	**NPV** (% 95 CI)
CATS	**ELISA 1**	**P**	11	0	**11**	100% (100.0%- 100.0%)	100% (100.0%- 100.0%)	100% (100.0%-100.0%)	100% (100.0%-100.0%)
		N	0	25	**25**				
			11	**2**	**36**				
	ELISA 2	**P**	8	0	**8**	72.70%* (46.4% - 99.9%) 90.90% (73.9% - 107.9%)	100% (100.0%- 100.0%)	100% (100.0%-100.0%)	89.3%* (77.8%- 100.7%) 96.2% (88.8%- 103.5%)
		N	3	25	**28**				
			11	**25**	**36**				
	ELISA 3	**P**	6	0	**6**	66.70% (35.9% - 97.5%)	100% (100.0%- 100.0%)	100% (100.0%-100.0%)	89.3% (77.8%- 100.7%)
		N	3	25	**28**				
			9	25	**34**				
DOGS	**ELISA 1**	**P**	12	0	**12**	100% (100.0% - 100.0%)	100% (100.0%- 100.0%)	100% (100.0%-100.0%)	100% (100.0%-100.0%)
		N	0	29	**29**				
			12	**29**	**41**				
	ELISA 2	**P**	2	0	**2**	16.70%* (-4.4%- 37.8%) 33.30% (6.70%- 60.0%)	100% (100.0%- 100.0%)	100% (100.0%-100.0%)	74.40% * (60.7%– 88.1%) 78.4% (65.1%- 91.6%)
		N	10	29	**39**				
			12	**29**	**41**				
	ELISA 3	**P**	4	0	**4**	33.30% (6.70%- 60.0%)	100% (100.0%- 100.0%)	100% (100.0%-100.0%)	78.40% (65.10%– 91.6%)
		N	8	29	**37**				
			12	**41**	**41**				

*The sensitivity and NPV increased from 16.70% to 33.30% and from 74.40% to 78.40%, respectively, when doubtful samples (n=2) of ELISA-2 were considered positive.

For the overall diagnostic performance of ELISA-1, ELISA-2, and ELISA-3, ROC analyses were performed, and the AUC was calculated for each assay (**Figure 7.3**). Data was evaluated according to the cut-off established for the pVNT (ID_{50} 60). The antibody assay with the highest AUC was ELISA-1 (AUC of 0.9964), followed by the ELISA-2 (AUC of 0.9732) and ELISA-3, which exhibited the lowest AUC (0.9435).

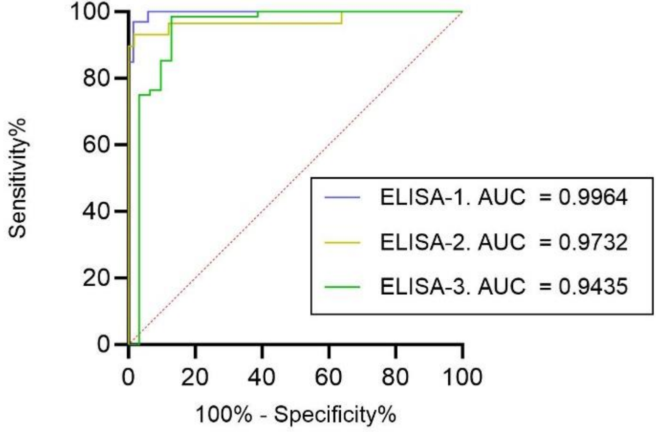

Figure 7.3 ROC curves of all evaluated ELISAs using the pVNT as a reference (cut-off ID_{50} 60). Associated AUC values are also represented.

Correlation of ELISAs detecting RBD nAbs (ELISA-1 vs ELISA-2)

A total of 87 out of 101 samples were analyzed by both ELISA-1 and ELISA-2, which similarly targeted RBD nAbs. A significant correlation (*P*-value < 0.05) between these assays ($r = 0.6734$; 95% CI = [0.5374 – 0.7767]) (**Figure 7.4**). From 56 samples that tested negative by ELISA-1, all tested negative by ELISA-2 (56/87; 64.37%). However, from 31 ELISA-1-positive samples (31/87; 35.63%), only 15 (15/31; 48.38%) samples tested positive

(% IH ≥35%), 4 (4/31; 12.90%) samples tested doubtful (%IH ≥ 20 to < 35), and 12 (12/31; 38.70%) samples tested negative (%IH < 20) by ELISA-2 (**Figure 7.4**). Generally, %IH values determined by ELISA-1 were slightly higher than those obtained by ELISA-2. Consequently, those %IH values close to the cut-off of ELISA-1 (%IH = 30%) were negative (%IH < 20) or doubtful (%IH ≥ 20 to < 35) by ELISA-2 (**Figure 7.4**).

Samples from cats (n = 36) and dogs (n = 41) were also analyzed separately to investigate the correlation between ELISA-1 and ELISA-2 for each species. Significant correlation (*P*-value < 0.05) was observed for both species (r (Spearman) = 0.6625 [%95 CI = 0.4187 – 0.8173] for cats and r (Spearman) = 0.6143 [%95 CI = 0.3700 – 0.7791] for dogs). All samples from cats and dogs considered negative by ELISA-1, were also negative by ELISA-2 (25/36 cats [71.42%]; 29/41 dogs [51.22%]) (**Figure 7.4**). However, a total of 11 seropositive cats (11/36; 30.55%) were detected by ELISA-1, from which only 8 (8/11; 72.73%) cats tested positive, 2 (2/11; 18.18%) tested doubtful, and 1 (1/11; 9.09%) tested negative by ELISA-2. Besides, a total of 12 seropositive dogs (12/41; 29.26%) were detected by ELISA-1, from which only 2 (2/12; 16.66%) dogs tested positive, 2 (2/12; 16.66%) dog samples were doubtful, and 8 (8/12; 66.66%) dogs tested negative by ELISA-2 (**Figure 7.4**).

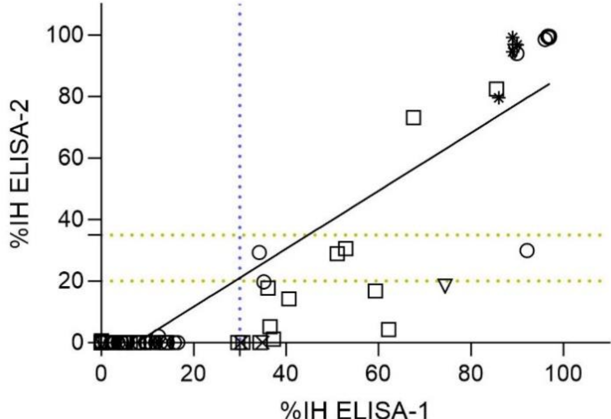

Figure 7.4 Spearman correlation analysis between ELISAs detecting nAbs against the RBD of SARS-CoV-2 (ELISA-1 and ELISA-2) (n = 87). Dashed lines indicate the cut-off for each serological assay (blue color for ELISA-1, and yellow color for ELISA-2). Negative values were represented as zero, and values exceeding 100 were represented as 100 for illustrative purposes. Species are indicated by different figures: circle (cats; n = 36), square (dogs; n = 41), downward triangle (ferret; n = 4), star (lions; n = 4), and cross (wild boar; n = 2). Spearman correlation coefficient value (r) = 0.6734 (95% CI = [0.5374 – 0.7767]).

Qualitative and quantitative correlation of RBD nAbs (ELISA-1) and N protein antibodies (ELISA-3)

To determine the correlation between the presence of RBD nAbs and N protein antibodies, the correlation between ELISA-1, the most sensitive ELISA for detection of RBD nAbs, and ELISA-3 was evaluated (n = 99). All samples that were negative for the presence of RBD nAbs (67/99; 67.89%), were also negative for the presence of N protein antibodies (S/P% ≥ 60). However, from 32 (32/99; 33.32%) samples exhibiting RBD nAbs, only 12 (12/32; 37.5%) were positive for the presence of N protein antibodies (S/P%

≥ 60) (**Figure 7.5A**). The two ELISAs showed a significant (*p-value* < 0.05) correlation (r = 0.5999; 95% CI = [0.4518 – 0.7158]) (**Figure 7.5B**).

Samples from cats (n = 34) and dogs (n = 41) were analyzed separately to investigate the correlation between ELISA-1 and ELISA-3 for each species. Significant correlation (*P*-value < 0.05) was observed for both species (r (Spearman) = 0.5585 [%95 CI = 0.2620-0.7587] for cats and r (Spearman) = 0.5318 [%95 CI = 0.2593 – 0.7259] for dogs). All cats and dogs that were negative for nAbs RBD detection, were also negative for N antibodies detection (25/34 cats [73.53%]; 29/41 dogs [51.22%]) (**Figure 7.5B**). However, from 9 (9/34; 26.47%) samples of cats exhibiting RBD nAbs (ELISA-1), 6 (6/9; 66.66%) were positive and 3 (3/9; 33.33%) were negative for N protein antibodies (ELISA-3). Besides, from 12 (12/41; 29.26%) positive dogs for RBD nAbs, only 4 (4/12; 33.33%) tested positive, and 8 (8/12; 66.66%) tested negative for N protein antibodies (ELISA-3) (**Figure 7.5B**).

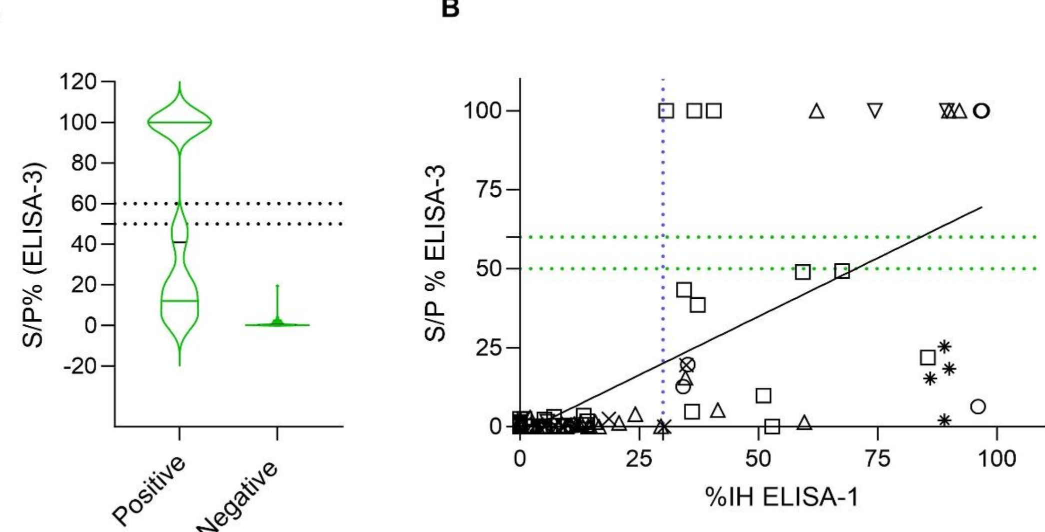

Figure 7.5 Qualitative and quantitative comparison analysis of ELISA-1 and ELISA-3 (N = 99). Negative values were represented as zero, and values exceeding 100 were represented as 100 for illustrative purposes. **(A)** Distribution of sample/positive % (S/P%) values of ELISA-3 within positive and negative ELISA-1 results (Cut-off IH % ≥30). Discontinuous lines indicate the cut-off of ELISA-2**.** **(B)** Correlation analysis between ELISA-1 and ELISA-3. Dashed lines indicate the cut-off for each serological assay (blue color for ELISA-1, and green color for ELISA-3). Species are indicated by different figures: circle (cats; n=34), square (dogs; n=41), downward triangle (ferret; n=4), upward triangle (goats; n=6), star (lions; n=4), and cross (wild boar; n=10). Spearman correlation coefficient value (r)= 0.5999 (95% CI = [0.4518 – 0.7158]).

Serological assays associated to RT-qPCR detection in animal samples

For most of animals included in this study, RT-qPCR was previously performed in each corresponding study, to assess whether they were suffering from a SARS-CoV-2 acute infection at the time of sampling. This includes all samples from pets (cats, dogs, and ferrets), although only one dog tested

positive by RT-qPCR (Dog 36 in **Supplementary Table 7.1**). This animal was infected by the Delta (B.1.617.2) variant. Dog 36 tested positive by pVNT (ID_{50} 884), ELISA-1 (%IH = 67.55%), and ELISA-2 (%IH = 73.13%), but negative by ELISA-3, with titers of N protein antibodies (S/P% = 49.27) close to the cut-off to consider it as doubtful (S/P% = 50-60%). The serum sample was collected two months after the RT-qPCR positive result (**Supplementary Table 7.1**), as previously described in *Chapter 3* (there referred as D2). Regarding domestic goats, the experimental infection with the Beta variant (B.1.351) was confirmed by RT-qPCR in all cases (*Chapter 6*); three goats (Goat 1, Goat 2, and Goat 3 in **Supplementary Table 7.1**) were sampled at 2 dpi, while the other three animals (Goat 4, Goat 5, and Goat 6 in **Supplementary Table 7.1**) were sampled at 18 dpi. All goats from 2 dpi tested negative for RBD nAbs by ELISA-1, and for N protein antibodies by ELISA-3. Goats' sera were not tested by ELISA-2. One goat from 2 dpi (Goat 3 in **Supplementary Table 7.1**) tested positive by pVNT with titers of ID_{50} 92.73, while the remaining ones tested negative. All goats from 18 dpi tested positive by both ELISA-1 and pVNT. With regard to the naturally infected lions, all tested positive by RT-qPCR (Pango lineage B.1.177) within 10 days (Lion 4 in **Supplementary Table 7.1**), 24 days (Lion 1 and Lion 3 in **Supplementary Table 7.1**), and 40 days (Lion 2 in **Supplementary Table 7.1**) before the blood sampling [165]. Also, all lions tested positive by pVNT and for RBD nAbs by ELISA-1 and ELISA-2, but negative for N protein antibodies by ELISA-3. None of the wild boar were tested by RT-qPCR.

7.4 Discussion

In the domain of human health, numerous serological assays have been developed to test exposure to SARS-CoV-2 [240,261,315]. Nevertheless, within the veterinary field, many serological tests were adapted from those

designed for humans without previous validation for animal samples [315]. Detection of acute infection by SARS-CoV-2 in animal species has more difficulties than in human population. Most of the animals do not exhibit clinical signs upon SARS-CoV-2 infection, they can be widely distributed, mainly free-range wild animals, making it very difficult to trace their contact chains, and sample accessibility can be complicated [313]. Thus, validation of serological assays for animal species is crucial for detecting SARS-CoV-2 antibodies, which demonstrates viral exposure and potential susceptibility to infection [329,330].

Given that this *PhD Thesis* used different ELISAs kits (ELISA-1, ELISA-2 and/or ELISA-3) to investigate humoral responses in animal species, the present study aimed to evaluate their correlation and compare them using the pVNT as a reference. ELISA-1 demonstrated the best diagnostic performance with the highest sensitivity (96.90%) in serum samples with pVNT titers >60, compared to ELISA-2 (51.72% - 65.51%), and ELISA-3 (35.70%). Consequently, by using ELISA-1, there is a higher probability that animals testing negative truly lack nAbs. Importantly, ELISA-2 was not able to detect positive samples with low titers of nAbs (<1,000), while ELISA-1 was able to detect positive samples with pVNT titers of 60 or higher. Considering that the main objective of our study was to determine which is the most accurate kit for initial screenings, it is crucial to prioritize obtaining the highest sensitivity possible. Thus, the ELISA-1 may be selected for this purpose, despite presenting lower specificity (97.05%) compared to the other tests (100.00%). This may be explained because ELISA-1 uses a lower cut-off value (%IH = 30%) than ELISA-2 (%IH = 35%) and ELISA-3 (S/P% = 60%). False-positive results could be identified by further analysis with pVNT or other techniques considered gold-standard

by the CDC (VNT or PRNT). Contrarily, ELISA-2 or ELISA-3 could potentially dismiss a significant number of seropositive samples.

ELISA-1 and ELISA-2 are both surrogate neutralization tests targeting RBD nAbs. Accordingly, pVNT also evaluates neutralizing response against the S glycoprotein, where RBD is located [24]. Considering that the ELISA-3 detects N protein antibodies, lowest diagnostic performance, and lowest correlation for this kit with pVNT was already expected. Both ELISA-1 and ELISA-2 use purified and recombinant RBD protein and the host cell receptor ACE2, and both evaluate the inhibition capacity of antibodies to neutralize RBD-ACE2 interaction [331]. This strategy gives clear advantages over conventional ELISAs which not differentiate between nAbs and bAbs, together with the fact that ELISA-1 and ELISA-2 are isotype- and species-independent [261,318]. Although these ELISAs are both competitive based, the design of each assay is different. ELISA-1 uses plates coated with the hACE2 extracellular domain and soluble RBD-HRP [315], whereas ELISA-2 has RBD pre-coated plates and uses soluble biotinylated ACE2 to dilute sera samples and further nAbs detection. Different orientation of the RBD and ACE2 proteins between kits may explain the differences observed between them.

A previous comparative study of various ELISAs testing human samples also observed higher sensitivity for ELISA-1 (93.65%) than for ELISA-2 (56.44%) using the VNT as a reference [261]. All ELISAs included herein have already been used in previous studies to investigate humoral responses in humans but also in different animal species [154,229,235,315,317,329,332]. However, both ELISA-1 and ELISA-2 were validated using human samples, and only ELISA-3 was validated for animal species including dogs, cats, cattle, horse, goat, and sheep. ELISA-3 used pre-pandemic samples for all mentioned animal species and exhibited an overall specificity of 99.1%, as

described by the manufacturer's protocol. Nevertheless, few positive samples were included to test sensitivity for each species. Moreover, serum samples from wildlife animals were not included for test validation. Assay sensitivity and specificity may differ between humans and animal samples, but also between animal species. In the present study, we observed that sensitivity for dog samples was strongly reduced in ELISA-2 (with values of 16.70% - 33.30%) and ELISA-3 (with a value of 33.30%). For cat samples, ELISA-2 exhibited a sensitivity of 72.70 – 90.90% and ELISA-3 exhibited a sensitivity of 66.70%. ELISA-1 is still the most accurate kit for both species with a sensitivity of 100%. Wildlife animal species are usually exposed to a major number of pathogens and their samples usually have lower quality than those from domestic animals [333]. Accordingly, samples from this group of species likely exhibit less specificity in serological tests.

In the present study, we detected two positive wild boars using ELISA-1 (with low percentage of inhibition), although they were negative by pVNT, ELISA-2, and ELISA-3. Worthy to note, both wild boars also tested negative by VNT, as previously described in *Chapter 5* (animals referred as Wb2 and Wb3). In a previous published study, sera from five out of fourteen wild boars collected during the COVID-19 pandemic were able to weakly neutralize SARS-CoV-2 by VNT (titers of nAbs from 10 to 50) [287]. Although potential exposure to SARS-CoV-2 of this animal species may not be entirely ruled out, the low titers of nAbs supports that their sera components may yield false positive results in VNT [287]. Here, we used a cut-off of ID_{50} = 60 for pVNT, which could have prevented potential false-positive results for this species and led to characterize the two ELISA-positive wild boars as negative for SARS-CoV-2 neutralization. Importantly, Hulst et al., observed that sera of juvenile pigs from the pre-pandemic period cross-reacted with recombinant S and N protein of SARS-CoV-2 by ELISA; the authors

suspected that these animals were previously infected with *Alpha*- and *Beta*-swine *CoVs,* which increased the likelihood of a potential cross-reactivity of antibodies [287]. A previous study assessing SARS-CoV-2 infection in wildlife species also suggested cross-reactivity in sera from wild boars using ELISA-3 (targeting the N protein), as they observed negative results when used ELISA-1 (targeting RBD nAbs) as a confirmatory test [334]. In the same line, another study confirmed two-way cross-reactivity for SARS-CoV and porcine CoV (transmissible gastroenteritis CoV - TGEV - and porcine respiratory CoV – PRCV) in ELISA, although it was demonstrated to be mediated by the N protein but not by the S glycoprotein [335]. Klompus et al., investigating cross-reactivity of SARS-CoV-2 with different peptides from animal CoVs also confirmed positive results regarding porcine CoV peptides [288]. Altogether highlights the importance to establish specific cut-offs for serological analysis for each animal species.

Despite the high specificity observed for ELISA-3 in our study, it is known that N protein gene is highly conserved among CoVs and can potentially lead to cross-reactivity of antibodies. Diezma-Diaz et al., used pre-pandemic samples from cats and dogs and observed cross-reactivity by N-protein based ELISAs [318]. Other studies also suggested cross-reactivity of N-protein antibodies from Canine-CoVs in serum samples from dogs, but also in serum samples from domestic cats previously infected by Feline-CoV (FCoV), using N-antigen ELISA assays [317,319,336]. Cross-reactivity to SARS-CoV-2 of antibodies targeting the RBD has been suggested in previous studies for some animal species, including domestic cats [268,288,318,337]. Importantly, RBD of SARS-CoV-2 exhibit higher antigenic distinctiveness from other animal CoVs compared to the N protein antigen or even when considering the whole S glycoprotein [140,318,319,321,327]. Previous studies using human samples already demonstrated cross-reactivity with SARS-CoV-2 of

antibodies targeting the N protein of hCoVs, principally *Alpha*-hCoV (229E and NL363), but also *Beta*-hCoV (HKU1 and OC43) [320]. Interestingly, cross-reactivity can be reduced using truncated versions from less conserved fragments of the N protein sequence instead of using the full length [320]. ELISA-3 is based on the truncated N recombinant antigen, which could affect the specificity and sensitivity of this test [320,336].

According to our results, previous studies supported that ELISAs based on the S glycoprotein and RBD protein are most accurate than those based on the N protein to diagnose seroconversion against SARS-CoV-2, especially in cats and dogs [318,319]. Diezma-Díaz et al. found that ELISA-3 (targeting N protein antibodies) did not correlate with VNT and had a poor diagnostic performance (AUC = 0.55) for dog samples, and a weakly but significant correlation and better diagnostic performance (AUC = 0.90) when analyzed cat samples [318]. Consistent with this, our data demonstrated that ELISA-3 had lower sensitivity for dog samples (33.33%) than for cat samples (66.70%). Other studies did not find correlation of ELISA-3 or other N-protein based assays with VNT or pVNT, neither for cats nor for dogs [317,319]. Into the human population, levels of N protein antibodies are positively correlated with COVID-19 severity [320]. Considering that dogs demonstrated lower susceptibility upon experimental and natural conditions compared to cats, they likely develop lower antibody titers which could be dismissed by the low sensitivity of ELISA-3 [64,66].

Moreover, assay sensitivity also may change depending on the time occurring from viral exposure or infection to sample collection, as it is known that measurable SARS-CoV-2 antibodies wane over time [338,339]. All pet animals included in this study were sampled owing to veterinary check-ups, and in some cases, due to clinical signs associated with SARS-CoV-2 infection. Despite evidence of viral exposure in some animals by presence of

nAbs, the timing between exposure and sampling was unknown. All pets tested negative for acute infection, except for Dog 36 (**Supplementary Table 7.1**), which tested positive by RT-qPCR. This animal was sampled for antibodies detection two months after acute infection and exhibited positive neutralization by pVNT, with nAbs against RBD according to ELISA-1 and ELISA-2. The animal exhibited N protein antibodies within the limit to consider it as doubtful. These results could be explained by the fact that N protein antibodies are known to persist for a shorter duration compared to RBD or S glycoprotein nAbs, at least in the human population [339]. This was also the case of the naturally infected lions, which were sampled for antibody detection at least 10 days, and maximum 40 days after detection of acute infection, and did not exhibit N protein antibodies but RBD nAbs [165]. Importantly, this fact could partially explain the negative results in ELISA-3 in most of the samples of this study, when presence of nAbs was confirmed by the other assays. Animals could have been potentially exposed to the virus long before the time of sampling.

Our analyses are mainly limited by the lack of previously validation of pVNT for animal samples. For this purpose, positive and negative controls for each species of interest should be included to establish appropriate cut-off values for this test. Considering that cross-reactivity with other CoVs is not uncommon, the use of serum samples from animals previously infected with other CoVs, as well as samples from the pre-pandemic period may be beneficial to validate the pVNT. Besides, a larger number of samples from each species from the present comparative study may be included to assess sensitivity, specificity, and an appropriated cut-off for specific species. Considering the advantages of pVNT over the VNT or PRNT (*e.g.,* time-consuming, sample processing capacity, more safety, and its use in BSL-2 facilities), validation of pVNT for animal samples could be very useful to use

it as a confirmatory test after previous ELISA screenings. The pVNT used here was developed and validated using human samples and the VNT as a reference. Interestingly, pVNT and VNT exhibited high qualitative and quantitative correlation (r [Spearman test] = 0.865) [240]. An additional trait of pVNT is its higher adaptability for testing newly emerging variants using pseudoviruses expressing the S glycoprotein of each variant of interest. In our study, all samples were tested with ELISAs that used the RBD of the ancestral (B.1 lineage) variant of SARS-CoV-2, as well as the pVNT with the pseudotype expressing the S protein only from the ancestral variant. Thus, this analysis may be adapted using the RBD or the S glycoprotein of the newly Omicron variants and check whether there is a similar correlation. Those animals that tested negative might test positive for antibodies against the Omicron variant or its sub-lineages. However, only 12 out of 101 serum samples from this study were collected between December 2021 and February 2022, when Omicron variant emerged in Spain.

In summary, ELISA-1 was the most suitable kit for initial screenings of animal samples, and specifically for cats and dogs, compared to ELISA-2 and ELISA-3. ELISA-3 may be useful to use it as an additional test for assessing presence of N-protein antibodies. ELISA-1 demonstrated to be not only effective but also efficient, requiring only ≈1.5 h from start to finish, while ELISA-2 and ELISA-3 required ≈2.5 h. However, despite ELISA-1's high diagnostic capacity, it cannot replace pVNT. ELISA-1 detects only nAbs against the RBD, while pVNT detects neutralization against the whole S glycoprotein [340]. Although the primary target of SARS-CoV-2 nAbs is the RBD, there are also nAbs targeting the NTD located in the S1 domain, or directly to the S2 domain of the S glycoprotein (Chen et al., 2023). Furthermore, pVNT would be necessary to accurately quantify antibody levels in serum samples.

Supplementary Table 7.1 Comparison of sensitivity and specificity of ELISA-1, ELISA-2 and ELISA-3 using the pVNT as a reference (Cut-off = ID_{50} 60). Seropositivity was defined by a cut-off of %IH ≥30% for ELISA-1, % IH ≥35% for ELISA-2, S/P% ≥ 60 for ELISA-3.

Species	Date of sampling	COVID-19 pandemic wave in Spain	ELISA-1 (%IH)	ELISA-2 (%IH)	ELISA-3 (S/P%)	pVNT ID_{50}	RT-qPCR
Cat 1	April 2020	Wuhan variant (B.1 lineage)	Positive 96.87	Positive 99.06	Positive 1393.43	Positive 6413	Negative
Cat 2	April 2020	Wuhan variant (B.1 lineage)	Positive 96.59	Positive 99.53	Positive 116.00	Positive 9315	Negative
Cat 3	May 2020	Wuhan variant (B.1 lineage)	Positive 96.87	Positive 99.19	NA	Positive 9570	Negative
Cat 4	June 2020	Wuhan variant (B.1 lineage)	Positive 96.67	Positive 99.53	Positive 1070.91	Positive 6051	Negative
Cat 5	May 2020	Wuhan variant (B.1 lineage)	Negative -14.68	Negative -6.89	Negative 0.38	Negative <60	Negative
Cat 6	May 2020	Wuhan variant (B.1 lineage)	Negative -19.27	Negative -27.91	Negative 2.21	Negative <60	Negative
Cat 7	June 2020	Wuhan variant (B.1 lineage)	Negative -16.65	Negative -15.69	Negative -4.05	Negative <60	Negative
Cat 8	January 2021	Alpha (B.1.1.7)	Positive 97.00	Positive 99.46	NA	Positive 5818	Negative
Cat 9	April 2021	Alpha (B.1.1.7)	Positive 96.06	Positive 98.46	Negative 6.36	Positive 2724	Negative

Cat 10	May 2021	Alpha (B.1.1.7)	Positive 96.83	Positive 99.19	Positive 263.45	Positive 3552	Negative
Cat 11	March 2021	Alpha (B.1.1.7)	Negative -26.81	Negative -22.81	Negative 0.10	Negative <60	Negative
Cat 12	April 2021	Alpha (B.1.1.7)	Negative -39.80	Negative -15.69	Negative 0.14	Negative <60	Negative
Cat 13	May 2021	Alpha (B.1.1.7)	Negative -55.62	Negative -10.31	Negative 2.07	Negative <60	Negative
Cat 14	June 2021	Alpha (B.1.1.7)/ Delta (B.1.617.2)	Positive 35.15	Negative 19.72	Negative 19.64	Positive 438	Negative
Cat 15	August 2021	Delta (B.1.617.2)	Positive 34.2	Doubtful 29.33	Negative 12.73	Positive 189	Negative
Cat 16	August 2021	Delta (B.1.617.2)	Negative 12.81	Negative -5.48	Negative 1.10	Negative <60	Negative
Cat 17	November 2021	Delta (B.1.617.2)	Negative 16.54	Negative -13.34	Negative 0.05	Negative <60	Negative
Cat 18	September 2021	Delta (B.1.617.2)	Negative -2.415	Negative -17.90	Negative 0.00	Negative <60	Negative
Cat 19	September 2021	Delta (B.1.617.2)	Negative 15.79	Negative -19.38	Negative 1.44	Negative <60	Negative
Cat 20	September 2021	Delta (B.1.617.2)	Negative 9.57	Negative -21.8	Negative -0.05	Negative <60	Negative
Cat 21	November 2021	Delta (B.1.617.2)	Negative 14.24	Negative -24.02	Negative -0.10	Negative <60	Negative
Cat 22	November 2021	Delta (B.1.617.2)	Negative	Negative	Negative	Negative	Negative

			-10.85	-15.96	0.05	<60	
Cat 23	November 2021	Delta (B.1.617.2)	Negative -20	Negative -22.34	Negative 1.54	Negative <60	Negative
Cat 24	November 2021	Delta (B.1.617.2)	Negative 10.65	Negative -23.68	Negative 0.48	Negative <60	Negative
Cat 25	November 2021	Delta (B.1.617.2)	Negative 9.73	Negative -24.03	Negative 0.05	Negative <60	Negative
Cat 26	November 2021	Delta (B.1.617.2)	Negative 2.13	Negative -9.57	Negative 2.93	Negative <60	Negative
Cat 27	November 2021	Delta (B.1.617.2)	Negative -1.59	Negative -15.08	Negative -0.31	Negative <60	Negative
Cat 28	November 2021	Delta (B.1.617.2)	Negative 5.68	Negative -15.08	Negative 0.05	Negative <60	Negative
Cat 29	November 2021	Delta (B.1.617.2)	Negative 11.78	Negative -16.90	Negative 0.63	Negative <60	Negative
Cat 30	December 2021	Delta (B.1.617.2)/ Omicron (BA.1)	Positive 92.15	Doubtful 29.93	Positive 110.36	Positive 682	Negative
Cat 31	December 2021	Delta (B.1.617.2)/ Omicron (BA.1)	Negative 8.23	Negative -19.25	Negative 0.05	Negative <60	Negative
Cat 32	December 2021	Delta (B.1.617.2)/ Omicron (BA.1)	Negative 3.63	Negative 21.93	Negative -0.19	Negative <60	Negative
Cat 33	December 2021	Delta (B.1.617.2)/ Omicron (BA.1)	Negative 12.36	Negative 2.05	Negative 0.62	Negative <60	Negative
Cat 34	December 2021	Delta (B.1.617.2)/ Omicron (BA.1)	Negative 2.88	Negative -4.00	Negative 0.34	Negative <60	Negative

Cat 35	December 2021	Delta (B.1.617.2)/ Omicron (BA.1)	Negative 4.43	Negative -12.19	Negative 0.24	Negative <60	Negative
Cat 36	January 2022	Omicron (BA.1)	Positive 89.93	Positive 93.95	Positive 544.73	Positive 2388	Negative
Dog 1	July 2020	Wuhan variant (B.1 lineage)	Positive 51.07	Doubtful 28.92	Negative 9.82	Positive 601	Negative
Dog 2	December 2020	Wuhan variant (B.1 lineage)	Positive 52.87	Doubtful 30.60	Negative -55.00	Positive 702	Negative
Dog 3	May 2020	Wuhan variant (B.1 lineage)	Negative 29.53	Negative -1.92	Negative 0.10	Negative <60	Negative
Dog 4	August 2020	Wuhan variant (B.1 lineage)	Negative -3.38	Negative 0.64	Negative 0.53	Negative <60	Negative
Dog 5	March 2020	Alpha (B.1.1.7)	Negative 7.09	Negative -7.63	Negative 0.29	Negative <60	Negative
Dog 6	March 2020	Alpha (B.1.1.7)	Negative -2.69	Negative -20.86	Negative 0.24	Negative <60	Negative
Dog 7	April 2021	Alpha (B.1.1.7)	Negative -12.22	Negative -15.62	Negative 0.34	Negative <60	Negative
Dog 8	April 2021	Alpha (B.1.1.7)	Negative 5.11	Negative -6.75	Negative 2.31	Negative <60	Negative
Dog 9	April 2021	Alpha (B.1.1.7)	Negative 7.11	Negative -10.65	Negative 3.17	Negative <60	Negative
Dog 10	April 2021	Alpha (B.1.1.7)	Negative -6.04	Negative -6.15	Negative 0.96	Negative <60	Negative
Dog 11	April	Alpha (B.1.1.7)	Negative	Negative	Negative	Negative	Negative

	2021		4.80	-9.91	-0.05	<60	
Dog 12	April 2021	Alpha (B.1.1.7)	Negative -8.64	Negative -15.55	Negative 0.34	Negative <60	Negative
Dog 13	April 2021	Alpha (B.1.1.7)	Negative 7.95	Negative -19.65	Negative 0.34	Negative <60	Negative
Dog 14	April 2021	Alpha (B.1.1.7)	Negative 13.37	Negative -20.93	Negative 3.55	Negative <60	Negative
Dog 15	April 2021	Alpha (B.1.1.7)	Negative 2.61	Negative -13.81	Negative 0.14	Negative <60	Negative
Dog 16	May 2021	Alpha (B.1.1.7)	Negative 14.10	Negative -9.84	Negative 1.68	Negative <60	Negative
Dog 17	May 2021	Alpha (B.1.1.7)	Negative 1.40	Negative -13.74	Negative 0.19	Negative <60	Negative
Dog 18	May 2021	Alpha (B.1.1.7)	Negative 4.12	Negative -15.08	Negative 0.14	Negative <60	Negative
Dog 19	April 2021	Alpha (B.1.1.7)	Negative -2.25	Negative -2.72	Negative 2.45	Negative <60	Negative
Dog 20	May 2021	Alpha (B.1.1.7)	Negative 13.81	Negative -8.50	Negative 0.58	Negative <60	Negative
Dog 21	May 2021	Alpha (B.1.1.7)	Negative 9.17	Negative -3.53	Negative 0.05	Negative <60	Negative
Dog 22	May 2021	Alpha (B.1.1.7)	Negative 0.96	Negative -29.26	Negative 2.46	Negative <60	Negative
Dog 23	April 2021	Alpha (B.1.1.7)	Negative 5.19	Negative -12.53	Negative 0.11	Negative <60	Negative

Dog 24	April 2021	Alpha (B.1.1.7)	Negative 9.98	Negative -7.09	Negative 0.42	Negative <60	Negative
Dog 25	April 2021	Alpha (B.1.1.7)	Negative -3.76	Negative -7.02	Negative 0.26	Negative <60	Negative
Dog 26	April 2021	Alpha (B.1.1.7)	Negative -10.24	Negative -7.36	Negative 0.52	Negative <60	Negative
Dog 27	April 2021	Alpha (B.1.1.7)	Negative 2.61	Negative -2.05	Negative 0.42	Negative <60	Negative
Dog 28	May 2021	Alpha (B.1.1.7)	Negative -13.30	Negative -18.37	Negative 0.19	Negative <60	Negative
Dog 29	April 2021	Alpha (B.1.1.7)	Negative -13.15	Negative -7.02	Negative 0.24	Negative <60	Negative
Dog 30	June 2021	Alpha (B.1.1.7)/ Delta(B.1.617.2)	Positive 30.62	Negative -5.34	Positive 861.82	Positive 519	Negative
Dog 31	June 2021	Alpha (B.1.1.7)/ Delta (B.1.617.2)	Positive 85.51	Positive 82.40	Negative 21.82	Positive 5599	Negative
Dog 32	June 2021	Alpha (B.1.1.7)/ Delta (B.1.617.2)	Negative -3.32	Negative -21.53	Negative 0.00	Negative <60	Negative
Dog 33	July 2021	Delta (B.1.617.2)	Positive 36.06	Negative 17.77	Negative 4.73	Positive 296	Negative
Dog 34	September 2021	Delta (B.1.617.2)	Positive 34.35	Negative -17.64	Negative 43.27	Positive 974	Negative
Dog 35	September 2021	Delta (B.1.617.2)	Positive 37.22	Negative 1.11	Negative 38.55	Positive 436	Negative
Dog 36	28/09/2021	Delta (B.1.617.2)	Positive	Positive	Negative	Positive	Positive

			67.55	73.13	49.27	884	27/07/2021
Dog 37	October 2021	Delta (B.1.617.2)	Positive 59.31	Negative 16.83	Negative 48.91	Positive 926	Negative
Dog 38	November 2021	Delta (B.1.617.2)	Positive 40.62	Negative 14.28	Negative 116.10	Positive 386	Negative
Dog 39	November 2021	Delta (B.1.617.2)	Positive 36.54	Negative 5.14	Positive 1238.55	Positive 378	Negative
Dog 40	July 2021	Delta (B.1.617.2)	Positive 62.21	Negative 4.27	Negative 617.82	Positive 166	Negative
Dog 41	August 2021	Delta (B.1.617.2)	Negative -5.48	Negative -15.62	Negative 0.77	Negative <60	Negative
Ferret 1	December 2020	Wuhan variant (B.1 lineage)	Negative 5.74	Negative -28.45	Negative 1.59	Negative <60	Negative
Ferret 2	December 2020	Wuhan variant (B.1 lineage)	Negative -1.61	Negative -27.24	Negative 1.39	Negative <60	Negative
Ferret 3	July 2021	Delta (B.1.617.2)	Positive 89.50	Positive 94.69	Positive 702.91	Positive 1247	Negative
Ferret 4	July 2021	Delta (B.1.617.2)	Positive 74.38	Negative 18.374	Positive 1145.45	Positive 767	Negative
Goat 1	2dpi	experimental / Beta (B.1.351)	Negative 14.71	NA	Negative -0.26	Negative <60	Positive
Goat 2	2dpi	experimental / Beta (B.1.351)	Negative 24.15	NA	Negative 3.97	Negative <60	Positive
Goat 3	2dpi	experimental / Beta (B.1.351)	Negative 20.72	NA	Negative 1.25	Positive 92.73	Positive

Goat 4	18 dpi	experimental / Beta (B.1.351)	Positive 59.59	NA	Negative 1.41	Positive 228.12	Positive
Goat 5	18 dpi	experimental / Beta (B.1.351)	Positive 41.45	NA	Negative 5.28	Positive 525.52	Positive
Goat 6	18 dpi	experimental / Beta (B.1.351)	Positive 34.70	NA	Negative 15.48	Positive 99.78	Positive
Lion 1	02.12.2020	Natural infection B.1.177	Positive 89.00	Positive 99.13	Negative 2.09	Positive 8769.50	Positive 09.11.2020
Lion 2	18.12.2020	Natural infection B.1.177	Positive 90.00	Positive 96.78	Negative 18.36	Positive 5491.64	Positive 09.11.2020
Lion 3	02.12.2020	Natural infection B.1.177	Positive 86.00	Positive 79.51	Negative 15.27	Positive 1224.93	Positive 09.11.2020
Lion 4	19.11.2020	Natural infection B.1.177	Positive 89.00	Positive 94.49	Negative 25.36	Positive 6238.82	Positive 09.11.2020
Wild boar 1	April 2020	Wuhan variant (B.1 lineage)	Negative 5.30	Negative NA	Negative -0.10	Negative <60	NA
Wild boar 2	November 2020	Wuhan variant /Alpha (B.1.1.7)	Negative 18.63	NA	Negative 2.56	Negative <60	NA
Wild boar 3	November 2020	Wuhan variant /Alpha (B.1.1.7)	Negative -10.11	NA	Negative 1.62	Negative <60	NA
Wild boar 4	May 2021	Alpha (B.1.1.7)	Positive 34.87	Negative -25.76	Negative 19.40	Negative <60	NA
Wild boar 5	August 2021	Delta (B.1.617.2)	Positive 34.87	Negative -20.32	Negative -0.36	Negative <60	NA
Wild boar	December 2021	Delta (B.1.617.2)/	Negative	Negative	Negative	Negative	NA

6		Omicron (BA.1)	11.74	NA	-0.05	<60	
Wild boar 7	December 2021	Delta (B.1.617.2)/ Omicron (BA.1)	Negative 8.88	Negative NA	Negative 0.10	Negative <60	NA
Wild boar 8	December 2021	Delta (B.1.617.2)/ Omicron (BA.1)	Negative 3.48	NA	Negative 0.68	Negative <60	NA
Wild boar 9	February 2022	Omicron (BA.1)	Negative 10.03	NA	Negative 0.10	Negative <60	NA
Wild boar 10	February 2022	Omicron (BA.1)	Negative 7.34	NA	Negative 0.37	Negative <60	NA

CHAPTER 8. GENERAL DISCUSSION

The COVID-19 pandemic has evidenced once again the globalized nature of the world and the close interconnection between animals and humans [3]. Following the initial cases of SARS-CoV-2 infections in China in late 2019, the virus quickly spread worldwide, leading to an unprecedented number of infections and fatalities [342]. This scenario prompted the implementation of measures such as lockdowns, movement restrictions, and the urgent development of vaccines and treatments to combat the disease. Considering the potential wildlife origin of SARS-CoV-2 and the ability of previous CoVs to infect animals [7,342], identifying animal species that could impact the epidemiology and evolution of the virus became of particular interest. Within this framework, the present *PhD Thesis* was started in early 2020 to assess the role of different animal species, spanning from domestic to wildlife environments, in the context of the COVID-19 pandemic. This investigation aligns with the One Health approach, emphasizing the need for a robust multidisciplinary collaboration to effectively manage global health threats [2,343].

When the first SARS-CoV-2 infections in companion animals (mainly cats and dogs) were reported [122,210], our objective was to determine the prevalence and seroprevalence of SARS-CoV-2 in the most common pets (cats, dogs, and ferrets) in North-Eastern Spain (*Chapter 3*). The monitoring study on SARS-CoV-2 extended from April 2020 until early 2022, a period during which different SARS-CoV-2 VOCs (*i.e.,* Alpha [B.1.1.7], Beta [B.1.351], Gamma [P.1], Delta [B.1.617.2], and Omicron [BA.1]) led to distinct pandemic waves and impacts on the human population [30,342]. Our findings align with numerous studies conducted across different countries, demonstrating instances of acute infection and/or exposure to SARS-CoV-2 and its VOCs in companion animals. The risk of infection in these animals primarily stems from close contact with humans [124–128,138,141,226,228,344].

Notably, the actively SARS-CoV-2 infected animals identified in our study (one cat and two dogs, representing a 0.3% frequency of detection) were from households with infected owners. Pets that had contact with COVID-19-positive owners exhibited significantly higher seroprevalence rates (23.80% in cats, 8.99% in dogs, and 40% in ferrets) compared to those without documented contact (~0-2% in all species).

While initial studies suggested that dogs had a low susceptibility to SARS-CoV-2 infection in experimental settings [64,66], various investigations, including our own and those of other authors, have demonstrated that dogs can indeed become infected. In some rare cases, they have developed clinical manifestations such as digestive and respiratory signs [122,125,128,141,226,345,346]. This highlights that, despite the apparent low susceptibility of specific species, their frequent interaction with humans can still pose risks for animal infection and disease transmission. Contrarily to dogs, cats, ferrets, and hamsters can transmit the virus and pose a threat to other animals in their proximity, such as those living at the same household [65,77,218]. However, the risk for sustained virus circulation and viral evolution in such scenarios is considered minimal [68,120]. Similarly, the risk of humans being infected by their pets is also deemed to be low [120,158,235]. However, this risk may increase for certain human populations frequently exposed to companion animals, such as veterinarians or pet shop workers [145,146].

So far, the genetic information of the virus obtained from infected companion animals does not exhibit phylogenetic clustering patterns, but rather spread among human sequences [120]. This suggests that there is no significant viral evolution in these species, indicating minimal animal-to-animal transmission and viral adaptation. The risk of viral persistence would be heightened in situations where viral circulation is facilitated. For instance, cats residing in shelters or colonies of stray cats may be exposed to infected

pet cats with outdoor access or viral contaminated sources such as human-produced waste [143,144]. Our study and other investigations have confirmed the exposure and infection of these group of cats, although the percentage of infected animals is lower compared to those in domestic settings [143,144,347,348]. These results confirm once again that humans are the primary source of infection in animals.

During the COVID-19 pandemic, particular focus has been placed on American minks (*Neovison vison*) farmed for fur production [154,156,349]. In contrast, other livestock species such as poultry, pigs, cattle, sheep, and goats have not been implicated in the epidemiology of the pandemic and currently do not pose risks regarding SARS-CoV-2 transmission events [57]. This assertion is supported by various experimental studies [57,87–89], including the *in vivo* SARS-CoV-2 infection experiment with the Beta (B.1.351) variant conducted on goats as part of this *PhD Thesis (Chapter 6).* Goats, cattle, and sheep are susceptible to SARS-CoV-2 and its VOCs, but typically exhibit subclinical infections with low viral loads, emphasizing their low susceptibility. Contrarily, American minks are considered to be at high risk of infection due to their heightened susceptibility and potential for severe disease leading to fatal outcomes in some cases [84,85,154,155]. The rapid spread of infection among mink populations is primarily attributed to the RZ transmission in a highly susceptible species that is raised in high numbers in close quarters. This combination has led to multiple outbreaks in mink farms across various countries [148,151,156,349]. Compared to companion animals, minks serve as efficient hosts for the rapid adaptation and transmission of SARS-CoV-2 [148,349]. Once the virus enters mink farms, counteracting its spread among the animals becomes a daunting task. Importantly, the number of outbreaks in mink farms has decreased over time, with the peak of infections occurring between 2020 and 2021 (involving over 400 mink farms reporting

outbreaks) [120,349]. From then on, the implementation of biosecurity measures has played a pivotal role in this decline, encompassing measures such as banning mink farming, culling of animals, improving disinfection protocols, and conducting passive and active surveillance of SARS-CoV-2 in animals [349]. Based on genetic sequencing data, instances of mink-to-human transmission have been documented on at least 43 farms during the pandemic [349]. Nevertheless, the risk of transmission from mink to the general population is considered low, particularly in comparison to residents living in the neighborhoods of mink farms or individuals working on them [158,159]. The risk of transmission between these hosts and humans is influenced by various factors [120,349], including: i) the prevailing epidemiology of SARS-CoV-2 in the human population, ii) the circulating variants, iii) the immunity of farmers against the virus through vaccination or previous infections, iv) the number of minks and workers within a farm, and v) the establishment of biosecurity measures (discussed further at the end of this section).

The interactions between livestock and wildlife animals have increased as a consequence of human population growth and anthropogenic factors such as intensive farming practices and changes in land usage [350,351]. The interface between livestock and wildlife also enhances the risk of spillover events of specific pathogens between these two groups of animals and between them and humans [350,351]. This is exemplified by the cross-species transmission of significant diseases, such as animal tuberculosis shared by various livestock and wildlife or African swine fever affecting both domestic pigs and wild boar [350,351]. As of May 2024, there is an unprecedented spread of highly pathogenic avian influenza (AI) virus (H5N1), primarily affecting domestic and wild birds, with recent infections in dairy herds and a further transmission to a human patient in the United States [352]. In the context of SARS-CoV-2, there is a considerable risk of transmission from infected

farmed American minks or escaped minks to surrounding wildlife, such as feral minks, cats, or other susceptible species, as previously described [189,190,353,354]. It is worth noting that the establishment of an animal reservoir in wildlife would pose significant challenges for controlling the disease, its spread, genetic evolution, and further spillover events [251,252]. For this reason, the development of strategies to reduce potential contact between livestock and wildlife is crucial [351,355]. For instance, small-scale fencing in farms may prevent animal escapees and mitigate SARS-CoV-2 transmission from minks to susceptible wildlife species [351,355].

Accordingly, in the present *PhD Thesis*, we have also monitored wildlife species from different regions of Spain throughout the pandemic period (*Chapter 4*). Our results suggested that this group of animals has not played a significant role in the epidemiology of SARS-CoV-2 so far. Similarly, reported infections in wildlife species have been relatively low on a global scale [57,120,187,192,193]. Considering that the primary route of animal infections is likely through contact with humans or human-contaminated areas, it is reasonable to assume that the risk of infection in free-ranging wildlife animals may be lower compared to domestic animals. Most infections in wildlife species have been observed in captive animals, particularly large felids and NHP [356], and in free-ranging WTD in North America, an urban species in close contact with human populations [357].

The SARS-CoV-2 surveillance in wildlife species performed in this *PhD Thesis* (*Chapter 4*) encompassed both captive and free-range animal species, mainly carnivores such as felids, mustelids, and canids, as well as cetaceans. Detecting infections in free-ranging animals entails greater challenges in comparison to animals with well-known distribution and location [177,313]. Additionally, achieving a representative sampling of the entire population poses many challenges due to limitations in obtaining samples for

this group of animals [177,313]. Among free-ranging animals, our primary focus was to test American minks and other mustelids for current acute SARS-CoV-2 infection. However, the lack of serum samples from these species prevented us from testing for past viral infections, which would have been of great interest. Conversely, we were able to obtain serum samples from captive animals, and only one bottlenose dolphin showed low titers of nAbs against SARS-CoV-2, suggesting potential exposure. Given that cetaceans were previously identified as susceptible to SARS-CoV-2 based on *in silico* and *in vitro* studies [37,249,259,260], the risk of infection cannot be ruled out, especially in captive settings with regular human contact.

In another study published by our research group in 2021, we detected acute SARS-CoV-2 infection (Pango lineage B.1.1777) in four captive lions at the Barcelona zoo, which had contracted the virus from zookeepers [165]. Large felids and NHP often develop mild to moderate severe disease, including interstitial pneumonia, similar to that seen in COVID-19 patients [131,356]. This has led to the consideration of vaccinating these animals against COVID-19 [358]. During the pandemic, an experimental vaccine originally designed for companion animals was administered to 260 animals from over 100 different species, primarily NHP and large felids, in various zoological parks and sanctuaries across North America [359,360]. The vaccine demonstrated its efficacy in eliciting significant immune responses in the animals against the virus [85,361,362]. Additional animal COVID-19 vaccines have also been designed [363]; nonetheless, the need for vaccinating animals still requires further evaluations. Considering the costs and challenges associated with animal vaccination, preventing animal exposure to SARS-CoV-2 through alternative preventive measures may be sufficient [120]. In zoological parks, as in intensively reared farms, animals are in close proximity to each other and caretakers, facilitating disease transmission,

especially among animals of the same species housed together [267,356]. However, the number of animals of the same species is not as high as on farms, which might suggest a lower likelihood of viral maintenance, adaptation, and evolution in zoo conditions [356]. Accordingly, while SARS-CoV-2 genetic sequences from wild felines show more clustering than those from companion animals, there appears to be no specific viral evolution [120,165,267]. Additionally, there is no significant evidence of animal-to-human transmission, thereby, it is not currently a major concern [356].

Assessing SARS-CoV-2 infection in animals is challenging due to the lack of previously validated assays for animal species, particularly wildlife [177,313]. In order to assess the diagnostic performance of the various ELISA kits used in this *PhD Thesis* for animal species, we conducted a comparative study (*Chapter 7*). Notably, the ELISAs used in the wildlife study (*Chapter 4*) demonstrated very low sensitivity. These ELISAs detect nAbs against the RBD (ELISA-2) and N protein (ELISA-3) of SARS-CoV-2, with overall sensitivities of 51.72% and 38.70%, respectively. It remains unclear whether using an alternative ELISA with higher sensitivity, such as ELISA-1 (96.60%), as employed in the other studies of this *PhD Thesis*, would have identified additional seropositive animals. These results highlighted the need to validate surveillance techniques for animal studies, especially in wildlife. However, from a broader perspective, SARS-CoV-2 infections in wild animal populations, though limited, appear to have minimal impact on the overall epidemiology of the COVID-19 pandemic.

In North America, the WTD (*Odocoileus virginianus*) is a species of special concern due to its high prevalence of SARS-CoV-2 infection [183,357,364]. In this region, WTD are significantly abundant in urban, suburban, and rural environments, where they frequently interact with humans, other wildlife species, domestic animals, and livestock species [183,357,364]. In fact, WTD are

already under scrutiny for various infectious diseases they share with livestock, including tuberculosis, epizootic hemorrhagic disease, and their role in the persistence of antibiotic-resistant pathogens [351]. Similar to mink, WTD have demonstrated a high susceptibility to SARS-CoV-2 and the ability to effectively transmit the virus, albeit with subclinical infections [96,357,364,365]. This fact allows the virus persist within WTD populations, making them a suitable reservoir host for SARS-CoV-2 [357]. Indeed, various VOCs that have already disappeared from the human population are still circulating in WTD in the United States [357].

In addition, SARS-CoV-2 variants adapted to WTD are circulating among their population, with proven transmission from these animals to humans [183]. Understanding the transmission route between humans and WTD is complex and may involve indirect pathways such as human-generated waste, contaminated fomites, food or water sources, hunting activities, and close contact feeding practices [183,357,364]. Our study (*Chapter 4*) did not include this ungulate species, as the population of WTD is scarce in Europe, with only a few individuals found in Czechia and Finland [120]. Contrarily, Europe is home to various deer species, including roe deer, fallow deer, and red deer [366,367]. These species usually inhabit wild environments rather than urban or peri-urban areas, except during the mating season when they may become more common in such environments. The risk of virus exposure in these European deer species may not be as concerning as for WTD in North America, but including them in surveillance studies across Europe, including Spain, is advisable [186]. Remarkably, the SARS-CoV-2 variants from mink and WTD, seem to have reduced the efficiency of viral transmission among humans [159]. Therefore, this has reduced the probability of these variants causing a significant impact on the human population worldwide [159].

However, it is unclear if novel variants emerging from these animal species will present greater concerns in the near future.

In this *PhD Thesis,* we included rodents and wild boars as part of our monitoring SARS-CoV-2 studies (*Chapter 5*). These species are among the most abundant urban and peri-urban species in Spain, including Catalonia, and are considered pests, making them targets for population control programs. Despite our efforts, we did not detect SARS-CoV-2 in these populations. Our research on wild boar, as well as prior *in vivo* studies [66,91,92], supports a low probability of SARS-CoV-2 infection in *Suidae* species, making them less relevant for further surveillance efforts. Experimental studies has previously demonstrated that rodent species are susceptible to certain SARS-CoV-2 variants, including the Omicron variants that are currently in circulation [106,107,280]. Additionally, few reports suggested potential SARS-CoV-2 natural exposure and/or infection in rodents[255,285]. Accurately determining the abundance, density, and distribution of rodent species, as well as wild boar, remains challenging and is still a limitation for surveillance studies [313]. The low volume of serum samples obtained from rodent species prevented us from drawing firm conclusions about whether some species have been exposed to SARS-CoV-2 throughout the pandemic. Therefore, continuous monitoring of SARS-CoV-2 in these species may be necessary. Rodents are already important animal reservoirs for zoonotic diseases, including CoVs [51,276,286,368]. This raises concerns about potential recombinant events between these CoVs and SARS-CoV-2, which could have significant threats to humans [51,277,279,369]. Indeed, after an initial RZ transmission event of a pre-omicron variant, there was speculation that the Omicron variant may have originated from rodents [281]. Notably, rats are pose important public health risks in the United States due to the widespread infestations in cities[370]. This is a direct consequence of urban expansion and the easy accessibility of

resources including food, water, and shelter. Considering global population growth and rising temperatures due to climate change, the proximity between rodents and humans is expected to increase [196,197]. Therefore, it is urgent to develop effective rodent management strategies and preventive measures in accordance with the principles of One Health.

From a general perspective and based on current knowledge, animals did not play a crucial role in the epidemiology of the COVID-19 pandemic. While there have been cases of transmission between humans and animals, these occurrences have not significantly altered the course of the pandemic[120]. However, the promiscuity of the virus underscores the need to remain alert for future global health threats [120,343]. Currently, the main mode of transmission and viral spread remains human-to-human. SARS-CoV-2 still continues to circulate and causes infections and fatalities worldwide [14]. The virus continues having opportunities to genetically evolve, giving rise to novel variants. This also implies the potential for the virus to evolve in animal species, principally American minks and WTD [349,357] or, eventually, in other unknown species so far. Importantly, these variants can progressively gain genetic distance from the ancestral variant, potentially effecting the effectiveness of existing COVID-19 vaccines and/or diminishing the protection offered by prior SARS-CoV-2 infections [371,372]. So far, herd immunity has been crucial for reducing the risk of severe disease in humans and, indirectly, in minimizing transmission and the risk of infection [16]. Not only is vaccination crucial, but also additional strategies are pivotal for controlling the spread and re-emergence of SARS-CoV-2. These strategies may be implemented via the One Health approach, which combines the expertise of public health professionals, medical professionals, veterinarians, virologists, ecologists, and experts from various fields.

Therefore, considering the current epidemiological situation, *what should we do from now on*? *In the context of the animal-human interface, should we consider specific preventive measures for the different species?*

For companion and captive animals, the primary preventive measure to take is to avoid close contact with them if we exhibit SARS-CoV-2-related symptoms or test positive for COVID-19. This may contribute prevent RZ transmission events. Instead of conducting regular SARS-CoV-2 monitoring studies in these animals, it may be sufficient to isolate and test animals showing clinical signs, especially if they have been in contact with SARS-CoV-2 infected humans [120,373].

Regular surveillance studies of SARS-CoV-2 and SARS-like CoVs may be more useful in populations considered to be reservoirs of these viruses and in synanthropic species, including rodents and bats [120]. In wildlife environments, it would be appropriate to establish surveillance programs to test SARS-CoV-2 infection in animals found dead or displaying specific clinical signs, especially stray cats, mustelids, felids, deer, or other potentially susceptible species. Using personal protective equipment (PPE) when handling animals during population control programs or when caring for sick and injured animals in wildlife conservation centers can help prevent cross-species transmission of SARS-CoV-2 [120,373]. In parallel, educating residents about the risks of feeding or interacting with wildlife can help prevent the congregation of animals such as WTD in United States parks, thereby limiting the spread of SARS-CoV-2. Focusing on urban and peri-urban species, proper waste management to reduce food sources for these animals would minimize disease transmission events and control the abundance of their populations in urban scenarios.

Although WTD and farmed minks are not included in monitoring studies of this *PhD Thesis*, it is important to highlight that harmful efforts may be addressed to control the SARS-CoV-2 spread within their populations. On mink farms, the principal aim may be the early detection of infections in animals, which may be achieved through regular SARS-CoV-2 testing of personnel, consistent use of face masks, and adherence to regular disinfection protocols, among other strategies [120,374]. Vaccination against COVID-19 in farmed minks may also be an effective approach to reduce both the risk of severe disease and disease transmission in these animals. Indeed, the above mentioned vaccine developed for pets was conditionally approved in 2021 for use in mink farms in North America [85]. This vaccine demonstrated a reduction in viral replication in the respiratory tract and prevent lung damage in minks [85]. Another alternative animal COVID-19 vaccine was administered to minks in mink farms in Finland after the Ministry of Agriculture and Forestry of Finland granted a conditional usage license [375]. Besides, for WTD, regular monitoring programs for SARS-CoV-2 should be established, especially in North America, to control the spread and evolution of the virus [120].

Finally, to universally implement genomic surveillance studies of all SARS-CoV-2 infected animals, as well as COVID-19 patients, could also contribute to controlling the epidemiology of the virus and the emergence of new variants [120]. Importantly, genome sequences should be deposited in public databases [120,373]. These efforts may be complemented by genomic surveillance studies of SARS-CoV-2 in wastewaters, which have already demonstrated their effectiveness in detecting new SARS-CoV-2 outbreaks in local populations and the early detection of novel variants [2,376].

What should we expect for the near future? Is it possible a new pandemic caused by new or old pathogens originated in animals? How can we be prepared to handle coming outbreaks of infectious diseases?

The advent of SARS-CoV-2 has reinforced what zoonotic viruses have previously demonstrated: EZDs can pose devastating repercussions across the world, profoundly impacting global health and economies, among other critical factors. The H1N1 influenza A virus (1918) and the HIV (identified in 1981 and currently ongoing) are other examples of originally zoonotic viruses that later adapted to humans, causing significant pandemics. In contrast, some pathogens of animal origin do not persist in humans, though they can still cause significant epidemics or health consequences (*e.g.,* Nipah virus, West Nile virus, Chikungunya, Ebola virus, Mpox, and hCoVs). The number of diseases shared by animals and humans has been rising in the recent times, currently reaching over 200 [377]. Approximately every decade, CoVs jump from animals to humans, and nothing leads us to believe this will not happen in the near future. Indeed, quite the opposite [3,377]. The disease transmission between species is supported by the human demographic growth, increased globalization and urbanization, destruction and alterations of ecosystems, intensive livestock farming and agricultural practices [2,3,343]. Altogether contributes to an increased interconnectivity between humans and animals, thereby facilitating zoonotic spillover events. Anthropogenic factors also contribute to climate change, which, in turn, further increases the risk of EZDs [196,197]. Global warming favors extreme precipitation events, droughts, fluctuations in temperature and humidity, and significant air pollution. This scenario brings pathogens closer to humans, making them more vulnerable. For instance, the distribution of vector-borne diseases (*e.g.,* Lyme disease, West Nile virus, and malaria), is associated with shifts in climate conditions due to the expansion of mosquitoes [196,197]. Also, natural disasters can increase

the prevalence of waterborne diseases (*e.g.,* cholera), which primarily affect low-income and mid-income countries [196,197]. Therefore, the intrinsic link between human, animal, and environmental health is undeniable [2,378].

The WHO has established the term "Disease X" to refer to the disease causing the next pandemic, which will be triggered by a pathogen that has not yet been characterized, known as "*Pathogen X" *[379,380]. This concept was introduced to enhance the preparedness for the new coming outbreaks caused by EZDs. Public health care systems, scientists, medical professionals, and economic entities must be involved in preparedness efforts [379,380]. *Pathogen X* may be an unknown pathogen or a previously identified pathogen that can end up causing catastrophic effects on the human population. An effective response against *Disease X* may be achieved by addressing several fundamental aspects [378–380]: i) major investment in scientific research and One Health activities, as well as the improvement of primary healthcare systems and infrastructure, including hospitals and laboratories, ii) establishing international guidelines to reduce the risk of spillover events of zoonotic pathogens, iii) developing effective surveillance and monitoring studies of circulating pathogens in humans, animals, and the environment, iv) sharing and managing data appropriately, v) gaining knowledge to characterize pathogens, which will consequently contribute to vi) the early development and production of vaccine and treatments. Furthermore, ensuring the equitable distribution of vaccines and therapeutics across low-, middle- and high-income countries is crucial, as local outcomes have global repercussions [381].

CHAPTER 9.
CONCLUSIONS

1) During the COVID-19 pandemic in North-Eastern Spain, companion animals -cats, dogs, and ferrets- were found to be exposed to and infected by SARS-CoV-2 and its variants. Pets residing in households with COVID-19-positive owners had a higher risk of infection. While the overall active infection rate was low (0.3%), pets in households with COVID-19 cases showed significantly higher antibody detection rates, with approximately 25% in cats, 10% in dog, and 40% in ferrets.

2) Stray cats in North-Eastern Spain were found to have antibodies to SARS-CoV-2, although their likelihood of exposure (2.34%) was lower than that of the overall domestic cat population (7.65%).

3) Captive and free-range wildlife animals from Spain appeared not to play a role in the epidemiology of the COVID-19 pandemic. However, close contact between humans and wildlife, including cetaceans, may pose risks for SARS-CoV-2 exposure in animals.

4) Urban and peri-urban species in Catalonia, including rodents and wild boar, exhibited no evidence of exposure to or infection by SARS-CoV-2 during the COVID-19 pandemic, suggesting a limited involvement in the spread of the virus.

5) Domestic goats, a common livestock species, showed low susceptibility to infection by the SARS-CoV-2 Beta (B.1.351) variant. They displayed low levels of viral genome and antigen in tissues, with evidence of seroconversion observed as early as 7 days post-infection. Goats are likely to be inefficient hosts for SARS-CoV-2.

6) The ELISA-1 kit, targeting RBD nAbs antibodies, proved to be a reliable tool for the initial screening of SARS-CoV-2 exposure in animal species and can serve as a confirmatory technique in the absence of gold-standard techniques. In contrast, ELISA-2 and ELISA-3, targeting RBD nAbs and N protein antibodies, respectively, exhibited poor diagnostic performance for detecting seropositive animals against SARS-CoV-2, particularly in samples from cats and dogs.

7) ELISAs that use the RBD protein offered higher accuracy in detecting SARS-CoV-2 exposure in animal species compared to the N-protein-based test. N-based ELISAs can be used as complementary tests

References

1. Jones, K. E. *et al.* Global trends in emerging infectious diseases. *Nature* **451**, 990–993 (2008).

2. Leifels, M. *et al.* The one health perspective to improve environmental surveillance of zoonotic viruses: lessons from COVID-19 and outlook beyond. *ISME COMMUN.* **2**, 107 (2022).

3. Baker, R. E. *et al.* Infectious disease in an era of global change. *Nat Rev Microbiol* **20**, 193–205 (2022).

4. Salata, C., Calistri, A., Parolin, C. & Palù, G. Coronaviruses: a paradigm of new emerging zoonotic diseases. *Pathog Dis* **77**, 9 (2019).

5. Alsafi, R. T. Lessons from SARS-CoV, MERS-CoV, and SARS-CoV-2 Infections: What We Know So Far. *Can J Infect Dis Med Microbiol* **2022**, 1156273 (2022).

6. Hu, B., Ge, X., Wang, L.-F. & Shi, Z. Bat origin of human coronaviruses. *Virol J* **12**, 221 (2015).

7. Ye, Z.-W. *et al.* Zoonotic origins of human coronaviruses. *Int J Biol Sci* **16**, 1686–1697 (2020).

8. Cui, J., Li, F. & Shi, Z.-L. Origin and evolution of pathogenic coronaviruses. *Nat Rev Microbiol* **17**, 181–192 (2019).

9. Zaki, A. M., van Boheemen, S., Bestebroer, T. M., Osterhaus, A. D. M. E. & Fouchier, R. A. M. Isolation of a novel coronavirus from a man with pneumonia in Saudi Arabia. *N Engl J Med* **367**, 1814–1820 (2012).

10. World Health Organization. *MERS Situation Update - January 2024*. https://applications.emro.who.int/docs/WHOEMCSR716E-eng.pdf?ua=1 (2024).

11. Zhou, P. *et al.* A pneumonia outbreak associated with a new coronavirus of probable bat origin. *Nature* **579**, 270–273 (2020).

12. Sohrabi, C. *et al.* World Health Organization declares global emergency: A review of the 2019 novel coronavirus (COVID-19). *Int J Surg* **76**, 71–76 (2020).

13. World Health Organization. Coronavirus disease (COVID-19) pandemic. *WHO* https://www.who.int/europe/emergencies/situations/covid-19 (2024).

14. WHO. WHO COVID-19 dashboard. *World Health Organization* https://data.who.int/dashboards/covid19/cases (2024).

15. Mathieu, E. *et al.* COVID-19 vaccinations. *Our World in Data* https://ourworldindata.org/covid-vaccinations (2024).

16. Mohammed, I. *et al.* The efficacy and effectiveness of the COVID-19 vaccines in reducing infection, severity, hospitalization, and mortality: a systematic review. *Hum Vaccin Immunother* **18**, e2027160 (2022).

17. Gorbalenya, A. E. *et al.* The species Severe acute respiratory syndrome-related coronavirus: classifying 2019-nCoV and naming it SARS-CoV-2. *Nat Microbiol* **5**, 536–544 (2020).

18. Zhou, Z., Qiu, Y. & Ge, X. The taxonomy, host range and pathogenicity of coronaviruses and other viruses in the Nidovirales order. *Animal Diseases* **1**, 5 (2021).

19. Brant, A. C., Tian, W., Majerciak, V., Yang, W. & Zheng, Z.-M. SARS-CoV-2: from its discovery to genome structure, transcription, and replication. *Cell Biosci* **11**, 136 (2021).

20. Gao, Y. *et al.* Structure of the RNA-dependent RNA polymerase from COVID-19 virus. *Science* **368**, 779–782 (2020).

21. Bai, C., Zhong, Q. & Gao, G. F. Overview of SARS-CoV-2 genome-encoded proteins. *Sci China Life Sci* **65**, 280–294 (2022).

22. Mingaleeva, R. N. *et al.* Biology of the SARS-CoV-2 Coronavirus. *Biochemistry Moscow* **87**, 1662–1678 (2022).

23. Cao, C. *et al.* The architecture of the SARS-CoV-2 RNA genome inside virion. *Nat Commun* **12**, 3917 (2021).

24. Kakavandi, S. *et al.* Structural and non-structural proteins in SARS-CoV-2: potential aspects to COVID-19 treatment or prevention of progression of related diseases. *Cell Commun Signal* **21**, 110 (2023).

25. Al-Qaaneh, A. M. *et al.* Genome composition and genetic characterization of SARS-CoV-2. *Saudi J Biol Sci* **28**, 1978–1989 (2021).

26. Jackson, C. B., Farzan, M., Chen, B. & Choe, H. Mechanisms of SARS-CoV-2 entry into cells. *Nat Rev Mol Cell Biol* **23**, 3–20 (2022).

27. Hoffmann, M. *et al.* SARS-CoV-2 Cell Entry Depends on ACE2 and TMPRSS2 and Is Blocked by a Clinically Proven Protease Inhibitor. *Cell* **181**, 271–280 (2020).

28. Yang, H. & Rao, Z. Structural biology of SARS-CoV-2 and implications for therapeutic development. *Nat Rev Microbiol* **19**, 685–700 (2021).

29. Holmes, E. C. *et al.* The origins of SARS-CoV-2: A critical review. *Cell* **184**, 4848–4856 (2021).

30. Lauring, A. S. & Hodcroft, E. B. Genetic Variants of SARS-CoV-2—What Do They Mean? *JAMA* **325**, 529–531 (2021).

31. Korber, B. *et al.* Tracking Changes in SARS-CoV-2 Spike: Evidence that D614G Increases Infectivity of the COVID-19 Virus. *Cell* **182**, 812–827 (2020).

32. Liu, H., Wei, P., Kappler, J. W., Marrack, P. & Zhang, G. SARS-CoV-2 Variants of Concern and Variants of Interest Receptor Binding Domain Mutations and Virus Infectivity. *Front Immunol* **13**, 825256 (2022).

33. ECDC. SARS-CoV-2 variants of concern. https://www.ecdc.europa.eu/en/covid-19/variants-concern (2024).

34. Men, K. *et al.* Estimate the incubation period of coronavirus 2019 (COVID-19). *Comput Biol Med* **158**, 106794 (2023).

35. Singh, D. D., Han, I., Choi, E.-H. & Yadav, D. K. A Clinical Update on SARS-CoV-2: Pathology and Development of Potential Inhibitors. *Curr Issues Mol Biol* **45**, 400–433 (2023).

36. Metzdorf, K. *et al.* TMPRSS2 Is Essential for SARS-CoV-2 Beta and Omicron Infection. *Viruses* **15**, 271 (2023).

37. Damas, J. *et al.* Broad host range of SARS-CoV-2 predicted by comparative and structural analysis of ACE2 in vertebrates. *Proc Natl Acad Sci U S A* **117**, 22311–22322 (2020).

38. Hikmet, F. *et al.* The protein expression profile of ACE2 in human tissues. *Mol Syst Biol* **16**, e9610 (2020).

39. Li, M.-Y., Li, L., Zhang, Y. & Wang, X.-S. Expression of the SARS-CoV-2 cell receptor gene ACE2 in a wide variety of human tissues. *Infect Dis Poverty* **9**, 45 (2020).

40. Deshmukh, V., Motwani, R., Kumar, A., Kumari, C. & Raza, K. Histopathological observations in COVID-19: a systematic review. *J Clin Pathol* **74**, 76–83 (2021).

41. Emrani, J. *et al.* SARS-COV-2, infection, transmission, transcription, translation, proteins, and treatment: A review. *Int J Biol Macromol* **193**, 1249–1273 (2021).

42. Pathangey, G., Fadadu, P. P., Hospodar, A. R. & Abbas, A. E. Angiotensin-converting enzyme 2 and COVID-19: patients, comorbidities, and therapies. *American Journal of Physiology-Lung Cellular and Molecular Physiology* **320**, L301–L330 (2021).

43. Zhang, J., Dong, X., Liu, G. & Gao, Y. Risk and Protective Factors for COVID-19 Morbidity, Severity, and Mortality. *Clinic Rev Allerg Immunol* **64**, 90–107 (2022).

44. Mlcochova, P. *et al.* SARS-CoV-2 B.1.617.2 Delta variant replication and immune evasion. *Nature* **599**, 114–119 (2021).

45. Twohig, K. A. *et al.* Hospital admission and emergency care attendance risk for SARS-CoV-2 delta (B.1.617.2) compared with alpha (B.1.1.7) variants of concern: a cohort study. *The Lancet Infectious Diseases* **22**, 35–42 (2022).

46. Suzuki, R. *et al.* Attenuated fusogenicity and pathogenicity of SARS-CoV-2 Omicron variant. *Nature* **603**, 700–705 (2022).

47. Paul, P. *et al.* Effectiveness of the pre-Omicron COVID-19 vaccines against Omicron in reducing infection, hospitalization, severity, and mortality compared to Delta and other variants: A systematic review. *Hum Vaccin Immunother* **19**, 2167410 (2023).

48. Alwine, J. C., Casadevall, A., Enquist, L. W., Goodrum, F. D. & Imperiale, M. J. A Critical Analysis of the Evidence for the SARS-CoV-2 Origin Hypotheses. *mBio* **14**, 2 (2023).

49. Worobey, M. *et al.* The Huanan Seafood Wholesale Market in Wuhan was the early epicenter of the COVID-19 pandemic. *Science* **377**, 951–959 (2022).

50. Temmam, S. *et al.* Bat coronaviruses related to SARS-CoV-2 and infectious for human cells. *Nature* **604**, 330–336 (2022).

51. Ruiz-Aravena, M. *et al.* Ecology, evolution and spillover of coronaviruses from bats. *Nat Rev Microbiol* **20**, 299–314 (2022).

52. Voskarides, K. SARS-CoV-2: tracing the origin, tracking the evolution. *BMC Med Genomics* **15**, 62 (2022).

53. Zhao, J., Cui, W. & Tian, B. The Potential Intermediate Hosts for SARS-CoV-2. *Frontiers in Microbiology* **11**, 580137 (2020).

54. Huang, X.-Y. *et al.* A pangolin-origin SARS-CoV-2-related coronavirus: infectivity, pathogenicity, and cross-protection by preexisting immunity. *Cell Discov* **9**, 1–13 (2023).

55. Wacharapluesadee, S. *et al.* Evidence for SARS-CoV-2 related coronaviruses circulating in bats and pangolins in Southeast Asia. *Nat Commun* **12**, 972 (2021).

56. Sharun, K. *et al.* SARS-CoV-2 in animals: potential for unknown reservoir hosts and public health implications. *Veterinary Quarterly* **41**, 181–201 (2021).

57. Meekins, D. A., Gaudreault, N. N. & Richt, J. A. Natural and Experimental SARS-CoV-2 Infection in Domestic and Wild Animals. *Viruses* **13**, 1993 (2021).

58. Carossino, M. *et al.* ACE2 and TMPRSS2 distribution in the respiratory tract of different animal species and its correlation with SARS-CoV-2 tissue tropism. *Microbiol Spectr* **12**, 2 (2024).

59. Conceicao, C. *et al.* The SARS-CoV-2 Spike protein has a broad tropism for mammalian ACE2 proteins. *PLoS Biol* **18**, e3001016 (2020).

60. Lean, F. Z. X. *et al.* Differential susceptibility of SARS-CoV-2 in animals: Evidence of ACE2 host receptor distribution in companion animals, livestock and wildlife by immunohistochemical characterisation. *Transboundary and Emerging Diseases* **69**, 2275–2286 (2022).

61. Huang, C., Jiang, Y. & Yan, J. Comparative analyses of ACE2 and TMPRSS2 gene: Implications for the risk to which vertebrate animals are susceptible to SARS-CoV-2. *Journal of Medical Virology* **93**, 5487–5504 (2021).

62. Bestion, E., Halfon, P., Mezouar, S. & Mège, J.-L. Cell and Animal Models for SARS-CoV-2 Research. *Viruses* **14**, 1507 (2022).

63. Doliff, R. & Martens, P. Cats and SARS-CoV-2: A Scoping Review. *Animals* **12**, 1413 (2022).

64. Bosco-Lauth, A. M. *et al.* Experimental infection of domestic dogs and cats with SARS-CoV-2: Pathogenesis, transmission, and response to reexposure in cats. *Proc. Natl. Acad. Sci. U.S.A.* **117**, 26382–26388 (2020).

65. Gaudreault, N. N. *et al.* SARS-CoV-2 infection, disease and transmission in domestic cats. *Emerging Microbes & Infections* **9**, 2322–2332 (2020).

66. Shi, J. *et al.* Susceptibility of ferrets, cats, dogs, and other domesticated animals to SARS–coronavirus 2. *Science* **368**, 1016–1020 (2020).

67. Halfmann, P. J. *et al.* Transmission of SARS-CoV-2 in Domestic Cats. *N Engl J Med* **383**, 592–594 (2020).

68. Bao, L. *et al.* Susceptibility and Attenuated Transmissibility of SARS-CoV-2 in Domestic Cats. *The Journal of Infectious Diseases* **223**, 1313–1321 (2021).

69. Chiba, S. *et al.* Protective Immunity and Persistent Lung Sequelae in Domestic Cats after SARS-CoV-2 Infection. *Emerging Infectious Diseases* **27**, 660–663 (2021).

70. Rudd, J. M. *et al.* Clinical and Histopathologic Features of a Feline SARS-CoV-2 Infection Model Are Analogous to Acute COVID-19 in Humans. *Viruses* **13**, 1550 (2021).

71. Tamil Selvan, M. *et al.* SARS CoV-2 (Delta Variant) Infection Kinetics and Immunopathogenesis in Domestic Cats. *Viruses* **14**, 1207 (2022).

72. Martins, M. *et al.* The Omicron Variant BA.1.1 Presents a Lower Pathogenicity than B.1 D614G and Delta Variants in a Feline Model of SARS-CoV-2 Infection. *J Virol* **96**, 17 (2022).

73. Kim, Y.-I. *et al.* Infection and Rapid Transmission of SARS-CoV-2 in Ferrets. *Cell Host Microbe* **27**, 704–709 (2020).

74. Richard, M. *et al.* SARS-CoV-2 is transmitted via contact and via the air between ferrets. *Nat Commun* **11**, 3496 (2020).

75. Barut, G. T. *et al.* The spike gene is a major determinant for the SARS-CoV-2 Omicron-BA.1 phenotype. *Nat Commun* **13**, (2022).

76. Ulrich, L. *et al.* Enhanced fitness of SARS-CoV-2 variant of concern Alpha but not Beta. *Nature* **602**, 307–313 (2022).

77. Pulit-Penaloza, J. A. *et al.* Comparative Assessment of Severe Acute Respiratory Syndrome Coronavirus 2 Variants in the Ferret Model. *mBio* **13**, (2022).

78. Brustolin, M. *et al.* Protection against reinfection with D614- or G614-SARS-CoV-2 isolates in golden Syrian hamster. *Emerging Microbes & Infections* **10**, 797–809 (2021).

79. Chan, J. F.-W. *et al.* Simulation of the Clinical and Pathological Manifestations of Coronavirus Disease 2019 (COVID-19) in a Golden Syrian Hamster Model: Implications for Disease Pathogenesis and Transmissibility. *Clinical Infectious Diseases* **71**, 2428–46 (2020).

80. Zhang, Y.-N. *et al.* Different pathogenesis of SARS-CoV-2 Omicron variant in wild-type laboratory mice and hamsters. *Sig Transduct Target Ther* **7**, 1–3 (2022).

81. Port, J. R. *et al.* Host and viral determinants of airborne transmission of SARS-CoV-2 in the Syrian hamster. *Elife* **12**, RP87094 (2024).

82. Plunkard, J. *et al.* SARS-CoV-2 Variant Pathogenesis Following Primary Infection and Reinfection in Syrian Hamsters. *mBio* **14**, 2 (2023).

83. Lyoo, K.-S. *et al.* Experimental Infection and Transmission of SARS-CoV-2 Delta and Omicron Variants among Beagle Dogs. *Emerg. Infect. Dis.* **29**, 782–785 (2023).

84. Adney, D. R. *et al.* Severe acute respiratory disease in American mink experimentally infected with SARS-CoV-2. *JCI Insight* **7**, e159573 (2022).

85. Shuai, L. *et al.* Replication, pathogenicity, and transmission of SARS-CoV-2 in minks. *National Science Review* **8**, nwaa291 (2021).

86. Virtanen, J. *et al.* Experimental Infection of Mink with SARS-COV-2 Omicron Variant and Subsequent Clinical Disease. *Emerg Infect Dis* **28**, 1286–1288 (2022).

87. Bosco-Lauth, A. M. *et al.* Susceptibility of livestock to SARS-CoV-2 infection. *Emerging Microbes & Infections* **10**, 2199–2201 (2021).

88. Gaudreault, N. N. *et al.* Susceptibility of sheep to experimental co-infection with the ancestral lineage of SARS-CoV-2 and its alpha variant. *Emerging Microbes & Infections* **11**, 662–675 (2022).

89. Ulrich, L., Wernike, K., Hoffmann, D., Mettenleiter, T. C. & Beer, M. Experimental Infection of Cattle with SARS-CoV-2. *Emerg. Infect. Dis.* **26**, 2979–2981 (2020).

90. Mykytyn, A. Z. *et al.* Susceptibility of rabbits to SARS-CoV-2. *Emerging Microbes & Infections* **10**, 1–7 (2021).

91. Schlottau, K. *et al.* SARS-CoV-2 in fruit bats, ferrets, pigs, and chickens: an experimental transmission study. *The Lancet Microbe* **1**, e218–e225 (2020).

92. Vergara-Alert, J. *et al.* Pigs are not susceptible to SARS-CoV-2 infection but are a model for viral immunogenicity studies. *Transboundary and Emerging Diseases* **68**, 1721–1725 (2021).

93. Pickering, B. S. *et al.* Susceptibility of Domestic Swine to Experimental Infection with Severe Acute Respiratory Syndrome Coronavirus 2. *Emerg. Infect. Dis.* **27**, 104–112 (2021).

94. Suarez, D. L. *et al.* Lack of Susceptibility to SARS-CoV-2 and MERS-CoV in Poultry. *Emerg Infect Dis* **26**, 3074–3076 (2020).

95. Cool, K. *et al.* Infection and transmission of ancestral SARS-CoV-2 and its alpha variant in pregnant white-tailed deer. *Emerging Microbes & Infections* **11**, 95–112 (2022).

96. Martins, M. *et al.* From Deer-to-Deer: SARS-CoV-2 is efficiently transmitted and presents broad tissue tropism and replication sites in white-tailed deer. *PLoS Pathog* **18**, e1010197 (2022).

97. Palmer, M. V. *et al.* Susceptibility of White-Tailed Deer (Odocoileus virginianus) to SARS-CoV-2. *Journal of Virology* **95**, e00083-21 (2021).

98. Chandrashekar, A. *et al.* SARS-CoV-2 infection protects against rechallenge in rhesus macaques. *Science* **369**, 812–817 (2020).

99. Deng, W. *et al.* Primary exposure to SARS-CoV-2 protects against reinfection in rhesus macaques. *Science* **369**, 818–823 (2020).

100. Munster, V. J. *et al.* Respiratory disease in rhesus macaques inoculated with SARS-CoV-2. *Nature* **585**, 268–272 (2020).

101. Rockx, B. *et al.* Comparative pathogenesis of COVID-19, MERS, and SARS in a nonhuman primate model. *Science* **368**, 1012–1015 (2020).

102. Francisco, R. *et al.* Experimental Susceptibility of North American Raccoons (Procyon lotor) and Striped Skunks (Mephitis mephitis) to SARS-CoV-2. *Front Vet Sci* **8**, 715307 (2022).

103. Freuling, C. M. *et al.* Susceptibility of Raccoon Dogs for Experimental SARS-CoV-2 Infection - Volume 26, Number 12—December 2020 - Emerging Infectious Diseases journal - CDC. *Emerging Infectious Diseases* **26**, 12 (2020).

104. Griffin, B. D. *et al.* SARS-CoV-2 infection and transmission in the North American deer mouse. *Nat Commun* **12**, 3612 (2021).

105. Zhao, Y. *et al.* Susceptibility of tree shrew to SARS-CoV-2 infection. *Sci Rep* **10**, 16007 (2020).

106. Tarrés-Freixas, F. *et al.* Heterogeneous Infectivity and Pathogenesis of SARS-CoV-2 Variants Beta, Delta and Omicron in Transgenic K18-hACE2 and Wildtype Mice. *Frontiers in Microbiology* **13**, 840757 (2022).

107. Shuai, H. *et al.* Emerging SARS-CoV-2 variants expand species tropism to murines. *eBioMedicine* **73**, 103643 (2021).

108. Chu, H., Chan, J. F.-W. & Yuen, K.-Y. Animal models in SARS-CoV-2 research. *Nat Methods* **19**, 392–394 (2022).

109. Fan, C. *et al.* Animal models for COVID-19: advances, gaps and perspectives. *Signal Transduct Target Ther* **7**, 220 (2022).

110. Muñoz-Fontela, C. *et al.* Animal models for COVID-19. *Nature* **586**, 509–515 (2020).

111. Imai, M. *et al.* Syrian hamsters as a small animal model for SARS-CoV-2 infection and countermeasure development. *Proceedings of the National Academy of Sciences* **117**, 16587–16595 (2020).

112. Rosa, R. B. *et al.* In Vitro and In Vivo Models for Studying SARS-CoV-2, the Etiological Agent Responsible for COVID-19 Pandemic. *Viruses* **13**, 379 (2021).

113. Zhao, Y. *et al.* Ferrets: A powerful model of SARS-CoV-2. *Zool Res* **44**, 323–330 (2023).

114. Muñoz-Fontela, C. *et al.* Advances and gaps in SARS-CoV-2 infection models. *PLOS Pathogens* **18**, e1010161 (2022).

115. Dong, W. *et al.* The K18-Human ACE2 Transgenic Mouse Model Recapitulates Non-severe and Severe COVID-19 in Response to an Infectious Dose of the SARS-CoV-2 Virus. *J Virol* **96**, e00964-21 (2022).

116. Oladunni, F. S. *et al.* Lethality of SARS-CoV-2 infection in K18 human angiotensin-converting enzyme 2 transgenic mice. *Nat Commun* **11**, 6122 (2020).

117. Gunasekara, S., Tamil Selvan, M., Miller, C. A. & Rudd, J. M. Thinking Outside the Box: Utilizing Nontraditional Animal Models for COVID-19 Research. *IJTM* **2**, 113–133 (2022).

118. Munster, V. J. *et al.* Respiratory disease in rhesus macaques inoculated with SARS-CoV-2. *Nature* **585**, 268–272 (2020).

119. World Organization for Animal Health. *SARS-CoV-2 in Animals – Situation Report 22*. (2023).

120. EFSA Panel on Animal Health and Welfare *et al.* SARS-CoV-2 in animals: susceptibility of animal species, risk for animal and public health, monitoring, prevention and control. *EFSA Journal* **21**, e07822 (2023).

121. Pappas, G., Vokou, D., Sainis, I. & Halley, J. M. SARS-CoV-2 as a Zooanthroponotic Infection: Spillbacks, Secondary Spillovers, and Their Importance. *Microorganisms* **10**, 2166 (2022).

122. Sit, T. H. C. *et al.* Infection of dogs with SARS-CoV-2. *Nature* **586**, 776–778 (2020).

123. Garigliany, M. *et al.* SARS-CoV-2 Natural Transmission from Human to Cat, Belgium, March 2020. *Emerg. Infect. Dis.* **26**, 3069–3071 (2020).

124. Curukoglu, A. *et al.* First direct human-to-cat transmission of the SARS-CoV-2 B.1.1.7 variant. *Australian Veterinary Journal* **99**, 482–488 (2021).

125. Galhardo, J. A. *et al.* Molecular detection and characterization of SARS-CoV-2 in cats and dogs of positive owners during the first COVID-19 wave in Brazil. *Sci Rep* **13**, 14418 (2023).

126. Gortázar, C. *et al.* Natural SARS-CoV-2 Infection in Kept Ferrets, Spain. *Emerg Infect Dis* **27**, 1994–1996 (2021).

127. Jara, L. M. *et al.* Evidence of neutralizing antibodies against SARS-CoV-2 in domestic cats living with owners with a history of COVID-19 in Lima – Peru. *One Health* **13**, 100318 (2021).

128. Miró, G. *et al.* SARS-CoV-2 Infection in One Cat and Three Dogs Living in COVID-19-Positive Households in Madrid, Spain. *Frontiers in Veterinary Science* **8**, 779341 (2021).

129. Račnik, J. *et al.* Transmission of SARS-CoV-2 from Human to Domestic Ferret. *Emerg Infect Dis* **27**, 2450–2453 (2021).

130. Segalés, J. *et al.* Detection of SARS-CoV-2 in a cat owned by a COVID-19−affected patient in Spain. *Proceedings of the National Academy of Sciences* **117**, 24790–24793 (2020).

131. Giraldo-Ramirez, S., Rendon-Marin, S., Jaimes, J. A., Martinez-Gutierrez, M. & Ruiz-Saenz, J. SARS-CoV-2 Clinical Outcome in Domestic and Wild Cats: A Systematic Review. *Animals (Basel)* **11**, 2056 (2021).

132. Jones, S. *et al.* SARS-CoV-2 in Domestic UK Cats from Alpha to Omicron: Swab Surveillance and Case Reports. *Viruses* **15**, 1769 (2023).

133. Piewbang, C. *et al.* SARS-CoV-2 Transmission from Human to Pet and Suspected Transmission from Pet to Human, Thailand. *J Clin Microbiol* **60**, e0105822 (2022).

134. Fritz, M. *et al.* High prevalence of SARS-CoV-2 antibodies in pets from COVID-19+ households. *One Health* **11**, 100192 (2021).

135. Hamer, S. A. *et al.* SARS-CoV-2 Infections and Viral Isolations among Serially Tested Cats and Dogs in Households with Infected Owners in Texas, USA. *Viruses* **13**, 938 (2021).

136. Kuhlmeier, E. *et al.* Detection and Molecular Characterization of the SARS-CoV-2 Delta Variant and the Specific Immune Response in Companion Animals in Switzerland. *Viruses* **15**, 245 (2023).

137. Kuroda, Y. *et al.* Pet Animals Were Infected with SARS-CoV-2 from Their Owners Who Developed COVID-19: Case Series Study. *Viruses* **15**, 2028 (2023).

138. Michelitsch, A. *et al.* SARS-CoV-2 Infection and Clinical Signs in Cats and Dogs from Confirmed Positive Households in Germany. *Viruses* **15**, 837 (2023).

139. Sánchez-Morales, L., Sánchez-Vizcaíno, J. M., Domínguez, L. & Barroso-Arévalo, S. A retrospective study of SARS-CoV-2 seroprevalence in dogs and cats in the Community of Madrid, Spain. *Frontiers in Microbiology* **14**, (2023).

140. Zhang, Q. *et al.* A serological survey of SARS-CoV-2 in cat in Wuhan. *Emerging Microbes & Infections* **9**, 2013–2019 (2020).

141. de Souza Barbosa, A. B. *et al.* Infection of SARS-CoV-2 in domestic dogs associated with owner viral load. *Research in Veterinary Science* **153**, 61–65 (2022).

142. Castillo, A. P. *et al.* Evidence of SARS-CoV-2 infection and co-infections in stray cats in Brazil. *Acta Trop* **249**, 107056 (2024).

143. Spada, E. *et al.* A pre- and during Pandemic Survey of Sars-Cov-2 Infection in Stray Colony and Shelter Cats from a High Endemic Area of Northern Italy. *Viruses* **13**, 618 (2021).

144. Villanueva-Saz, S. *et al.* A cross-sectional serosurvey of SARS-CoV-2 and co-infections in stray cats from the second wave to the sixth wave of COVID-19 outbreaks in Spain. *Vet Res Commun* **47**, 615–629 (2023).

145. Yen, H.-L. *et al.* Transmission of SARS-CoV-2 delta variant (AY.127) from pet hamsters to humans, leading to onward human-to-human transmission: a case study. *Lancet* **399**, 1070–1078 (2022).

146. Sila, T. *et al.* Suspected Cat-to-Human Transmission of SARS-CoV-2, Thailand, July-September 2021. *Emerg Infect Dis* **28**, 1485–1488 (2022).

147. Oreshkova, N. *et al.* SARS-CoV-2 infection in farmed minks, the Netherlands, April and May 2020. *Eurosurveillance* **25**, 2001005 (2020).

148. Lu, L. *et al.* Adaptation, spread and transmission of SARS-CoV-2 in farmed minks and associated humans in the Netherlands. *Nat Commun* **12**, 6802 (2021).

149. Hammer, A. S. *et al.* SARS-CoV-2 Transmission between Mink (Neovison vison) and Humans, Denmark. *Emerg Infect Dis* **27**, 547–551 (2021).

150. Boklund, A. *et al.* SARS-CoV-2 in Danish Mink Farms: Course of the Epidemic and a Descriptive Analysis of the Outbreaks in 2020. *Animals* **11**, 164 (2021).

151. Oude Munnink, B. B. *et al.* Transmission of SARS-CoV-2 on mink farms between humans and mink and back to humans. *Science* **371**, 172–177 (2021).

152. Larsen, H. D. *et al.* Preliminary report of an outbreak of SARS-CoV-2 in mink and mink farmers associated with community spread, Denmark, June to November 2020. *Eurosurveillance* **26**, 2100009 (2021).

153. Badiola, J. J. *et al.* SARS-CoV-2 Outbreak on a Spanish Mink Farm: Epidemiological, Molecular, and Pathological Studies. *Front. Vet. Sci.* **8**, (2022).

154. Chaintoutis, S. C. *et al.* Outbreaks of SARS-CoV-2 in naturally infected mink farms: Impact, transmission dynamics, genetic patterns, and environmental contamination. *PLoS Pathog* **17**, e1009883 (2021).

155. Eckstrand, C. D. *et al.* An outbreak of SARS-CoV-2 with high mortality in mink (Neovison vison) on multiple Utah farms. *PLOS Pathogens* **17**, e1009952 (2021).

156. Fenollar, F. *et al.* Mink, SARS-CoV-2, and the Human-Animal Interface. *Front Microbiol* **12**, 663815 (2021).

157. Ghai, R. R. *et al.* Epidemiologic and Genomic Evidence for Zoonotic Transmission of SARS-CoV-2 among People and Animals on a Michigan Mink Farm, United States, 2020. *Viruses* **15**, 2436 (2023).

158. Zhou, J. *et al.* Mutations that adapt SARS-CoV-2 to mink or ferret do not increase fitness in the human airway. *Cell Rep* **38**, 110344 (2022).

159. Tan, C. C. S. *et al.* Transmission of SARS-CoV-2 from humans to animals and potential host adaptation. *Nat Commun* **13**, 2988 (2022).

160. Hoffmann, M. *et al.* SARS-CoV-2 mutations acquired in mink reduce antibody-mediated neutralization. *Cell Reports* **35**, 109017 (2021).

161. Fiorito, F. *et al.* First Description of Serological Evidence for SARS-CoV-2 in Lactating Cows. *Animals* **12**, 1459 (2022).

162. Wernike, K. *et al.* Antibodies against SARS-CoV-2 Suggestive of Single Events of Spillover to Cattle, Germany. *Emerg. Infect. Dis.* **28**, 1916–1918 (2022).

163. Pusterla, N., Lawton, K. & Barnum, S. Investigation of the seroprevalence to equine coronavirus and SARS-CoV-2 in healthy adult horses recently imported to the United States. *Veterinary Quarterly* **44**, 1–6 (2023).

164. Bartlett, S. L. *et al.* SARS-COV-2 INFECTION AND LONGITUDINAL FECAL SCREENING IN MALAYAN TIGERS (PANTHERA TIGRIS JACKSONI), AMUR TIGERS (PANTHERA TIGRIS ALTAICA), AND AFRICAN LIONS (PANTHERA LEO KRUGERI) AT THE BRONX ZOO, NEW YORK, USA. *zamd* **51**, 733–744 (2021).

165. Fernández-Bellon, H. *et al.* Monitoring Natural SARS-CoV-2 Infection in Lions (Panthera leo) at the Barcelona Zoo: Viral Dynamics and Host Responses. *Viruses* **13**, 1683 (2021).

166. Koeppel, K. N. *et al.* SARS-CoV-2 Reverse Zoonoses to Pumas and Lions, South Africa. *Viruses* **14**, 120 (2022).

167. McAloose, D. *et al.* From People to Panthera: Natural SARS-CoV-2 Infection in Tigers and Lions at the Bronx Zoo. *mBio* **11**, e02220-20 (2020).

168. Mishra, A. *et al.* SARS-CoV-2 Delta Variant among Asiatic Lions, India. *Emerging Infectious Diseases* **27**, 2723–2725 (2021).

169. Mitchell, P. K. *et al.* SARS-CoV-2 B.1.1.7 Variant Infection in Malayan Tigers, Virginia, USA. *Emerging Microbes & Infections* **27**, 12 (2021).

170. Wang, L. *et al.* Detection of SARS-CoV-2 clade B.1.2 in three snow leopards. *Transboundary and Emerging Diseases* **69**, e3346–e3351 (2022).

171. Bartlett, S. L. *et al.* GLOBAL RETROSPECTIVE REVIEW OF SEVERE ACUTE RESPIRATORY SYNDROME SARS COV-2 INFECTIONS IN NONDOMESTIC FELIDS: MARCH 2020–FEBRUARY 2021. *zamd* **54**, 607–616 (2023).

172. Drozd, M. *et al.* Mortality associated with SARS-CoV-2 in nondomestic felids. *Vet Pathol* **0**, 1–12 (2024).

173. Cushing, A. C. *et al.* DURATION OF ANTIGEN SHEDDING AND DEVELOPMENT OF ANTIBODY TITERS IN MALAYAN TIGERS (PANTHERA TIGRIS JACKSONI) NATURALLY INFECTED WITH SARS-CoV-2. *J Zoo Wildl Med* **52**, 1224–1228 (2021).

174. Nagy, A. *et al.* Reverse-zoonotic transmission of SARS-CoV-2 lineage alpha (B.1.1.7) to great apes and exotic felids in a zoo in the Czech Republic. *Arch Virol* **167**, 1681–1685 (2022).

175. Tavera Gonzales, A. *et al.* Possible Spreading of SARS-CoV-2 from Humans to Captive Non-Human Primates in the Peruvian Amazon. *Animals* **14**, 732 (2024).

176. Allender, M. C. *et al.* Multi-species outbreak of SARS-CoV-2 Delta variant in a zoological institution, with the detection in two new

families of carnivores. *Transbound Emerg Dis* **69**, e3060–e3075 (2022).

177. Italiya, J., Bhavsar, T. & Černý, J. Assessment and strategy development for SARS-CoV-2 screening in wildlife: A review. *Vet World* **16**, 1193–1200 (2023).

178. Vercammen, F. *et al.* SARS-CoV-2 Infection in Captive Hippos (Hippopotamus amphibius), Belgium. *Animals (Basel)* **13**, (2023).

179. Chandler, J. C. *et al.* SARS-CoV-2 exposure in wild white-tailed deer (Odocoileus virginianus). *Proceedings of the National Academy of Sciences* **118**, e2114828118 (2021).

180. Hale, V. L. *et al.* SARS-CoV-2 infection in free-ranging white-tailed deer. *Nature* **602**, 481–486 (2022).

181. Palermo, P. M., Orbegozo, J., Watts, D. M. & Morrill, J. C. SARS-CoV-2 Neutralizing Antibodies in White-Tailed Deer from Texas. *Vector Borne Zoonotic Dis* **22**, 62–64 (2022).

182. Marques, A. D. *et al.* Multiple Introductions of SARS-CoV-2 Alpha and Delta Variants into White-Tailed Deer in Pennsylvania. *mBio* **13**, e02101-22 (2022).

183. Kuchipudi, S. V. *et al.* Multiple spillovers from humans and onward transmission of SARS-CoV-2 in white-tailed deer. *Proceedings of the National Academy of Sciences* **119**, e2121644119 (2022).

184. Vandegrift, K. J. *et al.* SARS-CoV-2 Omicron (B.1.1.529) Infection of Wild White-Tailed Deer in New York City. *Viruses* **14**, 2770 (2022).

185. Pickering, B. *et al.* Divergent SARS-CoV-2 variant emerges in white-tailed deer with deer-to-human transmission. *Nat Microbiol* **7**, 2011–2024 (2022).

186. Encinas, P. *et al.* SARS-CoV-2 Neutralizing Antibodies in Free-Ranging Fallow Deer (Dama dama) and Red Deer (Cervus elaphus) in

Suburban and Rural Areas in Spain. *Transboundary and Emerging Diseases* **2023**, 1–11 (2023).

187. Aguiló-Gisbert, J. *et al.* First Description of SARS-CoV-2 Infection in Two Feral American Mink (Neovison vison) Caught in the Wild. *Animals (Basel)* **11**, 1422 (2021).

188. Padilla-Blanco, M. *et al.* The Finding of the Severe Acute Respiratory Syndrome Coronavirus (SARS-CoV-2) in a Wild Eurasian River Otter (Lutra lutra) Highlights the Need for Viral Surveillance in Wild Mustelids. *Frontiers in Veterinary Science* **9**, 826991 (2022).

189. Shriner, S. A. *et al.* SARS-CoV-2 Exposure in Escaped Mink, Utah, USA. *Emerg. Infect. Dis.* **27**, 988–990 (2021).

190. van Aart, A. E. *et al.* SARS-CoV-2 infection in cats and dogs in infected mink farms. *Transboundary and Emerging Diseases* **69**, 3001–3007 (2022).

191. Davoust, B. *et al.* Evidence of antibodies against SARS-CoV-2 in wild mustelids from Brittany (France). *Transboundary and Emerging Diseases* **69**, e3400–e3407 (2022).

192. Mahajan, S. *et al.* Detection of SARS-CoV-2 in a free ranging leopard (Panthera pardus fusca) in India. *Eur J Wildl Res* **68**, 59 (2022).

193. Pereira, A. H. *et al.* Natural SARS-CoV-2 Infection in a Free-Ranging Black-Tailed Marmoset (Mico melanurus) from an Urban Area in Mid-West Brazil. *J Comp Pathol* **194**, 22–27 (2022).

194. Lefrançois, T. *et al.* After 2 years of the COVID-19 pandemic, translating One Health into action is urgent. *Lancet (London, England)* **401**, 789–794 (2023).

195. Esposito, M. M., Turku, S., Lehrfield, L. & Shoman, A. The Impact of Human Activities on Zoonotic Infection Transmissions. *Animals* **13**, 1646 (2023).

196. Carlson, C. J. *et al.* Climate change increases cross-species viral transmission risk. *Nature* **607**, 555–562 (2022).

197. Mora, C. *et al.* Over half of known human pathogenic diseases can be aggravated by climate change. *Nat. Clim. Chang.* **12**, 869–875 (2022).

198. Johnson, C. K. *et al.* Global shifts in mammalian population trends reveal key predictors of virus spillover risk. *Proc Biol Sci* **287**, 20192736 (2020).

199. CDC. *SAVING LIVES BY TAKING A ONE HEALTH APPROACH: Connecting Human, Animal, and Environmental Health*. https://www.cdc.gov/onehealth/who-we-are/one-health-office-fact-sheet.html (2023).

200. FAO, UNEP, WHO & WOAH. One Health Joint Plan of Action, 2022–2026. Working together for the health of humans, animals, plants and the environment. https://doi.org/10.4060/cc2289en (2022).

201. OHEJP. OHEJP Final Report 2023. ohejp-final-report-september-2023.pdf (2023).

202. Ministerio de Sanidad. Plan Estratégico de Salud y Medioambiente. https://www.sanidad.gob.es/ciudadanos/pesma/docs/241121_PESMA.pdf (2021).

203. Chen, Y. *et al.* Rhesus angiotensin converting enzyme 2 supports entry of severe acute respiratory syndrome coronavirus in Chinese macaques. *Virology* **381**, 89–97 (2008).

204. Martina, B. E. E. *et al.* SARS virus infection of cats and ferrets. *Nature* **425**, (2003).

205. Kuiken, T. *et al.* Newly discovered coronavirus as the primary cause of severe acute respiratory syndrome. *Lancet* **362**, 263–270 (2003).

206. McAuliffe, J. *et al.* Replication of SARS coronavirus administered into the respiratory tract of African Green, rhesus and cynomolgus monkeys. *Virology* **330**, 8–15 (2004).

207. Roberts, A. *et al.* Severe acute respiratory syndrome coronavirus infection of golden Syrian hamsters. *J Virol* **79**, 503–511 (2005).

208. Subbarao, K. *et al.* Prior Infection and Passive Transfer of Neutralizing Antibody Prevent Replication of Severe Acute Respiratory Syndrome Coronavirus in the Respiratory Tract of Mice. *J Virol* **78**, 3572–3577 (2004).

209. Li, W. *et al.* Angiotensin-converting enzyme 2 is a functional receptor for the SARS coronavirus. *Nature* **426**, 450–454 (2003).

210. Sailleau, C. *et al.* First detection and genome sequencing of SARS-CoV-2 in an infected cat in France. *Transbound Emerg Dis* **67**, 2324–2328 (2020).

211. Liu, Y. *et al.* Functional and genetic analysis of viral receptor ACE2 orthologs reveals a broad potential host range of SARS-CoV-2. *Proc Natl Acad Sci U S A* **118**, e2025373118 (2021).

212. Hassell, J. M., Begon, M., Ward, M. J. & Fèvre, E. M. Urbanization and Disease Emergence: Dynamics at the Wildlife-Livestock-Human Interface. *Trends Ecol Evol* **32**, 55–67 (2017).

213. Pascual, J., Franco, S., Bueno-Marí, R., Peracho, V. & Montalvo, T. Demography and ecology of Norway rats, Rattus norvegicus, in the sewer system of Barcelona (Catalonia, Spain). *J Pest Sci* **93**, 711–722 (2020).

214. Colomer, J., Rosell, C., Navàs, F., Pericas, B. & Colomer, A. *Programa de Seguiment de Les Poblacions de Senglar a Catalunya-Serralada Litoral*. https://senglar.cat/programa-de-seguiment/ (2023).

215. Kapczynski, D. R., Sweeney, R., Spackman, E., Pantin-Jackwood, M. & Suarez, D. L. Development of an in vitro model for animal species susceptibility to SARS-CoV-2 replication based on expression of ACE2 and TMPRSS2 in avian cells. *Virology* **569**, 1–12 (2022).

216. Lytras, S. *et al.* Exploring the Natural Origins of SARS-CoV-2 in the Light of Recombination. *Genome Biology and Evolution* **14**, evac018 (2022).

217. Pekar, J. E. *et al.* The molecular epidemiology of multiple zoonotic origins of SARS-CoV-2. *Science* **377**, 960–966 (2022).

218. Sia, S. F. *et al.* Pathogenesis and transmission of SARS-CoV-2 in golden hamsters. *Nature* **583**, 834–838 (2020).

219. CCVC. Arxiu d'Identificació d'Animals de Companyia. *CCVC - Consell de col·legis veterinaris de Catalunya* https://www.veterinaris.cat/aiac/aiac-particulars/ (2021).

220. Corman, V. M. *et al.* Detection of 2019 novel coronavirus (2019-nCoV) by real-time RT-PCR. *Euro Surveill* **25**, 2000045 (2020).

221. Rodon, J. *et al.* Identification of Plitidepsin as Potent Inhibitor of SARS-CoV-2-Induced Cytopathic Effect After a Drug Repurposing Screen. *Front Pharmacol* **12**, (2021).

222. Pillay, S. *et al.* Whole Genome Sequencing of SARS-CoV-2: Adapting Illumina Protocols for Quick and Accurate Outbreak Investigation during a Pandemic. *Genes (Basel)* **11**, (2020).

223. Pradenas, E. *et al.* Clinical course impacts early kinetics,magnitude, and amplitude of SARS-CoV-2 neutralizing antibodies beyond 1 year after infection. *Cell Rep Med* **3**, 100523 (2022).

224. Díez, J. M. *et al.* Anti-Severe Acute Respiratory Syndrome Coronavirus 2 Hyperimmune Immunoglobulin Demonstrates Potent Neutralization and Antibody-Dependent Cellular Cytotoxicity and Phagocytosis Through N and S Proteins. *The Journal of Infectious Diseases* **225**, 938–946 (2022).

225. SIVIC - COVID-19 - Seqüenciació. *Sistema d'informació per a la Vigilància d'Infeccions a Catalunya* https://sivic.salut.gencat.cat/covid_sequenciacio?ftipusdada=2&ftemporada=11 (2024).

226. Barroso-Arévalo, S. *et al.* Large-scale study on virological and serological prevalence of SARS-CoV-2 in cats and dogs in Spain. *Transbound Emerg Dis* **69**, e759–e774 (2022).

227. Ferasin, L. *et al.* Infection with SARS-CoV-2 variant B.1.1.7 detected in a group of dogs and cats with suspected myocarditis. *Veterinary Record* **189**, e944 (2021).

228. Patterson, E. I. *et al.* Evidence of exposure to SARS-CoV-2 in cats and dogs from households in Italy. *Nat Commun* **11**, (2020).

229. Udom, K. *et al.* Serological survey of antibodies against SARS-CoV-2 in dogs and cats, Thailand. *Transbounding Emerging Dis* **69**, 2140–2147 (2022).

230. Ruiz-Arrondo, I. *et al.* Detection of SARS-CoV-2 in pets living with COVID-19 owners diagnosed during the COVID-19 lockdown in Spain: A case of an asymptomatic cat with SARS-CoV-2 in Europe. *Transboundary and Emerging Diseases* **68**, 973–976 (2021).

231. Barrs, V. R. *et al.* SARS-CoV-2 in Quarantined Domestic Cats from COVID-19 Households or Close Contacts, Hong Kong, China. *Emerg Infect Dis* **26**, 3071–3074 (2020).

232. Hamer, S. A. *et al.* SARS-CoV-2 B.1.1.7 variant of concern detected in a pet dog and cat after exposure to a person with COVID-19, USA. *Transboundary and Emerging Diseases* **69**, 1656–1658 (2022).

233. Hosie, M. J. *et al.* Detection of SARS-CoV-2 in respiratory samples from cats in the UK associated with human-to-cat transmission. *Vet Rec* **188**, e247 (2021).

234. Bienzle, D. *et al.* Risk Factors for SARS-CoV-2 Infection and Illness in Cats and Dogs. *Emerging Infectious Diseases* **28**, 6 (2022).

235. Neira, V. *et al.* A household case evidences shorter shedding of SARS-CoV-2 in naturally infected cats compared to their human owners. *Emerging Microbes & Infections* **10**, 376–383 (2021).

236. Yang, J. *et al.* Prevalence of comorbidities and its effects in patients infected with SARS-CoV-2: a systematic review and meta-analysis. *International Journal of Infectious Diseases* **94**, 91–95 (2020).

237. Giner, J. *et al.* Seroprevalence of anti-SARS-CoV-2 antibodies in household domestic ferrets (Mustela putorius furo) in Spain, 2019–2023. *Vet Res Commun* **48**, 533–540 (2024).

238. Pomorska-Mól, M. *et al.* A cross-sectional retrospective study of SARS-CoV-2 seroprevalence in domestic cats, dogs and rabbits in Poland. *BMC Vet Res* **17**, 322 (2021).

239. Scully, E. P., Haverfield, J., Ursin, R. L., Tannenbaum, C. & Klein, S. L. Considering how biological sex impacts immune responses and COVID-19 outcomes. *Nat Rev Immunol* **20**, 442–447 (2020).

240. Trinité, B. *et al.* SARS-CoV-2 infection elicits a rapid neutralizing antibody response that correlates with disease severity. *Sci Rep* **11**, 2608 (2021).

241. Yang, Q., Syed, A. A. S., Fahira, A. & Shi, Y. Structural Analysis of the SARS-CoV-2 Omicron Variant Proteins. *Research* **2021**, 9769586 (2021).

242. Vogt, A.-C. S. *et al.* Increased Receptor Affinity and Reduced Recognition by Specific Antibodies Contribute to Immune Escape of SARS-CoV-2 Variant Omicron. *Vaccines* **10**, 743 (2022).

243. Zhai, X. *et al.* Comparison of Severe Acute Respiratory Syndrome Coronavirus 2 Spike Protein Binding to ACE2 Receptors from Human, Pets, Farm Animals, and Putative Intermediate Hosts. *Journal of Virology* **94**, e00831-20 (2020).

244. Klein, C. *et al.* Dogs and Cats Are Less Susceptible to the Omicron Variant of Concern of SARS-CoV-2: A Field Study in Germany, 2021/2022. *Transboundary and Emerging Diseases* **2023**, e1868732 (2023).

245. Sánchez-Morales, L., Sánchez-Vizcaíno, J. M., Pérez-Sancho, M., Domínguez, L. & Barroso-Arévalo, S. The Omicron (B.1.1.529) SARS-CoV-2 variant of concern also affects companion animals. *Front. Vet. Sci.* **9**, 940710 (2022).

246. Tyson, G. B. *et al.* Increase in SARS-CoV-2 Seroprevalence in UK Domestic Felids Despite Weak Immunogenicity of Post-Omicron Variants. *Viruses* **15**, 1661 (2023).

247. Friant, S. Human behaviors driving disease emergence. *Evolutionary Anthropology: Issues, News, and Reviews* e22015 (2023) doi:10.1002/evan.22015.

248. Fernández-Bastit, L., Vergara-Alert, J. & Segalés, J. Transmission of severe acute respiratory syndrome coronavirus 2 from humans to animals: is there a risk of novel reservoirs? *Current Opinion in Virology* **63**, 101365 (2023).

249. Martínez-Hernández, F. *et al.* Assessing the SARS-CoV-2 threat to wildlife: Potential risk to a broad range of mammals. *Perspectives in Ecology and Conservation* **18**, 223–234 (2020).

250. Tan, Z. H., Yong, K. Y. & Shu, J.-J. Predicting potential SARS-CoV-2 spillover and spillback in animals. *Journal of Microbiology, Immunology and Infection* **57**, 225–237 (2024).

251. Delahay, R. J. *et al.* Assessing the risks of SARS-CoV-2 in wildlife. *One Health Outlook* **3**, 7 (2021).

252. Gryseels, S. *et al.* Risk of human-to-wildlife transmission of SARS-CoV-2. *Mammal Review* **51**, 272–292 (2021).

253. Feng, A. *et al.* Transmission of SARS-CoV-2 in free-ranging white-tailed deer in the United States. *Nat Commun* **14**, 4078 (2023).

254. Takemura, T. *et al.* SARS-CoV-2 Infection in Beaver Farm, Mongolia, 2021. *Emerg Infect Dis* **30**, 391–394 (2024).

255. Wang, Y. *et al.* SARS-CoV-2 Exposure in Norway Rats (Rattus norvegicus) from New York City. *mBio* **0**, e03621-22 (2023).

256. Latorre, D., Campos Llach, M., Dalmau, G., Cicres, A. & Tubert, A. First detection of invasive coypu, Myocastor coypus Molina, 1782 (Mammalia: Roentia: Myocastoridae) into lake Banyoles (Catalonia, north-east Iberian Peninsula). *Butlletí de la Institució Catalana d'Història Natural (ICHN)* 253–256 (2020) doi:10.2436/20.1502.01.59.

257. Palazón, S. *et al.* Situation of feral American mink (Neovison vison) in Catalonia: expansion, distribution, ecology and population control. *de la Institució Catalana d'Història Natural (ICHN)* **80**, 145–154 (2016).

258. Vada, R. *et al.* Feral American mink Neogale vison continues to expand its European range: time to harmonise population monitoring and coordinate control. *Mammal Review* **53**, 158–176 (2023).

259. Stone, H. M., Unal, E., Romano, T. A. & Turner, P. E. Beluga whale and bottlenose dolphin ACE2 proteins allow cell entry mediated by spike protein from three variants of SARS-CoV-2. *Biology Letters* **19**, 20230321 (2023).

260. Audino, T. *et al.* Potential SARS-CoV-2 Susceptibility of Cetaceans Stranded along the Italian Coastline. *Pathogens* **11**, 1096 (2022).

261. Graninger, M. *et al.* Comprehensive Comparison of Seven SARS-CoV-2-Specific Surrogate Virus Neutralization and Anti-Spike IgG Antibody Assays Using a Live-Virus Neutralization Assay as a Reference. *Microbiology Spectrum* **11**, e02314-22 (2023).

262. Bewley, K. R. *et al.* Quantification of SARS-CoV-2 neutralizing antibody by wild-type plaque reduction neutralization, microneutralization and pseudotyped virus neutralization assays. *Nat Protoc* **16**, 3114–3140 (2021).

263. Wang, L. *et al.* Detection and Characterization of New Coronavirus in Bottlenose Dolphin, United States, 2019. *Emerg Infect Dis* **26**, 1610–1612 (2020).

264. Hemalatha, M. *et al.* Surveillance of SARS-CoV-2 spread using wastewater-based epidemiology: Comprehensive study. *Sci Total Environ* **768**, 144704 (2021).

265. Joseph-Duran, B. *et al.* Assessing wastewater-based epidemiology for the prediction of SARS-CoV-2 incidence in Catalonia. *Sci Rep* **12**, 15073 (2022).

266. Qiu, X., Liu, Y. & Sha, A. SARS-CoV-2 and natural infection in animals. *Journal of Medical Virology* **95**, e28147 (2022).

267. Tewari, D. *et al.* SARS-CoV-2 Infection Dynamics in the Pittsburgh Zoo Wild Felids with Two Viral Variants (Delta and Alpha) during the 2021–2022 Pandemic in the United States. *Animals* **13**, 3094 (2023).

268. Yamamoto, J. K. *et al.* Both Feline Coronavirus Serotypes 1 and 2 Infected Domestic Cats Develop Cross-Reactive Antibodies to SARS-CoV-2 Receptor Binding Domain: Its Implication to Pan-CoV Vaccine Development. *Viruses* **15**, 914 (2023).

269. Tinto, B. *et al.* Monitoring SARS-CoV-2 Seroprevalence in Domestics and Exotic Animals in Southern France. *Tropical Medicine and Infectious Disease* **8**, 426 (2023).

270. Gómez, J. C. *et al.* Exposure to severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) in the endangered Iberian lynx (Lynx pardinus). *Veterinary Microbiology* **290**, 110001 (2024).

271. Ryser-Degiorgis, M.-P. Wildlife health investigations: needs, challenges and recommendations. *BMC Veterinary Research* **9**, 223 (2013).

272. Mackenstedt, U., Jenkins, D. & Romig, T. The role of wildlife in the transmission of parasitic zoonoses in peri-urban and urban areas.

International Journal for Parasitology: Parasites and Wildlife **4**, 71–79 (2015).

273. Capizzi, D., Bertolino, S. & Mortelliti, A. Rating the rat: global patterns and research priorities in impacts and management of rodent pests. *Mammal Review* **44**, 148–162 (2014).

274. Galán-Puchades, M. T. *et al.* One Health Approach to Zoonotic Parasites: Molecular Detection of Intestinal Protozoans in an Urban Population of Norway Rats, Rattus norvegicus, in Barcelona, Spain. *Pathogens* **10**, 311 (2021).

275. Bartak, M., Słońska, A., Bańbura, M. W. & Cymerys, J. SDAV, the Rat Coronavirus—How Much Do We Know about It in the Light of Potential Zoonoses. *Viruses* **13**, 1995 (2021).

276. Han, B. A., Schmidt, J. P., Bowden, S. E. & Drake, J. M. Rodent reservoirs of future zoonotic diseases. *Proceedings of the National Academy of Sciences* **112**, 7039–7044 (2015).

277. Li, X. *et al.* A Novel Potentially Recombinant Rodent Coronavirus with a Polybasic Cleavage Site in the Spike Protein. *Journal of Virology* **95**, e01173-21 (2021).

278. Tsoleridis, T. *et al.* Discovery of Novel Alphacoronaviruses in European Rodents and Shrews. *Viruses* **8**, 84 (2016).

279. Wang, W. *et al.* Discovery, diversity and evolution of novel coronaviruses sampled from rodents in China. *Virology* **474**, 19–27 (2015).

280. Wang, Q. *et al.* Key mutations on spike protein altering ACE2 receptor utilization and potentially expanding host range of emerging SARS-CoV-2 variants. *Journal of Medical Virology* **95**, 1 (2023).

281. Wei, C. *et al.* Evidence for a mouse origin of the SARS-CoV-2 Omicron variant. *Journal of Genetics and Genomics* **48**, 1111–1121 (2021).

282. Fredriksson-Ahomaa, M. Wild Boar: A Reservoir of Foodborne Zoonoses. *Foodborne Pathogens and Disease* **16**, 153–165 (2019).

283. Meng, X. J., Lindsay, D. S. & Sriranganathan, N. Wild boars as sources for infectious diseases in livestock and humans. *Philos Trans R Soc Lond B Biol Sci* **364**, 2697–2707 (2009).

284. Wang, Q., Vlasova, A. N., Kenney, S. P. & Saif, L. J. Emerging and re-emerging coronaviruses in pigs. *Current Opinion in Virology* **34**, 39–49 (2019).

285. Fisher, A. M. *et al.* The ecology of viruses in urban rodents with a focus on SARS-CoV-2. *Emerg Microbes Infect* **12**, e22179940 (2023).

286. Morand, S., Jittapalapong, S. & Kosoy, M. Rodents as Hosts of Infectious Diseases: Biological and Ecological Characteristics. *Vector Borne Zoonotic Dis* **15**, 1–2 (2015).

287. Hulst, M. *et al.* Cross-Reactivity of Human, Wild Boar, and Farm Animal Sera from Pre- and Post-Pandemic Periods with Alpha- and Beta-Coronaviruses (CoV), including SARS-CoV-2. *Viruses* **16**, 34 (2023).

288. Klompus, S. *et al.* Cross-reactive antibodies against human coronaviruses and the animal coronavirome suggest diagnostics for future zoonotic spillovers. *Science Immunology* **6**, eabe9950 (2021).

289. Sikkema, R. S. *et al.* Experimental and field investigations of exposure, replication and transmission of SARS-CoV-2 in pigs in the Netherlands. *Emerg Microbes Infect* **11**, 91–94 (2022).

290. Chu, H. *et al.* Comparative tropism, replication kinetics, and cell damage profiling of SARS-CoV-2 and SARS-CoV with implications for clinical manifestations, transmissibility, and laboratory studies of COVID-19: an observational study. *Lancet Microbe* **1**, e14–e23 (2020).

291. Meekins, D. A. *et al.* Susceptibility of swine cells and domestic pigs to SARS-CoV-2. *Emerging Microbes & Infections* **9**, 2278–2288 (2020).

292. Sobsey, M. D. Absence of virological and epidemiological evidence that SARS-CoV-2 poses COVID-19 risks from environmental fecal waste, wastewater and water exposures. *Journal of Water and Health* **20**, 126–138 (2022).

293. Colombo, V. C. *et al.* SARS-CoV-2 surveillance in Norway rats (Rattus norvegicus) from Antwerp sewer system, Belgium. *Transboundary and Emerging Diseases* **69**, 3016–3021 (2022).

294. Miot, E. F. *et al.* Surveillance of Rodent Pests for SARS-CoV-2 and Other Coronaviruses, Hong Kong. *Emerg. Infect. Dis.* **28**, 467–470 (2022).

295. Huang, H., Zhu, Y., Niu, Z., Zhou, L. & Sun, Q. SARS-CoV-2 N501Y variants of concern and their potential transmission by mouse. *Cell Death Differ* **28**, 2840–2842 (2021).

296. Mohandas, S. *et al.* SARS-CoV-2 Delta Variant Pathogenesis and Host Response in Syrian Hamsters. *Viruses* **13**, 1773 (2021).

297. Pan, T. *et al.* Infection of wild-type mice by SARS-CoV-2 B.1.351 variant indicates a possible novel cross-species transmission route. *Sig Transduct Target Ther* **6**, 1–12 (2021).

298. Tegally, H. *et al.* Detection of a SARS-CoV-2 variant of concern in South Africa. *Nature* **592**, 438–443 (2021).

299. Liu, Y. *et al.* The N501Y spike substitution enhances SARS-CoV-2 infection and transmission. *Nature* **602**, 294–299 (2022).

300. Wang, R. *et al.* Analysis of SARS-CoV-2 variant mutations reveals neutralization escape mechanisms and the ability to use ACE2 receptors from additional species. *Immunity* **54**, 1611–1621 (2021).

301. Wang, W. B. *et al.* E484K mutation in SARS-CoV-2 RBD enhances binding affinity with hACE2 but reduces interactions with neutralizing antibodies and nanobodies: Binding free energy calculation studies. *J Mol Graph Model* **109**, 108035 (2021).

302. Wibmer, C. K. *et al.* SARS-CoV-2 501Y.V2 escapes neutralization by South African COVID-19 donor plasma. *Nat Med* **27**, 622–625 (2021).

303. Jori, F., Hernandez-Jover, M., Magouras, I., Dürr, S. & Brookes, V. J. Wildlife–livestock interactions in animal production systems: what are the biosecurity and health implications? *Animal Frontiers* **11**, 8–19 (2021).

304. Gilbert, M. *et al.* Global distribution data for cattle, buffaloes, horses, sheep, goats, pigs, chickens and ducks in 2010. *Sci Data* **5**, 180227 (2018).

305. Šali, A. & Blundell, T. L. Comparative Protein Modelling by Satisfaction of Spatial Restraints. *Journal of Molecular Biology* **234**, 779–815 (1993).

306. Schymkowitz, J. *et al.* The FoldX web server: an online force field. *Nucleic Acids Research* **33**, W382–W388 (2005).

307. Wölfel, R. *et al.* Virological assessment of hospitalized patients with COVID-2019. *Nature* **581**, 465–469 (2020).

308. REED, L. J. & MUENCH, H. A SIMPLE METHOD OF ESTIMATING FIFTY PER CENT ENDPOINTS12. *American Journal of Epidemiology* **27**, 493–497 (1938).

309. Zhang, H.-L. *et al.* Evaluating angiotensin-converting enzyme 2-mediated SARS-CoV-2 entry across species. *J Biol Chem* **296**, 100435 (2021).

310. Niles, M. A. *et al.* Macrophages and Dendritic Cells Are Not the Major Source of Pro-Inflammatory Cytokines Upon SARS-CoV-2 Infection. *Frontiers in Immunology* **12**, 647824 (2021).

311. Perez-Zsolt, D. *et al.* SARS-CoV-2 interaction with Siglec-1 mediates trans-infection by dendritic cells. *Cell Mol Immunol* **18**, 2676–2678 (2021).

312. Li, L. *et al.* Broader-species receptor binding and structural bases of Omicron SARS-CoV-2 to both mouse and palm-civet ACE2s. *Cell Discov* **8**, 1–16 (2022).

313. Barroso, P., López-Olvera, J. R., Kiluba, T. K. wa & Gortázar, C. Overcoming the limitations of wildlife disease monitoring. *Research Directions: One Health* **2**, 1–14 (2024).

314. Murray, J. & Cohen, A. L. Infectious Disease Surveillance. *International Encyclopedia of Public Health* **4**, 222–229 (2017).

315. Tan, C. W. *et al.* A SARS-CoV-2 surrogate virus neutralization test based on antibody-mediated blockage of ACE2–spike protein–protein interaction. *Nat Biotechnol* **38**, 1073–1078 (2020).

316. Xiang, Q., Li, L., Wu, J., Tian, M. & Fu, Y. Application of pseudovirus system in the development of vaccine, antiviral-drugs, and neutralizing antibodies. *Microbiol Res* **258**, 126993 (2022).

317. Barua, S. *et al.* Antibodies to SARS-CoV-2 in dogs and cats, USA. *Emerging Microbes & Infections* **10**, 1669–1674 (2021).

318. Diezma-Díaz, C. *et al.* A comparative study of eight serological methods shows that spike protein-based ELISAs are the most accurate tests for serodiagnosing SARS-CoV-2 infections in cats and dogs. *Front Vet Sci* **10**, 1121935 (2023).

319. Zhao, S. *et al.* Serologic Screening of Severe Acute Respiratory Syndrome Coronavirus 2 Infection in Cats and Dogs during First Coronavirus Disease Wave, the Netherlands. *Emerging Infectious Diseases* **27**, 5 (2021).

320. Dobaño, C. *et al.* Immunogenicity and crossreactivity of antibodies to the nucleocapsid protein of SARS-CoV-2: utility and limitations in seroprevalence and immunity studies. *Transl Res* **232**, 60–74 (2021).

321. Premkumar, L. *et al.* The receptor-binding domain of the viral spike protein is an immunodominant and highly specific target of antibodies in SARS-CoV-2 patients. *Science Immunology* **5**, eabc8413 (2020).

322. Khoury, D. S. *et al.* Neutralizing antibody levels are highly predictive of immune protection from symptomatic SARS-CoV-2 infection. *Nat Med* **27**, 1205–1211 (2021).

323. Qi, H., Liu, B., Wang, X. & Zhang, L. The humoral response and antibodies against SARS-CoV-2 infection. *Nat Immunol* **23**, 1008–1020 (2022).

324. Rogers, T. F. *et al.* Isolation of potent SARS-CoV-2 neutralizing antibodies and protection from disease in a small animal model. *Science* **369**, 956–963 (2020).

325. Bahnan, W. *et al.* Spike-Dependent Opsonization Indicates Both Dose-Dependent Inhibition of Phagocytosis and That Non-Neutralizing Antibodies Can Confer Protection to SARS-CoV-2. *Frontiers in immunology* **12**, 808932 (2022).

326. Chakraborty, S. *et al.* Early non-neutralizing, afucosylated antibody responses are associated with COVID-19 severity. *Sci Transl Med* **14**, eabm7853 (2022).

327. Meyer, B., Drosten, C. & Müller, M. A. Serological assays for emerging coronaviruses: Challenges and pitfalls. *Virus Research* **194**, 175–183 (2014).

328. Chia, W. N. *et al.* Serological differentiation between COVID-19 and SARS infections. *Emerging Microbes & Infections* **9**, 1497–1505 (2020).

329. Perera, R. A. P. M. *et al.* Evaluation of a SARS-CoV-2 Surrogate Virus Neutralization Test for Detection of Antibody in Human, Canine, Cat, and Hamster Sera. *Journal of Clinical Microbiology* **59**, e02504-20 (2021).

330. Müller, K., Girl, P., von Buttlar, H., Dobler, G. & Wölfel, R. Comparison of two commercial surrogate ELISAs to detect a neutralising antibody response to SARS-CoV-2. *Journal of Virological Methods* **292**, 114122 (2021).

331. Taylor, S. C. *et al.* A New SARS-CoV-2 Dual-Purpose Serology Test: Highly Accurate Infection Tracing and Neutralizing Antibody Response Detection. *J Clin Microbiol* **59**, e02438-20 (2021).

332. Embregts, C. W. E. *et al.* Evaluation of a multi-species SARS-CoV-2 surrogate virus neutralization test. *One Health* **13**, 100313 (2021).

333. Miller, M. R., Braun, E., Ip, H. S. & Tyson, G. H. Domestic and wild animal samples and diagnostic testing for SARS-CoV-2. *Vet Q* **43**, 1–11 (2023).

334. Ly, H. Assessing the Prevalence of SARS-CoV-2 in Free-Living and Captive Animals. *Pathogens* **11**, 1405 (2022).

335. Vlasova, A. N. *et al.* Two-Way Antigenic Cross-Reactivity between Severe Acute Respiratory Syndrome Coronavirus (SARS-CoV) and Group 1 Animal CoVs Is Mediated through an Antigenic Site in the N-Terminal Region of the SARS-CoV Nucleoprotein. *J Virol* **81**, 13365–13377 (2007).

336. Laidoudi, Y. *et al.* SARS-CoV-2 antibodies seroprevalence in dogs from France using ELISA and an automated western blotting assay. *One Health* **13**, 100293 (2021).

337. Hancock, T. J. *et al.* Possible Cross-Reactivity of Feline and White-Tailed Deer Antibodies against the SARS-CoV-2 Receptor Binding Domain. *J Virol* **96**, e0025022 (2022).

338. Fenwick, C. *et al.* Changes in SARS-CoV-2 Spike versus Nucleoprotein Antibody Responses Impact the Estimates of Infections in Population-Based Seroprevalence Studies. *J Virol* **95**, e01828-20 (2021).

339. Van Elslande, J. *et al.* Lower persistence of anti-nucleocapsid compared to anti-spike antibodies up to one year after SARS-CoV-2 infection. *Diagnostic Microbiology and Infectious Disease* **103**, 115659 (2022).

340. Nie, J. *et al.* Quantification of SARS-CoV-2 neutralizing antibody by a pseudotyped virus-based assay. *Nat Protoc* **15**, 3699–3715 (2020).

341. Chen, Y. *et al.* Broadly neutralizing antibodies to SARS-CoV-2 and other human coronaviruses. *Nat Rev Immunol* **23**, 189–199 (2023).

342. Pagani, I., Ghezzi, S., Alberti, S., Poli, G. & Vicenzi, E. Origin and evolution of SARS-CoV-2. *Eur. Phys. J. Plus* **138**, 157 (2023).

343. Lefrançois, T. *et al.* After 2 years of the COVID-19 pandemic, translating One Health into action is urgent. *Lancet* **401**, 789–794 (2023).

344. Kuhlmeier, E. *et al.* A Risk Factor Analysis of SARS-CoV-2 Infection in Animals in COVID-19-Affected Households. *Viruses* **15**, 731 (2023).

345. Fernández-Figueroa, E. A. *et al.* Evidence of SARS-CoV-2 infection in companion animals from owners who tested positive for COVID-19 in the Valley of Mexico. *Mol Biol Rep* **51**, 186 (2024).

346. Wendling, N. M. *et al.* Transmission of SARS-CoV-2 Delta variant (B.1.617.2) from a fully vaccinated human to a canine in Georgia, July 2021. *Zoonoses Public Health* **69**, 587–592 (2022).

347. Castillo, A. P. *et al.* Evidence of SARS-CoV-2 infection and co-infections in stray cats in Brazil. *Acta Trop* **249**, 107056 (2024).

348. Stranieri, A. *et al.* Absence of SARS-CoV-2 RNA and anti-SARS-CoV-2 antibodies in stray cats. *Transbound Emerg Dis* **69**, 2089–2095 (2022).

349. Jahid, M. J., Bowman, A. S. & Nolting, J. M. SARS-CoV-2 Outbreaks on Mink Farms—A Review of Current Knowledge on Virus Infection, Spread, Spillover, and Containment. *Viruses* **16**, 81 (2024).

350. Barroso, P. & Gortázar, C. The coexistence of wildlife and livestock. *Anim Front* **14**, 5–12 (2024).

351. Karmacharya, D., Herrero-García, G., Luitel, B., Rajbhandari, R. & Balseiro, A. Shared infections at the wildlife–livestock interface and their impact on public health, economy, and biodiversity. *Animal Frontiers* **14**, 20–29 (2024).

352. National Center for Immunization and Respiratory Diseases. Human Infection with Highly Pathogenic Avian Influenza A(H5N1) Virus in Texas | CDC. *CDC* https://www.cdc.gov/ncird/whats-new/human-infection-H5N1-bird-flu.html (2024).

353. Amman, B. R. *et al.* GPS Tracking of Free-Roaming Cats (Felis catus) on SARS-CoV-2-Infected Mink Farms in Utah. *Viruses* **14**, 2131 (2022).

354. Strang, T. *et al.* SARS-CoV-2 wildlife surveillance surrounding mink farms in British Columbia, Canada. *Can Commun Dis Rep* **48**, 252–260 (2022).

355. Gortázar, C. *et al.* The Wild Side of Disease Control at the Wildlife-Livestock-Human Interface: A Review. *Front. Vet. Sci.* **1**, 27 (2015).

356. Nederlof, R. A., de la Garza, M. A. & Bakker, J. Perspectives on SARS-CoV-2 Cases in Zoological Institutions. *Veterinary Sciences* **11**, 78 (2024).

357. Caserta, L. C. *et al.* White-tailed deer (Odocoileus virginianus) may serve as a wildlife reservoir for nearly extinct SARS-CoV-2 variants of concern. *Proceedings of the National Academy of Sciences* **120**, e2215067120 (2023).

358. Sharun, K., Tiwari, R., Saied, A. A. & Dhama, K. SARS-CoV-2 vaccine for domestic and captive animals: An effort to counter

COVID-19 pandemic at the human-animal interface. *Vaccine* **39**, 7119–7122 (2021).

359. Daly, N. First great apes at U.S. zoo receive COVID-19 vaccine made for animals. https://www.nationalgeographic.com/animals/article/first-great-apes-at-us-zoo-receive-coronavirus-vaccine-made-for-animals (2021).

360. Zoetis. Zoetis Donates COVID-19 Vaccines to Help Support the Health of Zoo Animals. https://news.zoetis.com/press-releases/press-release-details/2021/Zoetis-Donates-COVID-19-Vaccines-to-Help-Support-the-Health-of-Zoo-Animals/default.aspx (2021).

361. Morozov, I. *et al.* Preliminary Study on the Efficacy of a Recombinant, Subunit SARS-CoV-2 Animal Vaccine against Virulent SARS-CoV-2 Challenge in Cats. *Vaccines* **11**, 1831 (2023).

362. Hoyte, A. *et al.* Experimental veterinary SARS-CoV-2 vaccine cross neutralization of the Delta (B.1.617.2) variant virus in cats. *Veterinary Microbiology* **268**, 109395 (2022).

363. Chavda, V. P., Feehan, J. & Apostolopoulos, V. A Veterinary Vaccine for SARS-CoV-2: The First COVID-19 Vaccine for Animals. *Vaccines (Basel)* **9**, 631 (2021).

364. Feng, A. *et al.* Transmission of SARS-CoV-2 in free-ranging white-tailed deer in the United States. *Nat Commun* **14**, 4078 (2023).

365. Cool, K. *et al.* Infection and transmission of ancestral SARS-CoV-2 and its alpha variant in pregnant white-tailed deer. *Emerg Microbes Infect* **11**, 95–112 (2022).

366. Bijl, H. & Csányi, S. Fallow Deer (Dama dama) Population and Harvest Changes in Europe since the Early 1980s. *Sustainability* **14**, 12198 (2022).

367. ENETWILD-consortium *et al.* Update of model for wild ruminant abundance based on occurrence and first models based on hunting

yield at European scale. *EFSA Supporting Publications* **19**, 7174E (2022).

368. Williams, E. P. *et al.* Common Themes in Zoonotic Spillover and Disease Emergence: Lessons Learned from Bat- and Rodent-Borne RNA Viruses. *Viruses* **13**, 1509 (2021).

369. Bartak, M., Słońska, A., Bańbura, M. W. & Cymerys, J. SDAV, the Rat Coronavirus—How Much Do We Know about It in the Light of Potential Zoonoses. *Viruses* **13**, 1995 (2021).

370. Lee, M. J. *et al.* Municipal urban rat management policies and programming in seven cities in the United States of America. *Journal of Urban Affairs* **46**, 667–681 (2024).

371. Shrestha, L. B., Foster, C., Rawlinson, W., Tedla, N. & Bull, R. A. Evolution of the SARS-CoV-2 omicron variants BA.1 to BA.5: Implications for immune escape and transmission. *Rev Med Virol* **32**, e2381 (2022).

372. Vogt, A.-C. S. *et al.* Increased Receptor Affinity and Reduced Recognition by Specific Antibodies Contribute to Immune Escape of SARS-CoV-2 Variant Omicron. *Vaccines* **10**, 743 (2022).

373. WOAH. Considerations on monitoring SARS-CoV-2 in animals. (2022).

374. Animal and Plant Health Inspection Service & CDC. *Guidance on Working with Farmed Animals of Species Susceptible to Infection with SARS-CoV-2*. https://www.aphis.usda.gov/sites/default/files/sars-cov-2-mink-guidance.pdf (2023).

375. Rautiainen, E. Notification of a conditional usage permit for COVID-19 subunit vaccine (Furovac) for minks. *European comission* https://food.ec.europa.eu/system/files/2021-12/reg-com_ahw_20211116_mink-fin.pdf (2021).

376. WHO. Environmental surveillance for SARS-CoV-2 to complement other public health surveillance. https://www.who.int/publications-detail-redirect/9789240080638 (2023).

377. Fauci, A. S. It Ain't Over Till It's Over…but It's Never Over — Emerging and Reemerging Infectious Diseases. *New England Journal of Medicine* **387**, 2009–2011 (2022).

378. Aarestrup, F. M., Bonten, M. & Koopmans, M. Pandemics– One Health preparedness for the next. *The Lancet Regional Health - Europe* **9**, 100210 (2021).

379. Banerjee, S. *et al.* Disease-X: Accounting for the unknown. *Health Sci Rep* **6**, e1173 (2023).

380. Mipatrini, D. *et al.* 'Disease X'—time to act now and prepare for the next pandemic threat. *European Journal of Public Health* **32**, 841–842 (2022).

381. Duroseau, B., Kipshidze, N. & Limaye, R. J. The impact of delayed access to COVID-19 vaccines in low- and lower-middle-income countries. *Front Public Health* **10**, 1087138 (2023).

List of scientific publications by Leira Fernández Bastit

As a 1st authr

Fernández-Bastit, L. et al. First Detection of SARS-CoV-2 Delta (B.1.617.2) Variant of Concern in a Dog with Clinical Signs in Spain. *Viruses* **13**, 2526 (2021).

1. Fernández-Bastit, L. et al. Severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) infection and humoral responses against different variants of concern in domestic pet animals and stray cats from North-Eastern Spain. *Transbound. Emerg. Dis*. **69**, 3518–3529 (2022).

2. Fernández-Bastit, L. et al. Susceptibility of Domestic Goat (*Capra aegagrus hircus*) to Experimental Infection with Severe Acute Respiratory Syndrome Coronavirus 2 (SARS-CoV-2) B.1.351/Beta Variant. *Viruses* **14**, 2002 (2022).

3. Fernández-Bastit, L., Vergara-Alert, J. & Segalés, J. Transmission of severe acute respiratory syndrome coronavirus 2 from humans to animals: is there a risk of novel reservoirs? *Curr. Opin. Virol*. **63**, 101365 (2023).

As a co-author

4. Fernández-Bellon, H. et al. Monitoring Natural SARS-CoV-2 Infection in Lions (*Panthera leo*) at the Barcelona Zoo: Viral Dynamics and Host Responses. *Viruses* **13,** (2021).

5. Katsande, P. M. et al. Heterologous Systemic Prime–Intranasal Boosting Using a Spore SARS-CoV-2 Vaccine Confers Mucosal Immunity and

Cross-Reactive Antibodies in Mice as well as Protection in Hamsters. *Vaccines* **10**, 1900 (2022).

6. Usai, C. et al. Agreement and differential use of laboratory methods for the detection and quantification of SARS-CoV-2 in experimentally infected animals. *Front. Microbiol.* **13,** (2022).

7. Barreiro, A. et al. Preclinical evaluation of a COVID-19 vaccine candidate based on a recombinant RBD fusion heterodimer of SARS-CoV-2. *iScience* **26**, 106126 (2023).

8. Gómez, J. C. et al. Exposure to severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) in the endangered Iberian lynx (Lynx pardinus). *Vet. Microbiol.* **290**, 110001 (2024).

9. Cano-Terriza, D. et al. SARS-CoV-2 in Captive Nonhuman Primates, Spain, 2020–2023. *Emerg. Infect. Dis*. **30,** (2024).

Acknowledgments

Vull aprofitar aquest espai per poder agrair a tothom que ha fet possible que hagi pogut realitzar i acabar aquesta tesi doctoral i a tothom que ha format part d'aquesta etapa tan bonica!

Vull donar les gràcies a tota la gent del CReSA que d'una manera o altra m'ha ajudat en algun moment, i que m'han fet molt més fàcil el camí. Primer, gràcies als meus "*veteranos*", sou la part més bonica d'aquesta tesi. Carla, has arribat cap al final d'aquest caminet, però has sigut tot aire fresc i alegria! Ja saps que "la vida no és fàcil", però amb tu ho ha sigut i molt! He tingut moltíssima sort que arribessis. Gràcies a l'Albert *pequeñín*, per els raïms incomptables, per la figura i pels milers de riures junts; et fas estimar molt! Carlooooos, hem tingut moltíssima sort de coincidir en aquesta etapa, no podia haver tingut millor company per tot aquests anys. Tinc molt guardada a la memòria la nostra súper escapada a Belfast (tot i que em deixessis dormint abans de la meva *flash talk,* mentre tu esmorzaves tot el *buffet*). David, solo espero que podamos jugar muchos partidos de pádel juntos y compartir muchos *boldús*, quién sabe si será en Madrid; estoy segura que con tu esfuerzo y empeño vas a sacar una tesis genial!!! Als partits de pàdel no hi pot faltar la Maria, has sigut un gran descobriment per mi i estic molt feliç de l'amistat que hem fet. Lau i Jud, vam arrencar aquest camí juntes i hem viscut coses molt bones i boniques. Em quedo sobretot amb les primeres festes del CReSA juntes, converses infinites i, òbviament, els gins. També vull agrair a tots els antics becaris que ara ja son doctors/es; ens van obrir les portes i ens van posar les coses molt fàcils per començar aquesta aventura: Álvaro, Uxi, Alejo, Perlas, Miguel, Carlos,...I clar, gràcies a tu JR, et diré el meu "sènior" per últim cop, m'has ajudat i ensenyat molt; hem compartit moments de tot tipus, però sobretot, moltes alegries! També,

gràcies al Guille, Rosa Valle i a tot el grup corona. Núr, ets una tia genial i hi ha molt per aprendre de tu! I en general, gràcies a tot l'ambient i família CReSA, que fa molt més fàcils els dies i et fa sentir com a casa. Vull agrair també als col·laboradors de tots els estudis i, sobretot, al Julià Blanco i a tot el seu grup d'Irsi Caixa; he après moltíssim amb vosaltres. Gràcies al Marcel Müller, per deixar-me gaudir de tres mesos a Berlín al seu laboratori, deixar-me aprendre tant a nivell científic i, també, per la seva hospitalitat!

Jefes, Jú i Quim, milions de gràcies per donar-me aquesta oportunitat, per fer-la més divertida, més enriquidora, i per voler que creixi sempre a nivell científic i personal. He tingut moltíssima sort amb vosaltres. Gràcies també per l'oportunitat que m'heu brindat de marxar a fer l'estada a Berlín i sempre estar disposats a obrir-me les portes a noves experiències. M'heu escoltat sempre i durant tots aquests anys no heu deixat mai de posar-me un somriure. Sou uns jefes i unes persones increïbles!

Als meus amics i amigues, que són el meu pilar fonamental. Les nenes Monte de sempre, les meves *we are the milk* i penya tigres, sou vida, alegria i tot el que una persona podria demanar tenir al costat! Les meves súper compis de pis durant aquests anys, Carla i Clau, ha sigut increïble poder-vos tenir al costat. Carlita, la meva germana que m'ha escoltat i recolzat sempre. Noa, a tu també, gràcies per el súper *team* que formem!

Òbviament gràcies a tu, Aniol. Per voler estar sempre al meu costat i, sobretot, de la manera en què ho has fet, des de la primera copa de vi al senyor Vermut. Gràcies per la teva bondat, empatia i generositat. M'ho has posat tot molt fàcil. He tingut i tinc molta sort de tenir-te. Ah! I gràcies per presentar-me en Pol, l'artista de la portada d'aquesta tesi; moltíssimes gràcies Pol!

Unes gràcies infiniiiiiiites als meus pares. Per estar sempre al meu costat, per recolzar-me a cada pas que faig. Tinc uns pares 10. De vosaltres he après i segueixo aprenent moltes coses, sobretot, de minimitzar els problemes i de compartir el millor de mi amb els meus. La meva valentia i fortalesa l'he tret de vosaltres dos. Gràcies per fer possible que hagi arribat fins aquí, a nivell professional i en tots els aspectes. No us podré agrair mai suficientment tot el vostre esforç! Molt important, també, gràcies als meus balulinus!!! Per rebre'm sempre feliços i per tot el carinyo i companyia que donen sempre.

I finalment a tu, Avi. Al meu número 1. A la persona que li dedico aquesta tesi i que dedicaria cada pas i etapa que segueixo. Gràcies per ensenyar-me tant, avi. Els teus consells, la teva mentalitat de voler estar sempre a dalt de tot i no rendir-te i les teves ganes de viure, ho tinc sempre present. M'hagués fet infinitament feliç poder compartir el final de la meva tesi amb tu i poder veure't per abraçar-te i donar-te les gràcies. Tinc clar que vas marxar orgullós de mi i sobretot de la nostra relació. Jo no puc estar més orgullosa de tu. He tingut i tinc el millor avi del món! T'estimo.