

UNIVERSIDADE DE LISBOA
FACULDADE DE MEDICINA VETERINÁRIA



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APPLICATION OF A DECISION TREE MODEL TO PREDICT THE OUTCOME OF
NEUTRALIZATION ASSAYS ON SARS-COV-2 INFECTED DOMESTIC CATS

TERESA DE JESUS PEREIRA CABRAL DA PENHA COUTINHO

ORIENTADOR:

Doutor Virgílio da Silva Almeida

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Faculdade de Medicina Veterinária da Universidade de Lisboa, 08 de janeiro de 2024

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Resumo

APLICAÇÃO DE MODELOS DE DECISÃO PARA PREVER O RESULTADO DOS ENSAIOS DE NEUTRALIZAÇÃO EM GATOS DOMÉSTICOS INFETADOS COM SARS-COV-2

Durante a pandemia de COVID-19 foram reportados casos de gatos domésticos que contraíram o vírus em agregados familiares com indivíduos positivos para o SARS-CoV-2, mantendo-se esses gatos frequentemente assintomáticos.

O objetivo deste estudo foi identificar fatores de risco associados à transmissão do SARS-CoV-2 aos gatos, identificar potenciais focos espaço-temporais de infeção e desenvolver um modelo de decisão para determinar a presença de anticorpos neutralizantes para o SARS-CoV-2 com base nos resultados do ELISA.

Entre maio e outubro de 2021, foram colhidas amostras de sangue de 523 gatos em consultas realizadas no Hospital Veterinário da Faculdade de Medicina Veterinária da Universidade de Lisboa e dos registos médicos, foram extraídos os dados de cada animal. Todas as amostras foram submetidas a testes ELISA e, posteriormente, as amostras positivas foram testadas para anticorpos neutralizantes do SARS-CoV-2.

Dos 523 gatos testados, 17,21% testaram positivo no ELISA e 3,63% no ensaio de neutralização. Nenhuma das características dos gatos investigados revelou uma associação estatística significativa com a positividade da amostra ($p > 0,05$).

Foram identificados 4 focos espaço-temporais usando os resultados do ELISA, e 4 usando os resultados do ensaio de neutralização, com riscos relativos entre 3,11 e 31,31. No entanto, apenas 1 dos focos, composto por 3 amostras positivas no ensaio de neutralização, mostrou significância estatística ($p < 0,05$).

As árvores de decisão desenvolvidas permitem prever o resultado do ensaio de neutralização a partir dos resultados do ELISA. Foram construídos 3 modelos de árvores de decisão, mas a árvore n.º 2 foi a que apresentou os melhores resultados. Quando a OD_{415nm} da *spike* foi superior a 0,992, o rácio entre a OD_{415nm} do RBD e a OD_{415nm} da *spike* igual ou inferior a 1,27 e a OD_{415nm} do RBD superior a 1,8, previu a deteção de anticorpos neutralizantes.

Palavras-chave: SARS-CoV-2, gato, ELISA, neutralização, modelo de decisão.

Abstract

APPLICATION OF A DECISION TREE MODEL TO PREDICT THE OUTCOME OF NEUTRALIZATION ASSAYS ON SARS-COV-2 INFECTED DOMESTIC CATS

During the COVID-19 pandemic instances of cats contracting SARS-CoV-2 in households with COVID-19 positive individuals were reported and these cats often remained asymptomatic. Within this framework, this study's purpose was to identify risk factors linked to the transmission of SARS-CoV-2 to cats, to uncover potential spatiotemporal clusters of infection and to develop a decision-making model to determine the presence of SARS-CoV-2 neutralizing antibodies based on the ELISA outcomes.

Between May and October 2021, blood samples were collected from 523 cats during appointments held at the Veterinary Hospital of the Faculty of Veterinary Medicine, University of Lisbon, and individual data of these cats was extracted from their medical records. All samples were submitted to ELISA tests and, subsequently, the positive samples were tested for SARS-CoV-2 neutralizing antibodies.

Out of the 523 sampled cats, 17.21% tested positive in the ELISA and 3.63% tested positive in the neutralization assay. None of the studied features revealed to have a significant statistical association with sample positivity ($p > 0.05$).

Four spatiotemporal clusters were identified using the results from the ELISA, and 4 using the results from the neutralization assay, with relative risks between 3.11 and 31.31, however, only 1 of the clusters, composed by 3 positive samples from the neutralization assay, showed statistical significance ($p < 0.05$).

The developed decision model trees allow predicting the neutralization outcome from the results of the ELISA test. Three decision models were built but Tree #2 presented the best results, with 100% in all performance metrics, indicating that the result of all investigated samples was correctly predicted. Based on this, when the OD_{415nm} for spike was greater than 0.992, the ratio between the OD_{415nm} of RBD and the OD_{415nm} of spike equal to or lower than 1.27, and the OD_{415nm} for RBD higher than 1.8, it predicted the detection of neutralizing antibodies.

Keywords: SARS-CoV-2, cat, ELISA, neutralization, decision model.

Resumo alargado

APLICAÇÃO DE MODELOS DE DECISÃO PARA PREVER O RESULTADO DOS ENSAIOS DE NEUTRALIZAÇÃO EM GATOS DOMÉSTICOS INFETADOS COM SARS-COV-2

O SARS-CoV-2 é uma doença emergente e, até 5 de maio de 2023, foram notificados mais de 764 milhões de casos, abrangendo os 7 continentes e causando mais de 6,9 milhões de mortes. As doenças virais emergentes são uma das principais ameaças à saúde pública e esta foi mais uma a acrescentar à lista de doenças infecciosas com grandes impactos financeiros e sanitários em todo o mundo. O surto de SARS-CoV-2 é o terceiro surto causado por um coronavírus nos últimos vinte anos.

A origem exata do surto de SARS-CoV-2 permanece pouco clara, mas propõe-se que tenha tido origem em morcegos e que tenha sido transmitido aos seres humanos através de um hospedeiro reservatório intermediário, muito provavelmente um pangolim.

O SARS-CoV-2 pertence à família *Coronaviridae*, cujos membros são vírus com envelope, positivos e de cadeia simples. O genoma é composto por ORFs (*open reading frames*) funcionais, incluindo a proteína *spike* (S), o envelope (E), a membrana (M) e a nucleocápside (N). As proteínas *spike* são glicoproteínas transmembranares responsáveis pela entrada do vírus nas células hospedeiras e, por esse motivo, são o principal alvo para o desenvolvimento de vacinas e antivirais. A entrada nas células hospedeiras depende da ligação da proteína *spike* a um recetor celular, a enzima conversora de angiotensina-2 (ACE2).

A disseminação do vírus de pessoa para pessoa é principalmente causada pela transmissão direta ou indireta através de gotículas respiratórias das mucosas nasais, conjuntivais ou orais, quando as partículas são inaladas ou depositadas nestas membranas mucosas, o que é reforçado pelo contacto próximo entre pessoas. A transmissão depende de diferentes fatores, como o padrão de contacto, o ambiente, a infecciosidade do hospedeiro e fatores socioeconómicos. O SARS-CoV-2 tem um período de incubação de 4 a 11 dias e a libertação do vírus pode ocorrer até 3 semanas após o início dos sintomas, tornando difícil o isolamento e a quarentena dos doentes infetados e dos seus contactos.

O SARS-CoV-2 tem a capacidade de infetar um amplo espectro de mamíferos, causando doenças de diferentes gravidades, desde a constipação comum a doenças respiratórias graves. Os sintomas predominantes são a febre, que é um sintoma-chave para o rastreio da COVID-19, o mal-estar, a falta de ar, a tosse, a dor de cabeça e a fadiga. A evolução clínica depende de fatores relacionados com o hospedeiro e com o vírus, nomeadamente a idade do hospedeiro, as condições médicas subjacentes, a competência imunitária do hospedeiro e a exposição anterior ao coronavírus.

O diagnóstico definitivo pode basear-se na deteção do gene viral, na deteção do antígeno ou na deteção da resposta imunitária do hospedeiro. O método mais utilizado para o diagnóstico da COVID-19 é a pesquisa do genoma viral. Os testes serológicos identificam a presença de uma resposta humoral do hospedeiro ao SARS-CoV-2 através da medição de anticorpos. Os dois tipos diferentes de anticorpos são os anticorpos neutralizantes e os anticorpos não neutralizantes ou de ligação, podendo ambos ser alvo de testes serológicos.

O SARS-CoV-2 tem uma grande propensão para mutações e ações de recombinação, passando por frequentes eventos de mudança de hospedeiro entre humanos e/ou espécies não humanas, e ocorrem eventos de *spill over* com as interfaces humana e animal a serem continuamente alteradas.

O primeiro caso de SARS-CoV-2 num gato ocorreu em março de 2020, na Bélgica, quando o RNA viral do vírus SARS-CoV-2 foi detetado num gato com sinais clínicos digestivos e respiratórios. Este gato pertencia a uma pessoa com uma infeção confirmada pelo SARS-CoV-2. Desconhece-se se os gatos podem transmitir o SARS-CoV-2 aos seres humanos, embora tenham sido notificadas infeções naturais de gatos em agregados familiares positivos para a COVID-19. O RNA do SARS-CoV-2 foi encontrado em amostras respiratórias e/ou fecais de gatos, com e sem sinais clínicos, e alguns estudos também demonstraram que a transmissão de gato para gato pode ocorrer através de gotículas respiratórias. No que diz respeito à deteção de anticorpos, certos estudos, utilizando o ELISA e o ensaio de neutralização, encontraram anticorpos contra o SARS-CoV-2 tanto em gatos inoculados como em gatos expostos. Os gatos infetados com SARS-CoV-2, geralmente permanecem assintomáticos.

O objetivo deste estudo foi identificar os fatores de risco associados à transmissão do SARS-CoV-2 aos gatos domésticos, descobrir potenciais *clusters* espaço-temporais de infeção e desenvolver um modelo de tomada de decisão para determinar a presença de anticorpos neutralizantes contra o SARS-CoV-2 com base nos resultados do ELISA.

Foram recolhidas amostras de sangue durante 6 meses, de 523 gatos observados no Hospital Veterinário da Faculdade de Medicina Veterinária da Universidade de Lisboa. A idade, sexo, código postal do tutor, acesso ao exterior, contactos com outros gatos, estado vacinal, positividade para o vírus da imunodeficiência felina (FIV), vírus da leucemia felina (FeLV) e peritonite infecciosa felina (PIF), e comorbilidades de cada gato amostrado foram obtidos a partir dos registos médicos dos gatos.

A existência de anticorpos IgG contra o SARS-CoV-2 foi avaliada utilizando ELISA e proteínas recombinantes purificadas do SARS-CoV-2 do tipo *spike* e RBD. Foram incluídos um controlo positivo e um controlo negativo e a densidade ótica a 415 nm (OD_{415nm}) foi medida aos 5, 15 e 30 minutos. As amostras com leituras de OD_{415nm} superiores a 0,5, tanto para a *spike* como para o RBD, foram classificadas como positivas.

Para o ensaio de neutralização, foi utilizado um kit SARS-CoV-2 *Surrogate Virus Neutralization Test* (sVNT) para identificar os anticorpos circulantes que bloqueavam a ligação do RBD do SARS-CoV-2 ao recetor ACE2. A OD_{450nm} e a taxa de inibição de cada amostra foram calculadas, e se a taxa de inibição fosse igual ou superior a 30%, revelava a presença de anticorpos neutralizantes do SARS-CoV-2.

Todas as análises foram executadas através de programação R utilizando o RStudio e para avaliar uma possível correlação entre as diferentes categorias de dados clínicos e uma amostra positiva, foi utilizado o teste do qui-quadrado de Pearson.

O pacote do RStudio "FFTrees" foi aplicado para construir um modelo de decisão que permitisse determinar se uma amostra teria um resultado positivo ou negativo no teste para a presença de anticorpos neutralizantes, utilizando apenas as amostras submetidas ao ensaio de neutralização. A identificação dos agrupamentos espaço-temporais foi efetuada utilizando o software SaTScan.

No primeiro ELISA, dos 523 gatos testados, 119 animais (22,75%) tiveram resultados positivos, 334 (63,86%) resultados negativos e os restantes 70 gatos (13,39%) resultados inconclusivos. Como passo de confirmação, as 119 amostras positivas foram testadas num ensaio ELISA independente e noventa amostras foram positivas (17,21%; 90/523).

Nestas 90 amostras, foram detetados anticorpos com atividade neutralizante contra o SARS-CoV-2 em dezanove amostras (21,11%; 19/90), o que significa que, entre os 523 gatos, 3,63% tinham anticorpos com capacidade para neutralizar o RBD da variante de *wild type* do SARS-CoV-2.

Com base nos resultados do teste do qui-quadrado de Pearson, não houve evidência que sugerisse uma associação estatística entre qualquer uma das características ou comportamentos avaliados dos gatos e a positividade no ensaio ELISA independente ou no ensaio de neutralização.

Para a avaliação dos *clusters* espaço-temporais, foram realizadas duas análises distintas. A primeira utilizou os resultados obtidos no ELISA independente, revelando 4 clusters e, embora alguns exibissem riscos relativos (RR) mais altos, nenhum deles alcançou significância estatística com base nos valores de p. A segunda análise utilizou os resultados dos sVNT e foram encontrados mais 4 clusters. Apenas o Cluster #1, localizado na Amadora, composto por 3 amostras positivas, com um raio de 0 km, RR de 31,31 e valor de p de 0,02, demonstrou significância estatística.

As árvores de decisão incorporaram três parâmetros de decisão decorrentes dos resultados do ELISA, incluindo os valores de OD_{415nm} para RBD e para a *spike*, bem como o rácio entre a OD_{415nm} para RBD e para *spike*. Foram desenvolvidas três árvores de decisão, mas a Árvore #2 foi a que apresentou valores excepcionais em todas as principais métricas de avaliação, classificando com precisão os eventos e garantindo um mínimo de falsos positivos e falsos

negativos. O primeiro critério de avaliação foi a OD_{415nm} para a *spike*. Se a medição fosse igual ou inferior a 0,992, esperar-se-ia um resultado negativo no teste de neutralização. O segundo critério considerou o rácio entre a OD_{415nm} do RBD e a OD_{415nm} da *spike*. Era de esperar que o teste de neutralização desse um resultado negativo quando este rácio fosse superior a 1,27. O último critério aplicado referia-se à OD_{415nm} do RBD e, quando o valor era inferior ou igual a 1,8, o resultado previsto para o teste de neutralização também seria negativo. Por outro lado, previa-se que as amostras apresentassem um resultado positivo no sVNT se a sua OD_{415nm} para RBD fosse superior a 1,8.

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List of abbreviations

2019-nCoV	2019 Novel Coronavirus
ABTS	2,2'-Azinobis (3-ethylbenzothiazoline-6-sulfonic acid)
ACE	Angiotensin-converting enzyme
ACE2	Angiotensin-converting enzyme 2
AFCD	Agriculture, Fisheries and Conservation Department
Ag-RDT	Antigen-detection rapid diagnostic test
ALT	Alanine aminotransferase
ARDS	Acute Respiratory Distress Syndrome
AST	Aspartate aminotransferase
BatCoV	Bat Coronavirus
BSA	Bovine serum albumin
CBC	Complete blood count
CLIA	Chemiluminescence Immunoassays
CoV	Coronavirus
COVID-19	Coronavirus Disease 2019
CT	Computer tomography
DNA	Deoxyribonucleic acid
E	Envelope protein
ECDC	European Centre for Disease Prevention and Control
ELISA	Enzyme-Linked Immunosorbent Assay
fAPN	Feline aminopeptidase-N
FASFC	Federal Agency for the Safety of Food Chain
FCoV	Feline Coronavirus
FECV	Feline Enteric Coronavirus
FeLV	Feline Leukemia Virus
FIP	Feline Infectious Peritonitis
FIPV	Feline Infectious Peritonitis Virus
FIV	Feline Immunodeficiency Virus
hACE2	Human angiotensin-converting enzyme 2
HRP	Horse radish peroxidase
HRP-RBD	HRP conjugated recombinant SARS-CoV-2 RBD wild type fragment
IgA	Immunoglobulin A
IGC	Instituto Gulbenkian da Ciência
IgG	Immunoglobulin G
IgM	Immunoglobulin M

km	Kilometres
LFA	Lateral Flow Assay
M	Membrane protein
MAL	Metropolitan area of Lisbon
MERS-CoV	Middle East Respiratory Syndrome Coronavirus
N	Nucleocapside protein
NK	Natural killer
OD	Optical density
OR	Odds ratio
ORF	Open reading frame
PBS	Phosphate buffered saline
PCR	Polymerase Chain Reaction
PPE	Personal Protective Equipment
RBD	Receptor binding domain
RNA	Ribonucleic acid
RR	Relative risk
RT	Room temperature
RT-PCR	Reverse Transcription Polymerase Chain Reaction
RT-qPCR	Quantitative Reverse Transcription Polymerase Chain Reaction
S	Spike protein
SARS-CoV	Severe Acute Respiratory Syndrome Coronavirus
SARS-CoV-2	Severe Acute Respiratory Syndrome Coronavirus-2
Se	Test sensibility
Sp	Test specificity
TD	Transmembrane domain
TH1	Type 1 T helper
TMB	Tetramethylbenzidine
TMPRSS2	Type II transmembrane serine protease 2
WAHIS	World Animal Health Information System
WHO	World Health Organization
WOAH	World Organization for Animal Health

1. LITERATURE REVIEW

1.1. Pandemic's evolution

SARS-CoV-2 is an emerging disease, defined by the WOAHA Terrestrial Animal Health Code as “a new occurrence in an animal of a disease, infection or infestation, causing a significant impact on animal or public health resulting from a change of a known pathogenic agent or its spread to a new geographic area or species or from a previously unrecognized pathogenic agent or disease diagnosed for the first time” (WOAHA 2021a).

Although the true number of cases is likely to be greater than the reported, until 5 May 2023 more than 764 million cases have been reported, reaching all 7 continents and causing more than 6,9 million deaths (Zhang and Holmes 2020; WHO 2023b). In the early phase of the pandemic, the cases were linked to the Huanan Seafood Wildlife Market in Wuhan, where mammalian species were present at the time of the outbreak (Li et al. 2020). However the majority of cases was caused by the transmission between people, even though the zoonotic origin is very likely (Zhang and Holmes 2020).

On the 31st of December 2019, the WHO China Country Office issued the public health alert after notifying the cases. On January 7, 2020, the new type of coronavirus was isolated and 5 days later, the genetic sequence shared with WHO and other countries. The first case outside of China happened in Thailand. By January 20, the WHO acknowledged human-to-human transmission and on the 30th of January, WHO declares SARS-CoV-2 outbreak a Public Health Emergency of International Concern (The College of Physicians of Philadelphia 2020).

In mid-January 2020, WHO temporarily named the new virus as the 2019 novel coronavirus (2019-nCoV), however, one month later, this name was replaced by coronavirus disease 2019 (COVID-19). On the same day, the International Committee on Taxonomy of Viruses named the virus as Severe Acute Respiratory Syndrome Coronavirus-2 (SARS-CoV-2) (Sun et al. 2020).

The World Health Organization (WHO) Director-General announced on 5 May 2023 that “COVID-19 is now an established and ongoing health issue which no longer constitutes a public health emergency of international concern”, starting the transition process to the long-term management of the virus alongside other infectious diseases (WHO 2023a).

1.2. Previous coronavirus outbreaks

Emerging viral diseases are one of the major threats to public health and the novel coronavirus is just one more to add to the list of infectious diseases with major financial and health impacts all over the world. SARS-CoV-2 outbreak is the third outbreak caused by a coronavirus in the last twenty years.

In 2002, the first Severe Acute Respiratory Syndrome Coronavirus (SARS-CoV) outbreak started in the province of Guangdong, China, and spread through 29 countries, summing 8098 reported cases and 774 deaths. It is believed that this outbreak was due to a transmission from bats, to civets, to humans (Wang and Eaton 2007). SARS-CoV spread until 2004 and after this year there were no more reports of infections (Peiris et al. 2003; Wang and Eaton 2007; D.S. Hui et al. 2020; Zhai et al. 2020; Hussain et al. 2021).

Ten years later, in Saudi Arabia, another coronavirus outbreak emerged, the Middle East Respiratory Syndrome Coronavirus (MERS-CoV). This time, around 2500 laboratory-confirmed cases and 866 deaths in 27 countries were reported to WHO. MERS-CoV was transmitted to humans by dromedary camels, which were subsequently infected by bats.

Bats are suspected to be the primary reservoir host for both SARS-CoV and MERS-CoV just like in other zoonotic viruses. Although unlike SARS-CoV, MERS-CoV has emerged and re-emerged through sporadic cases, clusters and nosocomial outbreaks (D.S. Hui et al. 2020; Hussain et al. 2021).

1.3. Probable origin

The exact origin of the outbreak remains unclear, however it is proposed that SARS-CoV-2 originated in bats and was transmitted to humans through an intermediate reservoir host, a pangolin most likely (Wardeh et al. 2021).

Zhou et al. (2020) showed that SARS-CoV-2 whole genome is 96.2% identical to a bat coronavirus (BatCoV RaTG13) detected in *Rhinolophus affinis*, from Yunnan Province. In this study, the samples were obtained from patients with severe pneumonia at the start of Wuhan outbreak and the majority of the patients were linked to the seafood market (Maurin et al. 2021). Because SARS-CoV and MERS-CoV are transmitted through intermediate hosts, it is suggested that also SARS-CoV-2 passed into other animals before reaching humans.

The pangolin coronavirus shares 91.2% whole-genome identity with SARS-CoV-2, being the second closest relative to SARS-CoV-2, behind BatCoV RaTG13, and sharing 90.55% similarity with it. The pangolin samples were obtained from the lung of two dead Malayan pangolins (*Manis javanica*) presenting frothy liquid in the lungs and pulmonary fibrosis. These findings support the theory proposed for the probable origin of SARS-CoV-2 by T. Zhang et al. (2020) and Zhou et al. (2020).

1.4. Coronavirus

The Severe Acute Respiratory Syndrome Coronavirus-2, responsible for the pandemic, belongs to the *Nidovirales* class, *Coronaviridae* family and *Orthocoronavirinae* subfamily (King et al. 2012). The members of *Coronavirinae* are enveloped, positive single-stranded viruses with a fringe of petal-shaped surface projections.

Coronaviruses (CoV) are a group of diverse viruses with the potential to infect a lot of different animal species and causing mild to severe respiratory infections in humans (Hu et al. 2020). CoV are homogeneous in size, distinctively spherical and their nucleocapsid has helical form designed to create a compact structure tighten to the envelope (King et al. 2012; Cui et al. 2019).

SARS-CoV-2 belongs to the *Betacoronavirus* genus, now called Sarbecovirus, being the third known zoonotic coronavirus disease that belongs to the Sarbecovirus cluster (Sun et al. 2020). Being a betacoronavirus, SARS-CoV-2 has 79% and 50% similarity with SARS-CoV and MERS-CoV genome sequence identity, respectively. The genome of betacoronaviruses is composed by six functional open reading frames (ORFs) organized from 5' to 3' in replicase (ORF1a/ORF1b), spike (S), envelope (E), membrane (M) and nucleocapsid (N) (Figure 1). In between the structural genes, there are seven presumed ORFs encoding accessory proteins. The SARS-CoV-2 protein is formed by 1.273 amino acids, sharing around 77% of amino acid sequence similarity with bat and human SARS-CoV (Hu et al. 2020; Nikolich-Zugich et al. 2020).

Viral particle

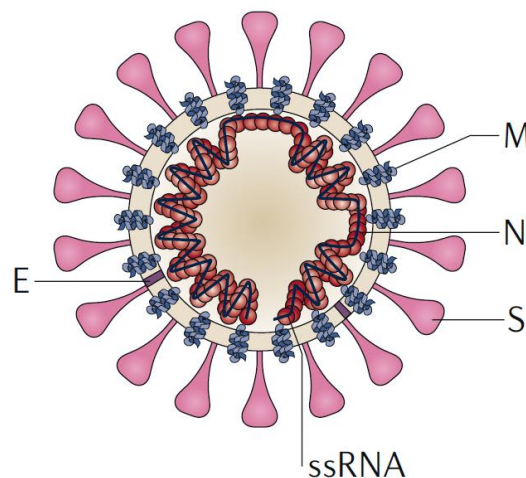


Figure 1. The coronavirus particle. Adapted from Cui et al. (2019).

The coronavirus is a spherical and enveloped particle with a diameter between 100-160nm. The genome is a positive and single strand of RNA (ssRNA) which encodes the envelope glycoproteins spike (S), the envelope (E), the membrane (M) and the nucleocapsid (N).

The S proteins are transmembrane glycoproteins responsible for the virus entry into host cells and for that reason they are the primary target for vaccines and antivirals development (Alenquer et al. 2021). The spike proteins create homotrimers protruding in the viral surface and are composed by two functional subunits: the distal one (S₁) is related with the binding to the host cell receptor and the second one (S₂) anchored to the membrane (Figure 2) and responsible for the fusion of the viral and cellular membranes (Walls et al. 2020). The

entry into host cells relies on binding the S1 unit to a cellular receptor, the angiotensin-converting enzyme 2 (ACE2), and it requires S protein priming by host cell proteases, furin and type II transmembrane serine protease 2 (TMPRSS2), causing the S protein cleavage in two different sites: S1/S2 and S2', respectively. This succession of actions allows the viral and host cell membranes fusion and the connection between the SARS-2-S and ACE2 determines the transmissibility. During the perfusion, the receptor binding domain (RBD) alternates its conformation between open and closed (Hoffmann et al. 2020; Alenquer et al. 2021).

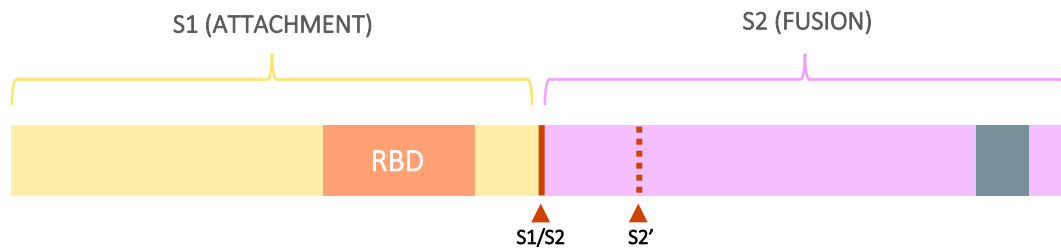


Figure 2. Schematic illustration of SARS-CoV-2 spike protein. Adapted from Hoffmann et al. (2020).

The SARS-S is formed by two units: the S1 (distal) unit which is responsible for the attachment of the virus to the host cells by the S protein and the S2 unit which allows the fusion of the viral membrane and the cellular membrane. The S1 unit includes the receptor binding domain (RBD) and the S2 unit includes the transmembrane domain (TD). The S protein cleavage by furin and TMPRSS2 happens in the S1/S2 and S2' sites, respectively.

1.5. SARS-CoV-2 in humans

1.5.1. Transmission dynamics

In the beginning of the SARS-CoV-2 outbreak, some events such as a cruise ship in Japan, Italian and Austrian skiing resorts and religious gatherings in South Korea, strongly contributed for the rapid spread of the virus. In comparison with SARS-CoV and MERS-CoV, the new coronavirus proved to be more transmissible but less deadly (Petersen et al. 2020). Even though, both SARS-CoV and SARS-CoV-2 have preference with the same receptor, the structural differences in SARS-CoV-2 surface proteins make the bind to the ACE2 receptor stronger (Cevik et al. 2020). These 2 aspects combined with the broad symptoms caused by SARS-CoV-2 and the long incubation period considerably challenged its control and eradication (Petersen et al. 2020).

The spread of the virus from person to person is mostly caused by direct or indirect transmission via respiratory droplets from nasal, conjunctival or oral mucosa, when the particles are inhaled or deposited in these mucous membranes, which is enhanced by close contact between people without personal protective equipment (PPE) (Rabenau et al. 2005; Cevik et al. 2020). The transmission relies on different factors, such as the contact pattern, environment, infectiousness of the host and socioeconomic factors (Cevik et al. 2020).

The SARS-CoV-2 replication competence and cellular tropism to the human respiratory tract and extrapulmonary tissue and cells makes the airways and the conjunctival epithelium probable gateways of infection. Due to its strong replication in human bronchus with alveolar epithelium infection, the transmission among humans is very efficient (K.P.Y. Hui et al. 2020). Although this is the most common way of transmission, the virus can also be transmitted by aerosol, fomites and faecal-oral via, which have been reported as the origin of several cases (Rabenau et al. 2005; Nikolich-Zugich et al. 2020). A recent study showed that SARS-CoV-2 remains viable in aerosols for hours and in fomites for days (van Doremalen et al. 2020). Having this in account, thermal and chemical inactivation should be put into practice in order to improve laboratory safety (Rabenau et al. 2005).

SARS-CoV-2 has an incubation period from 4 to 11 days and viral shedding can occur until up to 3 weeks after symptom onset, making isolation and quarantine of infected patients and their contacts difficult, as it must be implemented as soon as possible (Table 1). In severe cases of COVID-19, the viral load is higher and lasts longer in the lower respiratory tract of the patients (Petersen et al. 2020).

Table 1. Comparison of SARS-CoV-2 and SARS-CoV characteristics. Adapted from Petersen et al. (2020).

	SARS-CoV-2	SARS-CoV
Transmissibility (R_0)	2-5	2-4
Incubation period	4-12 days	2-7 days
Interval between symptom onset and maximum infectivity	0 days	5-7 days
Proportion of mild illness	High	Low
Proportion of patients requiring hospitalization	Low (20%)	High (>70%)
Proportion of patients requiring intensive care	Very low (1/16.000)	High (40/100)
Proportion of deaths in people under 65 years old out of all deaths	0.6-2.8%	Unknown

1.5.2. Pathogenesis and clinical signs

Coronaviruses have the ability to infect a wide spectrum of mammals causing diseases of different severities, ranging from common cold-like to severe respiratory diseases, as it is the case of SARS-CoV-2. The accessory proteins encoded by the ORFs that shape the virus play an active role in the immune evasion and inflammation, inhibiting type I interferons, inducing apoptosis, modulating cellular DNA synthesis, inducing chemokine production and stimulating inflammation routes (Nikolich-Zugich et al. 2020).

The ACE2 presence inhibits acute lung injury so the pathogenesis is enhanced by the downregulation of ACE2 following viral entry. This decrease of action caused by the binding

between the virus and the host cell receptor (ACE2 receptor) leads to an increase in angiotensin II by ACE which, in turn, stimulates type 1a receptors, increasing lung vascular permeability and potentiating lung pathogenesis (Cevik et al. 2020; Nikolich-Zugich et al. 2020).

Non-specific symptoms like fever, myalgia, headache and dyspnoea are due to the active replication and release of the virus in lung cells. Besides the lungs, SARS-CoV-2 has affinity for the upper respiratory tract and conjunctiva, infecting ciliated, mucus-secreting and club cells of bronchial epithelium, type 1 pneumocytes in the lung and the conjunctival mucosa (Cevik et al. 2020; K.P.Y. Hui et al. 2020). The broad locations of ACE2 receptors over different tissues and organs, such as the intestine, endothelial cells present in the kidneys and blood vessels, contribute for the wide range of symptoms and explaining possibly the gastrointestinal symptoms and cardiovascular complications reported (Cevik et al. 2020; Huang et al. 2020).

Following viral entry, the first inflammatory reaction calls specific T cells to the infection site, eliminating the infected cells before viral spreading. In most cases, this leads to recovery. However, SARS-CoV-2 is known for its broad clinical picture, including asymptomatic, mild and severe disease (American Thoracic Society 2020; Cevik et al. 2020; Petersen et al. 2020). In patients suffering severe illness, the virus induces an aberrant host immune response. Some histologic findings comprise bilateral diffuse alveolar damage, interstitial mononuclear inflammatory infiltrates, and desquamation consistent with Acute Respiratory Distress Syndrome (ARDS), formation of hyaline-membrane and presence of mucus plugs with fibrinous exudate in the respiratory tract. This last mentioned feature results probably from the accumulation of pro-inflammatory cytokines in the lungs, potentially causing in lung parenchyma damage (Cevik et al. 2020). Moreover, the illness may evolve to septic shock and multi-organ dysfunction because of the tissue damage triggered by the accumulation of cytokines and exacerbation of the immune response in the organs or due to cytokine release syndrome leading to capillary leak, formation of thrombus and organ dysfunction (Cevik et al. 2020). In summary, the responses based on pro-inflammatory cytokine activity perpetuate the pathogenesis and contribute for the severity of the disease.

The predominant symptoms are fever, which is a key symptom for COVID-19 screening, malaise, shortness of breath, cough, headache and fatigue. Gastrointestinal symptoms such as diarrhoea, nausea and vomit may occur but are less frequent (American Thoracic Society 2020; Huang et al. 2020; Nikolich-Zugich et al. 2020). The clinical development relies on factors related to the host and to the virus, namely host age, underlying medical conditions, host-immune competence and previous exposure to coronavirus (Table 2) (Cevik et al. 2020).

Table 2. Risk factors associated with severe disease cases, intensive care necessity and mortality (Cevik et al. 2020; Nikolich-Zugich et al. 2020; Zheng et al. 2020).

Comorbidity or underlying medical conditions	Presentation on admission
• Older age (> 70 years) • Sex (men) • Hypertension • Cardiovascular disease • Chronic obstructive pulmonary disease • Diabetes • Obesity • Immunodeficiency	• High fever ($\geq 39^{\circ}\text{C}$) • Dyspnoea

1.5.3. Diagnosis

SARS-CoV-2 induces a very heterogeneous clinical presentation, varying from asymptomatic individuals to severe cases and sometimes death, makes the diagnosis very challenging and, for these reasons, the diagnosis exclusively based on the clinical appearance is unreliable. The serious implications that a wrong diagnosis might have in the individuals' lives reinforce the need of complementary tests with good specificity and sensibility to obtain a definitive diagnosis, which may be based on the detection of the viral gene, antigen's detection or the host's immune response (Goudouris 2021; Yüce et al. 2021).

1.5.3.1. Viral genome detection method

The most commonly used method for COVID-19 diagnosis are viral genome detection tests, however, to perform these tests it is necessary technology available only on medical diagnostic laboratories and research centres. Currently, the gold standard for COVID-19 diagnosis is reverse transcription polymerase chain reaction (RT-PCR) due to its reliability, relatively fast technique, relatively fast results and ability to quantify viral genomic portions instead of secondary biomarkers, such as antigens or antibodies (Goudouris 2021; Yüce et al. 2021). Different kits may use different regions of the SARS-CoV-2 genome for detection depending on the manufacturer. Virus mutations can lead to false-negative results. Other factors, such as timing of the sample collection, sample collection technique, type of sample and disease severity affect the test result (Goudouris 2021).

RT-PCR can test samples of different origins including, oropharyngeal swabs, nasopharyngeal swabs, nasal swabs, upper and lower respiratory tract aspirates, bronchoalveolar lavage, sputum, anal swabs, urine samples and blood samples (W. Zhang et al. 2020; Zheng et al. 2020; Goudouris 2021; Yüce et al. 2021). Different studies showed that the viral load dynamics varies according to the biological sample and the disease severity. Xu et al. (2020), Zheng et al. (2020) and Zhang et al. (2020) reported that the virus is detectable

in stool samples for longer than in respiratory samples. Therefore, anal swabs should be considered during epidemics or pandemics. Zheng et al. (2020) described in patients with severe illness, a longer persistence of SARS-CoV-2 in respiratory samples, a higher viral load and a later shedding peak comparing with patients with mild disease.

Regardless of the method, RT-PCR never achieves maximum sensitivity (Se) and specificity (Sp). Moreover, RT-PCR can only be made in laboratories equipped with specialized instruments and a limited number of tests may be performed daily. Therefore, the use of this technique for rapid screening or surveillance is not suitable (Yüce et al. 2021).

1.5.3.2. Antigen detection methods

The antigen tests came as an alternative to the molecular tests to cope with the challenges that most countries faced in increasing testing capacity, delivering results in a short period and finding supplies and reagents. These difficulties have a key impact in the individuals' isolation and, consequently, in the pandemic's evolution (Peeling et al. 2021).

Unlike PCR based tests, the antigen detection methods identify SARS-CoV-2 viral components, such as spike proteins and membrane proteins. On the other hand, they share with PCR based tests the capacity of detecting active infections and unlike serological tests they do not recognize recovered individuals (Peeling et al. 2021; Yüce et al. 2021).

Currently, antigen assays resort to two different techniques: lateral flow assay (LFA) and ELISA. The most used is a single use, lateral flow assay allowing for a rapid diagnosis, but it has a lower Se than the ELISA. Furthermore, the ELISA has the advantage of testing 96 samples at once.

The available antigen-detection rapid diagnostic tests (Ag-RDTs) anywhere, processed and read in small portable devices, using nasal or nasopharyngeal swabs as samples, and the results are available within 15 to 20 minutes. These kits offer a rapid and cheap detection of SARS-CoV-2 (Peeling et al. 2021; Yüce et al. 2021).

Ag-RDTs performance is enhanced in individuals with high viral load and when performed in the early stage of infection. It is recommended that only tests with at least 80% Se and 97% Sp should be used. In individuals with COVID-19 like symptoms or at high risk of infection, like contacts of confirmed cases and health-care workers, Ag-RDTs should be used, especially when molecular tests are not available. One of the most critical factors for the test performance is the proper collection of respiratory fluids samples as inadequate sampling may result in false-negative results (Peeling et al. 2021; WHO 2021).

1.5.3.3. Antibody detection methods

The purpose of the serological tests is to identify the presence of a host's humoral response to SARS-CoV-2 through the measurement of virus proteins specific antibodies, also

called immunoglobulins (Ig), using enzyme-linked immunosorbent assay (ELISA), chemiluminescence immunoassays (CLIA), rapid diagnostic or neutralization assay techniques (Ghaffari et al. 2020; Goudouris 2021; Yüce et al. 2021). Antibodies are glycoproteins produced by the immune system towards specific pathogens, in response to infection or immunizations, and that bind to the pathogens, neutralize them, or prepare them for phagocyte intake and destruction (A Janeway Jr et al. 2001). Although the priority response to the virus is a robust cellular response by the action of NK cells and T lymphocytes, the cellular response also plays an important role in the immune response and even in the pathogenesis of COVID-19, facilitating the virus penetration into cells (Ghaffari et al. 2020; Goudouris 2021; Yüce et al. 2021).

Each antibody has sites that can attach to a specific antigen and there are two different types of antibodies: neutralizing antibodies and non-neutralizing or binding antibodies. Both types may be targeted in serological tests; however, they have different functions. The binding antibodies signal the presence of a pathogen but do not interfere with its infectivity, while neutralizing antibodies block the pathogen entry into cells (Liu and Rusling 2021; Yüce et al. 2021). Antibodies targeting the spike protein, where the receptor-binding domain (RBD) is situated, have a high specificity for SARS-CoV-2 and their levels are correlated with their capacity of virus neutralization. On the contrary, as referred above, antibodies against other virus proteins may promote COVID-19 pathogenesis (Goudouris 2021).

IgM is the first antibody produced in case of infection and it is recognized from the fifth day of symptoms onset, being considered an indicator of early-stage infection (Figure 3).

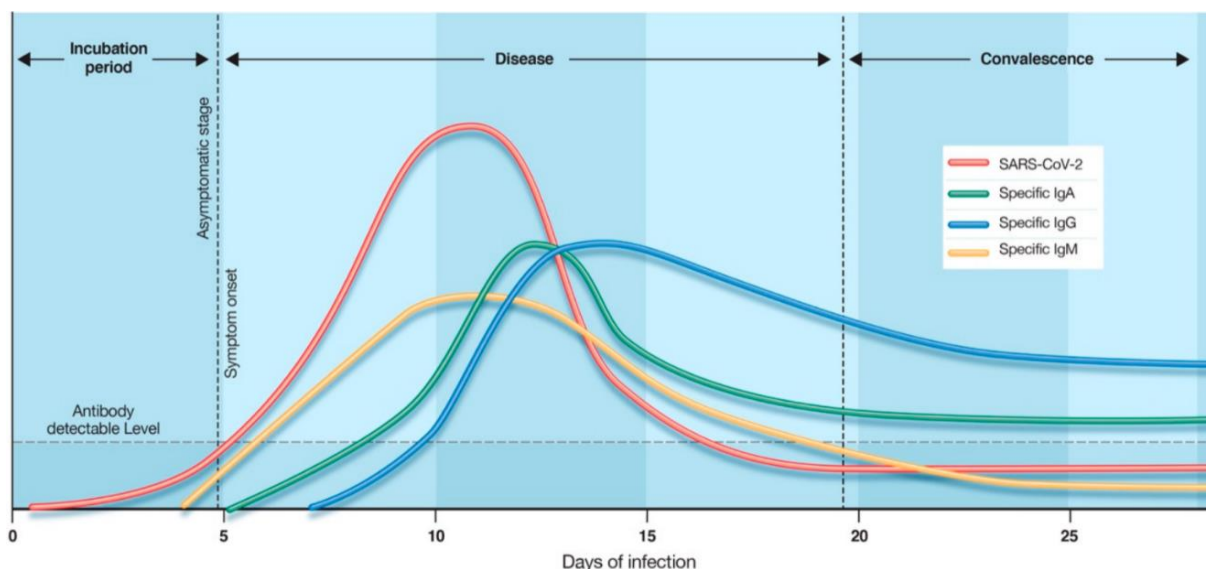


Figure 3. Time kinetics of COVID-19 antibody response. Adapted from Ghaffari et al. (2020).

Following IgM, the second antibody produced is IgG, which is the most common and abundant antibody in the serum. IgG start to be detectable on day 10 of symptomatology and

more significantly from day 14 onwards. IgG marks a current or prior infection and can persist in the body for more than 3 months. IgM and IgA have a similar specificity against SARS-CoV-2, being both lower than the specificity of IgG (Ghaffari et al. 2020; Goudouris 2021; Liu and Rusling 2021; Yüce et al. 2021).

Antibody detection methods can provide useful information and have multiple advantages comparing to viral genome and antigen detection tests, even though they are not able to confirm the presence of the virus. Having such a wide window of detection (Figure 4) serological tests can play a supplementary but very important role in the diagnosis: (1) suspected cases of COVID-19 with a negative viral RNA test, (2) assessment of a previous infection, (3) epidemiological surveillance and risk management, (4) monitoring of the immune responses to describe the disease course, (5) measuring degree and duration of immunity, (6) development and evaluation of vaccines, (7) identification of COVID-19 geographical clusters (Liu and Rusling 2021).

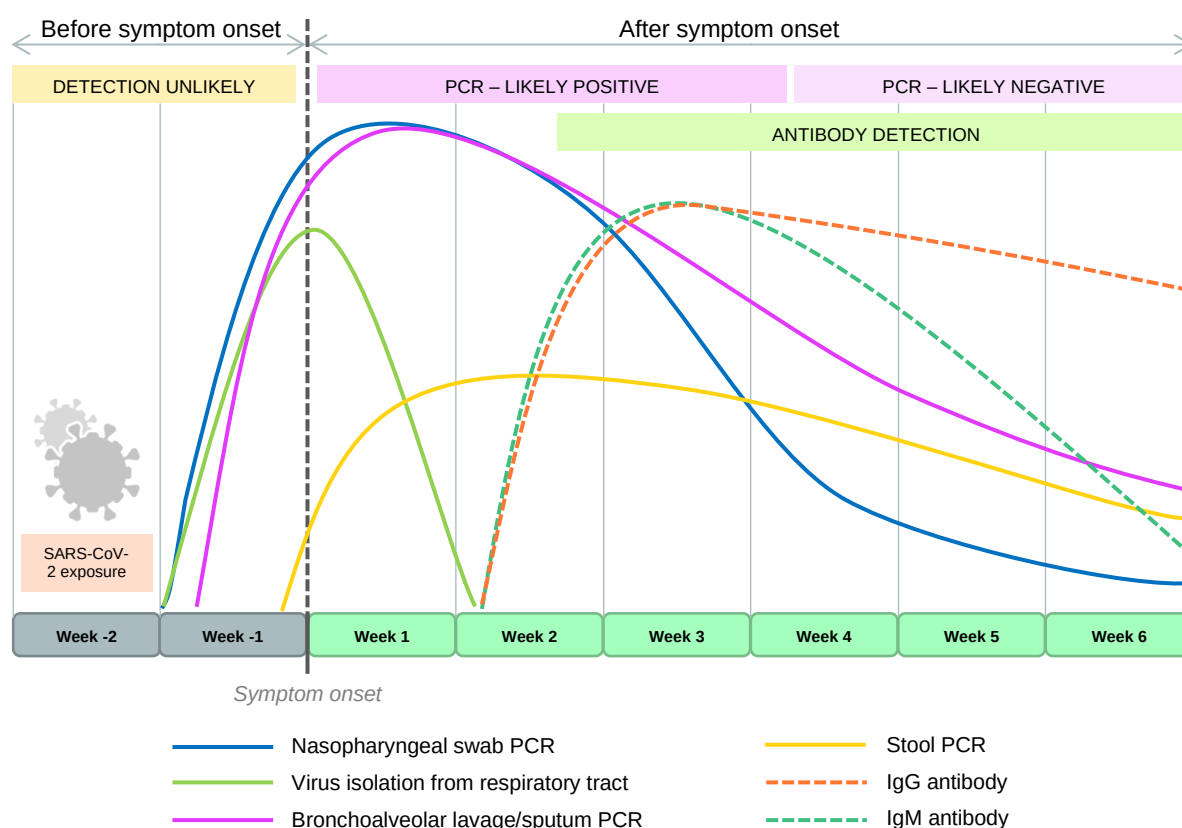


Figure 4. Estimated window for detection of SARS-CoV-2 using different diagnostic tests. Adapted from Sethuraman et al. (2020).
The estimated time windows should be considered approximations due to the high variability of values. PCR indicates polymerase chain reaction.

1.5.3.4. Support tests

The diagnosis of COVID-19 can use some complementary tests, such as laboratory tests and imaging tests. With these, it is possible to reveal clinical manifestations, complications and, eventually, predict future difficulties based on the presence of risk factors (Goudouris 2021).

The complementary laboratory tests requested as routine for COVID-19 patients include a complete blood count (CBC), biochemical factors analysis, coagulation parameters investigation and assays for inflammatory markers. In CBC, most individuals present lymphopenia and thrombocytopenia related to a worse prognosis, although there might be an increase in the neutrophils count (American Thoracic Society 2020; Pourbagheri-Sigaroodi et al. 2020; Goudouris 2021). Lymphopenia is caused by the virus cytopathic action, provoking apoptosis and suppression of the bone marrow (Goudouris 2021). In the biochemical factors, elevated creatinine, AST, ALT and total bilirubin have been reported in patients with SARS-CoV-2 (American Thoracic Society 2020; Pourbagheri-Sigaroodi et al. 2020). As for the coagulation parameters, D-dimer and prothrombin time are usually elevated, which can cause coagulopathy and thromboembolic complications. In the inflammation markers assessment, it's common to find high values of C-reactive proteins, ferritin and procalcitonin (American Thoracic Society 2020; Pourbagheri-Sigaroodi et al. 2020; Goudouris 2021).

Because etiological detection methods are not 100% reliable, imaging tests for COVID-19 diagnosis have shown to be relevant. Even though, these tests are not specific for SARS-CoV-2, when accompanied by a history of contact with a positive case and/or clinical signs compatible with COVID-19, they are very useful (Goudouris 2021). Chest X-rays may show bilateral ground glass opacities along with sparse consolidations, mostly in the peripheral, posterior and lower lung lobes (American Thoracic Society 2020; Nikolich-Zugich et al. 2020; Goudouris 2021). Chest computer tomography (CT) is more sensitive than chest X-rays and may evidence multifocal, bilateral, peripheral or subpleural ground glass opacities with or without consolidation and pulmonary crazy paving (American Thoracic Society 2020; Goudouris 2021). B-lines, consolidations with air bronchograms and pleural line thickening can also be shown through pulmonary ultrasonography (American Thoracic Society 2020; Goudouris 2021).

1.6. SARS-CoV-2 in animals

1.6.1. Zoonotic origin

Coronaviruses belong to a complex group of viruses capable of causing disease in a wide spectrum of species (Stout et al. 2020). A study shown that SARS-CoV-2 is 96% identical to a bat coronavirus (BatCoV RaTG13) and that also the ACE2 receptor is used to enter the

cell, suggesting that SARS-CoV-2 RBD mediates the infection not only in humans, but also in animals as ACE2 is present in almost all vertebrates (Zhou et al. 2020). Besides the probable origin from bats, it is also proposed that the virus shifted from bats to humans through an intermediate reservoir host, Malayan pangolin sharing 90.55% and 91.02% identity of overall genome sequence with BatCoV RaTG13 and SARS-CoV-2, respectively (Andersen et al. 2020; T. Zhang et al. 2020; Alexander et al. 2020).

SARS-CoV-2 has a great propensity for mutations and recombination actions, undergoing frequent host-shifting events between humans and/or non-human species, causing various changes to the infected cells and tissues (Ghai et al. 2021; Wardeh et al. 2021). For virus spill over to happen, it is need an interspecies contact, virus binding to the new host's cells, cell entry and replication, and the virus must be able to evade the host's immune system. Even with all these required conditions, spill over events do occur and the human and animal interfaces are continuously shifting (Neff 2021).

Just like with other zoonotic diseases, applying a One Health approach is an effective way to prevent and control the disease's spread, to diminish the risks of future zoonosis emergence and to avoid possible spill over events back to humans (Ghai et al. 2021).

1.6.2. Reported cases in animals

Animal infections arising from close contact between people and animals are a reflection of the characteristics of this zoonosis, as well as its global spread and vulnerability of certain species to infection (WOAH 2022a). On the other hand, close contact with infected animals, has been shown to be a possible source of infection in humans (Oreshkova, Molenaar, Vreman, Harders, Oude Munnink, et al. 2020).

The first case of SARS-CoV-2 in animals reported to the World Organization for Animal Health (WOAH) was a dog from Hong Kong on the 29th of February 2020. Since then, another 34 countries reported animals cases, summing a total of 663 outbreaks affecting 19 different species, including cats, dogs, mink, otter, ferrets, lions, tigers, pumas, snow leopards, gorillas, white-tailed deer, fishing cat, Binturong, South American coati, spotted hyena, Eurasian lynx, Canada lynx, hippopotamus and hamster (Figure 5) (WOAH 2022a).

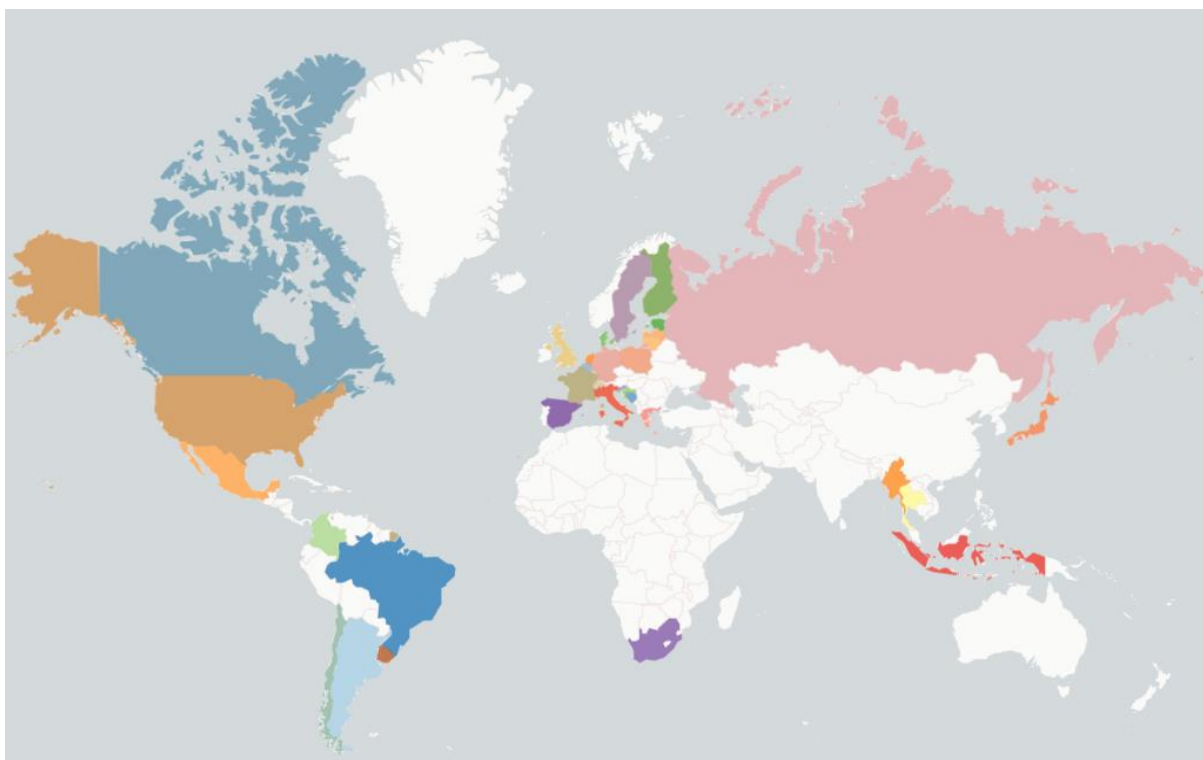


Figure 5. Distribution of reported SARS-CoV-2 cases in animals to the WOAH between 29 February 2020 and 14 March 2022 (WOAH 2022b).

The 35 countries reporting SARS-CoV-2 confirmed cases in animals to WOAH were Argentina, Belgium, Bosnia and Herzegovina, Brazil, Canada, Croatia, Chile, Colombia, Denmark, Estonia, France, Finland, Germany, Greece, Hong Kong, Italy, Indonesia, Japan, Latvia, Lithuania, Mexico, Myanmar, Netherlands, Poland, Russia, Singapore, Slovenia, South Africa, Spain, Sweden, Switzerland, Thailand, United Kingdom, United States of America and Uruguay.

The WOAH has been releasing monthly reports since 31 May 2021 with updates about the total number of outbreaks in animals, the number of outbreaks in the previous month, animal species affected, the geographical location of the outbreak and relevant comments about the occurrences (WOAH 2021b; WOAH 2022a).

Being an emerging infectious disease, it is required that the WOAH Members notify SARS-CoV-2 detection in animals, following the Terrestrial Animal Health Code (WOAH 2021a; WOAH 2021c). The notification may be sent through the World Animal Health Information System (WAHIS) or by fax or email. The Veterinary Authorities of the country should also send to the WOAH Headquarters, periodic reports regarding the original notification and a final report (WOAH 2021a; WOAH 2021c).

For notification purposes, the WOAH recommends treating each cluster of infected animals as an individual occurrence being every cluster epidemiologically distinct, possibly with different animal species involved. These cases may indicate diverse exposure hazards

that would be difficult to distinguish if reported as a group, thus the WOAHA guidance to reported them as individual occurrences.

WOAH country members should consider attaching other relevant information in the notification, in addition to the needed information for an immediate notification (WOAH 2021c). The complementary information may include the zoonotic potential, such as any factors that could favour zoonotic transmission, if the transmission is suspected to be from an animal to a person or the other way around, onset of symptoms in people. Countries' General Directorates for Food and Veterinary should disclose any elements that may favour inter-species transmission in outbreaks affecting numerous species and disease symptoms and onset times should be provided for each species and individual, if applicable. A description of the affected population should be part of the additional details comprising a characterization of clinical signs, samples collected (material collected, number of samples), type of tests applied, positive results, known contacts with COVID-19 infected people/animals, site description and environmental conditions. In case, there is a suspected source of the outbreak and if risk factors were identified, these may as well be reported. Epidemiological comments should include control measures, and available resources. Its goal is to give a broad overview of the entire event in a national/international context (WOAH 2021a; WOAHA 2021c).

1.6.2.1. First reported cases of COVID-19 in cats

The first reported case of SARS-CoV-2 infection in domestic animals, happened in Hong Kong by the end of February 2020 (Sit et al. 2020; WOAHA 2020a). The notification concerned a dog, which was placed under quarantine on the 26th of February 2020 after his owner tested positive for COVID-19. In Hong Kong, when a person was diagnosed with COVID-19, they were hospitalized, and their close contacts isolated in special facilities. The Hong Kong Agriculture, Fisheries and Conservation Department (AFCD) offered affected pet owners the option of having their dogs and cats looked after and quarantined. Specimens from these animals were obtained to check if they were infected with SARS-CoV-2 and to establish proper ways for handling animals under isolation, including the timing when they should be released back to their owners (Sit et al. 2020).

On 18 March 2020, in Belgium, the Federal Agency for the Safety of Food Chain (FASFC) was notified by the Faculty of Veterinary Medicine of the University of Liège that viral RNA of SARS-CoV2 virus was detected in the faeces, vomit and nasopharyngeal swab samples of a cat with digestive and respiratory clinical signs (Garigliany et al. 2020; WOAHA 2020b). The cat belonged to a person with a confirmed infection by SARS-CoV-2. It became the first case of COVID-19 in a cat reported to the WOAHA and since this first notification, 20 more countries have notified WOAHA regarding cases in cats, including Argentina, Brazil, Canada, Croatia, Chile, France, Finland, Germany, Greece, Hong Kong, Italy, Japan, Latvia,

Russia, Spain, Switzerland, Thailand, United Kingdom, United States of America and Uruguay (WOAH 2020b; WOA 2022b).

In a study made in Wuhan, China by Zhang et al. (2020), SARS-CoV-2 serological prevalence in cats from COVID-19 patient families was investigated, detecting specific antibodies for the virus in 14.7% of the tested cats. In Hong Kong, on March 30, 2020, viral RNA was detected on nasal, oral and rectal swabs specimens collected from a clinically healthy cat, whose owners were infected with COVID-19 (Barrs et al. 2020). A serological assay was also carried out to test for neutralizing antibodies and the serum sample indicated a positive result. In the United States, the first companion animals infected were reported in April 22, 2020, in New York State. The animals were two cats from different households, both with epidemiologic connection to suspected or confirmed human COVID-19 in their households (Newman et al. 2020). In France, a study to investigate the possibility of SARS-CoV-2 infection in cats whose owners had previously been suspected of infected with COVID-19 was conducted and one cat tested positive by RT-qPCR on a rectal swab (Sailleau et al. 2020; WOA 2020c). Also Spain (Ministerio de Agricultura Pesca y Alimentación 2020; Segalés et al. 2020; Ruiz-Arrondo et al. 2021; Villanueva-Saz et al. 2021), Germany (Federal Ministry of Food and Agriculture 2020; Michelitsch et al. 2020; WOA 2020d), Italy (Istituto Zooprofilattico Sperimentale delle Venezie 2020; Musso et al. 2020; Patterson et al. 2020), among other countries (Calvet et al. 2021; WOA 2022b) reported cases and published studies of companion animals infected with SARS-CoV-2.

1.6.3. Other animal species susceptibility

The recent emergence of respiratory coronavirus from mammalian reservoirs into human populations, reaching pandemic status, suggests that one of the most pressing current research questions is *From which reservoirs could the next novel coronaviruses be generated and emerge in the future?* It will be possible to limit the risk of emergence into human populations by tight surveillance and enforced separation of the identified hosts at live animal markets, farms, and other close-quarters environments, or we may be able to design potential mitigations in advance, armed with this knowledge?

To answer these questions, the susceptibility to SARS-CoV-2 of different laboratory animal species has been tested, as well as companion and domestic animals. In the study by Shi et al. (2020), different species of animals, including cats, dogs, ferrets, chickens, pigs and ducks, were inoculated with SARS-CoV-2 to assess virus replication. Viral RNA was detected in the ferrets' nasal turbinate, soft palate, tonsils and trachea, indicating that SARS-CoV-2 can replicate in the upper respiratory tract of ferrets without causing severe sickness or death. As for adult cats, viral RNA was found in nasal turbinate, soft palates, tonsils, tracheas, lungs, small intestines, and faeces. Juvenile cats aging between 70 and 100 days were more

vulnerable to COVID-19 and virus replication was more efficient, causing extensive lesions in the nasal and tracheal mucosa epitheliums and lungs. In dogs, only rectal swabs revealed viral RNA, indicating that dogs have low susceptibility to SARS-CoV-2. In pigs, chickens and ducks none of the samples tested positive for the virus.

In North America, from April to December 2020, a study was made to test the hypothesis that the virus spread to white-tailed deer (*Odocoileus virginianus*). Two hundred and eighty-three white-tailed deer, including free-living and captive animals, were assessed by RT-PCR and 94, around 30%, of the samples were positive for SARS-CoV-2 RNA. The genomes of SARS-CoV-2 detected in all positive samples were sequenced and revealed numerous lineages corresponding to virus genotypes prevalent in humans at that time. These findings indicate the prospect of reverse zoonosis, particularly in exurban areas populated by white-tailed deer. These results emphasize the potential hazards and knowledge gaps related with SARS-ongoing CoV-2's evolution in animal hosts, and urges the implementation of improved surveillance programs to detect possible reservoir species at the animal-human interface (Kuchipudi et al. 2021).

1.6.3.1. Minks

During the first wave of the COVID-19 pandemic, in April 2020, SARS-CoV-2 was detected in the Netherlands in farmed minks (*Neogale vison*) of two farms closely located. On 19 and 20 of April 2020, respiratory clinical signs were detected in minks and in both farms; SARS-CoV-2 was identified in at least one worker. Viral RNA was detected in all throat swabs and in the majority of rectal swabs of the necropsied animals. The overall mortality in these farms reached values of 2.4% and 1.2% in each farm, exceeding the expected mortality of 0.6% in farmed minks. The most common respiratory clinical sign was watery nasal discharge, however respiratory distress was also observed in the minks. At necropsy, lesions compatible with interstitial pneumonia were observed in the lungs and no significant macroscopic changes were found in other organs. Histologically, the lungs with macroscopic alterations showed signs of severe diffuse interstitial pneumonia with hyperaemia and alveolar damage (Oreshkova et al. 2020).

Lu et al. (2021) proved that workers employed on the mink farms were infected with mink strains rather than human strains circulating in the same community, implying that SARS-CoV-2 transmitted from animal to human in these farms. At least 43 zoonotic transmissions from minks to people were thought to have occurred across different farms. Some human cases, nevertheless, may have been caused by human-to-human infections between mink-farm employees or farm-owner family members. As a result, the true number of mink-to-human jumps may be smaller. SARS-CoV-2 infections in minks is an aspect of concern because the virus's evolution in an animal reservoir could lead to the establishment of new zoonotic

reservoirs, potentially resulting in repeated spill over occurrences of novel pathogens. The Netherlands was the first country to report SARS-CoV-2 in a mink and detected outbreaks in 69 mink farms between April 2020 and November 2020. Canada, Denmark, France, Greece, Italy, Lithuania, Poland, Spain, Sweden and the United States of America also notified cases in mink (European Centre for Disease Prevention and Control (ECDC) 2020; WOA 2022b).

1.6.4. Coronaviruses in cats

Viral infections play a critical role in feline medicine, and research on feline viruses and viral diseases is a well-established subject providing critical information to protect the health and welfare of domestic cats and wild felids (Beatty and Hartmann 2021). Despite various research projects attempting to comprehend it, the epidemiological role of cats in the coronavirus disease pandemic remains unknown; in other words, it is unknown whether cats may transmit Severe Acute Respiratory Syndrome Coronavirus-2 (SARS-CoV-2) to humans. Again, this reinforces the need to fully integrate the paradigm of One Health into studies and practices of epidemiological surveillance of infectious diseases by multidisciplinary teams in order to obtain a better understanding of diseases' transmission with zoonotic potential (Teixeira and Cantarino 2021).

SARS-CoV-2 and feline coronavirus (FCoV) are coronaviruses that infect cats and humans, respectively. However, SARS-CoV-2 has been shown to infect cats, and FCoV has been confirmed to infect humans (Gao et al. 2022).

1.6.4.1. Feline coronavirus in cats

Infection with the feline coronavirus (FCoV) is quite frequent in cats. The FCoV is the causative agent of feline infectious peritonitis (FIP), which infects both domestic and wild felids. The first case of FIP was reported in the beginning of the 60s. However, the viral aetiology was just hypothesized in 1966, when healthy cats were experimentally infected using tissues from diseased cats, and was only confirmed two years after, in 1968. Natural FCoV infections tend to be temporary, while a number of infections may become persistent (Dye et al. 2007; Jaimes and Whittaker 2018).

FCoV has two serotypes based on antigenicity: FCoV I and FCoV II (Gao et al. 2022). Serotype I viruses are common in the field but difficult to isolate and multiply in cell culture; serotype II viruses are less common but replicate well in cell culture (Dye et al. 2007). For the clinical forms of FCoV, two separate clinical forms, the biotypes, have been described: feline enteric coronavirus (FECV) and feline infectious peritonitis virus (FIPV) (Jaimes and Whittaker 2018). Both serotypes, I, and II, include FECV and FIPV. FIPV is more virulent than FECV, and it can kill cats within 1 to 3 weeks of infection, whereas FECV usually only causes minor

digestive clinical signs. The two biotypes have diverse tropisms, in addition to variances in pathogenicity *in vivo*. During natural infection, FECV spread is restricted to intestinal mucosal epithelial cells or mesenteric lymph nodes, causing mild self-limiting infections, whilst FIPV invades feline peritoneal macrophages and can therefore infect other types of cells, such as monocytes, plasma cells, lymphocytes, and neurocytes, leading to high titres of progeny virus and causing a systemic and fatal illness (Gao et al. 2022).

The association of the viral surface glycoprotein with a cellular receptor is an essential factor of coronavirus infection *in vivo* and in cell culture. The identification of a viable receptor on the surface of the host cell establishes coronavirus species specificity largely. Coronaviruses use a range of cellular receptors, and various coronaviruses use different receptors even within the same host species (Dye et al. 2007). The feline aminopeptidase-N (fAPN) acts as a receptor for FCoV strains that differ considerably in their ability to develop in cultured feline cells and in their pathogenicity in cats, including FECV and FIPV. Also, fAPN can act as a receptor for transmissible gastroenteritis virus (TGEV), porcine respiratory coronavirus (PRCV), canine corona virus (CCV) and human coronavirus 229E (HCoV-229E) (Tresnan and Holmes 1998). Moreover, the S protein can bind fAPN for all FCoV II serotypes, including FECV and FIPV, being the major viral mediator for virus entry into the host cell (Jaimes and Whittaker 2018).

1.6.4.1.1. Feline Infectious Peritonitis Virus

Feline Infectious Peritonitis (FIP) is one of the few animal viral illnesses in which a virus acquires a limited number of mutations that significantly alter its pathogenicity during the course of a generally harmless persistent infection, ultimately culminating in death. These mutations are more prone to happen throughout primary infection in kittens, due to increased FECV replication leading to diminished immune resistance to the mutation once it occurs.

FIP can infect cats of every age, but it is most frequent in felines with less than three years old, and especially with ages between three and sixteen months. Catteries (pedigreed cats), shelters, kitten foster/rescue facilities, and dense free-roaming colonies are places where FIP may be present. FIP's incidence varies considerably over time, as is typically an endemic disease. Once clinical signs are manifested, the mortality is exceptionally high, yet some animals can live with the condition for weeks, months, and perhaps even years (Pedersen 2014; Tekes and Thiel 2016).

Most apparently, healthy cats shed FECV, and transmission occurs through faeces, contaminated litter, and other fomites, meaning that healthy carriers of FECV play a key role in the epidemiology of this disease. Only a small proportion of cats infected with these mutant viruses develop FIP. FIP resistance is complex, combining genetic predisposition, age at the exposure time, and a multitude of stress factors that occur simultaneously with infection and

weaken the infected cat's capacity to eliminate the virus. The duration between first FECV exposure and clinical signs onset might range from 2-3 weeks to several months or, in exceptional cases, years. This time frame may represent the time it takes for mutated FIPVs to evolve, or for the illness to develop from a subclinical to a symptomatic stage (Kipar and Meli 2014; Pedersen 2014).

FECV and FIPV cannot be differentiated serologically or morphologically, and the hunt for markers to distinguish the two pathotypes is inconclusive for many years (Kipar and Meli 2014). The age, origin, clinical indicators, and physical examination of the cat should all be taken into account when diagnosing FIP. This disease should be suspected in cats with less than 3 years old, living in high-density conditions, and with persistent but fluctuating antibiotic unresponsive fever. Other than FIP, few infectious diseases exhibit this clinical picture (Pedersen 2014).

The more prevalent variant of FIP is the effusive form ("wet", non-parenchymatous), which is a contagious inflammatory disorder of the visceral serosa and omentum causing exudation into the abdomen. Granulomatous involvement of parenchymatous organs such as the kidneys, mesenteric lymph nodes, bowel wall, liver, central nervous system, and eyes characterizes the second form of FIP. Because there is no inflammatory exudation into bodily cavities, granulomatous FIP is described to as non-effusive ("dry", parenchymatous) (Pedersen 2009; Paltrinieri et al. 2021). Cats rarely show both kinds of the condition simultaneously, and if they do, it is at a stage of transition from effusive to non-effusive or the other way around (Pedersen 2009).

The diagnostic options can be more narrowed even more if other indicators of FIP are recognized by the tutor, or during physical examination. In animals either with the effusive ('wet') or non-effusive ('dry') form of FIP, abdominal distension with ascites, dyspnoea with pleural effusion, jaundice, hyperbilirubinuria, discernible masses on the kidneys and/or on mesenteric lymph nodes, uveitis, and a wide assortment of neurological signs allied with brain and/or spinal cord involvement are all frequent. At this stage, the diagnosis of FIP can be established with considerable assurance, yet, given the high mortality of FIP, many veterinarians and pet owners are apprehensive of a diagnosis based on "considerable assurance" (Pedersen 2014).

Despite the fact that FIP was once a disease with no treatment options accessible for decades, effective treatments have been developed with promising results in recent years (Paltrinieri et al. 2021). Most cats die from FIP within weeks to months after diagnosis if treatment is not received (Ritz et al. 2007). Traditionally, cats with FIP were given only supportive or anti-inflammatory medication, which improved their quality of life, according to anecdotal reports, but did not eliminate the infection or stop the disease's immunopathogenesis (Paltrinieri et al. 2021). The drug investigations for FIP in cats took a

variety of different approaches. Treatment trials with antiviral compounds either showed limited efficacy or were toxic for cats, such as ribavirin (Weiss et al. 1993), but there are promising news regarding the nucleoside GS-441524, a derivative of remdesivir, shown to be beneficial in combating FIPV (Jones et al. 2021; Taylor et al. 2023). According to Taylor et al. (2023), treatment regimens involving remdesivir were found to be as effective as oral GS-441524 monotherapy. Therefore, it appears that oral GS-441524 should constitute the primary treatment for most felines with FIP, with injectable remdesivir being used in just two instances: in cases where cats come late with advanced illness and oral administration of GS-441524 is not feasible or trustworthy, demanding an injectable medication; or when remdesivir is the only available nucleoside analogue or the most economical choice. Other strategy is to use immunomodulators like feline interferon-omega to suppress crucial components of the inflammatory response. This strategy is rarely successful on its own (Ritz et al. 2007) but shown to be effective when paired with certain antiviral drugs, for example, an oral multi-component drug containing GS-441524, called Xraphconn (Krentz et al. 2021). Unfortunately, the drug is not currently legally available for veterinary use in many countries, such as Portugal. A third strategy is to boost the immune response in an unspecific form expecting that it will be able to fight the infection (Hartmann and Ritz 2008; Pedersen 2014).

Table 3. Comparison between FCoV and SARS-CoV-2. Adapted from Paltrinieri et al. (2020).

		FCoV/FIP	SARS-CoV-2/COVID-19
EPIDEMIOLGY	Transmissibility	High	High
	Reinfections	Frequent	Rarely reported (up to now)
PATHOGENESIS	Route of infection	Faecal – Oral	Respiratory (oral not excluded)
	Cellular tropism	Enterocytes, monocytes/macrophages	Alveolar macrophages, enterocytes
	Main target organs	FECV: intestine FIPV: multiple organs/ tissues	Lung (less frequently GI tract or other organs)
	Lesions	Granulomatous lesions, vasculitis and effusions, lymphoplasmacytic infiltrates	Cytopathic effect on lung cells, multinucleated syncytial cells, mononuclear infiltrates
PREVENTION	Vaccination	Available but not recommended (risk of antibody-dependent enhancement)	Not available

	Quarantine/isolation	May eradicate the disease from catteries	May reduce the prevalence of infection/disease
TREATMENT	Symptomatic drugs	Effective as a support therapy	Effective, curative in mild forms
	Anti-inflammatory drugs	Effective as a support therapy	Possibly curative
	Anti-cytokine drugs	Not tested	Effective, curative in mild forms
	Hyper-immune plasma	Not tested	Possibly curative
	Interferon or TH1 cytokine modulators	Rarely effective	Studies ongoing
	Antiviral drugs	Effective	Possibly effective

1.6.4.2. SARS-CoV-2 in cats

Recently, felines have been discovered to be susceptible hosts for the human SARS-CoV-2 virus (Stout et al. 2020; Bessi re et al. 2021; Allendorf et al. 2022). The incidence of infection in the cat population, as well as the severity of disease and clinical signs, or even the ability for transmission among cats or to other species, including people, remain unknown (Stout et al. 2020). Many people live in close proximity to their cats, especially in the Western countries, and natural infections of cats in COVID-19-positive households have been reported in multiple regions (Hosie et al. 2021). The considerable degree of similarity between the human and feline variants of the SARS-CoV-2 receptor, ACE2, is one viable explanation for this susceptibility. Full-length ACE2 protein sequence alignment and phylogenetic analysis in selected mammalian species demonstrate that simian ACE2 from macaques (*Macaca mulatta*) and chimpanzees (*Pan troglodytes*) are the identical to human ACE2 with 94.9-99% sequence identity. Remarkably, the next most closely related mammalian ACE2 sequence investigated is the domestic cat (*Felis catus*), sharing 85.2% overall identity with human ACE2 (Stout et al. 2020). Human-to-cat transmission may indeed be overlooked, and a better comprehension of cats' involvement in COVID-19 epidemiological cycle is essential (Bessi re et al. 2021). Throughout the first COVID-19 pandemic wave, seroprevalence levels in domestic cats ranged from 0.4% in the Netherlands to 23% in COVID-19 positive households in France, according to Schulz et al. (2021). Furthermore, overall SARS-CoV-2 seroprevalence among cats was 4.2% in Germany, 3.3% in the United Kingdom, 4.2% in Italy, and 6.4% in Spain (Schulz et al. 2021). Close seroprevalence rates in cats and humans in the same regions suggest that

SARS-CoV-2 infections in cats are attributable to human-to-cat contact transmission in the absence of another valid source (Schulz et al. 2021).

1.6.4.2.1. Transmission, detection, and clinical signs

Cats have occasionally been infected with SARS-CoV-2. According to these reports, SARS-CoV-2 RNA was found in respiratory and/or faecal samples from cats, whether or not there were clinical signs (Patterson et al. 2020). In a few experimental studies, viral RNA was detected in different tissues, including the nasal turbinate, soft palates, tonsils, tracheas, lungs, oesophagus, small intestines and faeces of cats previously inoculated with the virus (Bosco-Lauth et al. 2020; Gaudreault et al. 2020; Halfmann et al. 2020; Shi et al. 2020). These studies also demonstrated that cat-to-cat transmission might occur through respiratory droplet. Uninfected cats were housed with inoculated/infected cats in order to access the transmission via respiratory droplets. Some days after, viral RNA was detected in the exposed cats in nasal or oral swabs. These results demonstrated the likelihood of aerosol transmission.

Regarding antibody detection, Gaudreault et al. (2020) and Shi et al. (2020), using respectively ELISA and neutralization assay, found antibodies against SARS-CoV-2 in both inoculated and exposed cats. Shi et al. (2020) inoculated young cats, aging between 70 and 100 days, and their findings suggest that juvenile cats are more susceptible than adult cats, showing extensive lesions in the nasal and tracheal mucosa, and in the lungs at histopathologic exams. In adult cats, histological alterations were circumscribed to the upper and lower airways (Gaudreault et al. 2020) and none of the cats showed gross lesions (Bosco-Lauth et al. 2020).

Cats infected with SARS-CoV-2, usually remain asymptomatic with no display of clinical signs (Bosco-Lauth et al. 2020; Gaudreault et al. 2020; Halfmann et al. 2020; Shi et al. 2020).

Cats may be viable models to study subclinical COVID-19 infections in people because they easily spread SARS-CoV-2 (Gaudreault et al. 2020).

2. OBJECTIVES

The objectives of this dissertation were the following three:

1. Detect potential risk factors associated with the transmission of SARS-CoV-2 to cats.
2. Identify possible clusters of infection in space and time.
3. Build a decision-making model that allows to identify a sample with SARS-CoV-2 neutralizing antibodies, based on the OD_{415nm} obtained from the ELISA assays.

3. MATERIALS AND METHODS

3.1. Sample collection and initial sample processing

Blood samples were collected for 6 months, between May and October 2021, by the clinical staff, from cats consulted at the Veterinary Hospital of the Faculty of Veterinary Medicine, University of Lisbon, for diverse motives, namely medical emergencies, pre-surgical consultations, referral appointments, medical check-ups, scheduled vaccinations, and follow-ups.

Seven hundred and fifty-one blood samples were collected from 523 cats.

Blood was collected through venepuncture and stored in Deltalab serum tubes containing clot accelerator and gel serum separator. Next, the tubes were submitted to a 2356 g centrifugation, for 5 minutes at a room temperature of 25°C (RT), allowing serum separation because of the inert gel placed between the clot and the serum (Figure 6).

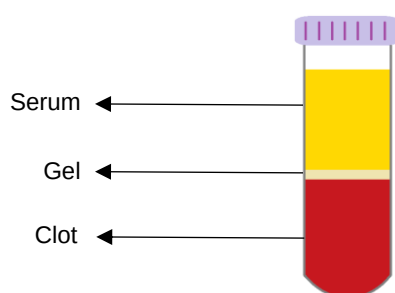


Figure 6. Schematic representation of a serum tube after centrifugation. Original.

After this procedure, the serum was picked from the Deltalab serum tube using a micropipette, and transferred to an Eppendorf tube, which was centrifuged for 10 minutes, at 241 g, at a temperature of 4 °C, and then stored at -80 °C.

The age, gender, tutor's postal code, outdoor access, contacts with other cats, vaccination status, Feline Immunodeficiency Virus (FIV), Feline Leukemia Virus (FeLV) and Feline infectious peritonitis (FIP) positivity, and comorbidities for of each sampled cat was retrieved from the cats' medical records kept in GURUVET, the information system of the Veterinary Hospital.

A database was developed with the gathered data, using Microsoft® Office Excel 365 for Windows® to entry, validate, store, and analyse data.

3.2. ELISA to detect SARS-CoV-2 antibodies

The existence of IgG antibodies to SARS-CoV-2 was assessed using ELISA and purified recombinant spike and RBD SARS-CoV-2 proteins. The Instituto Gulbenkian da Ciência (IGC) supplied the spike and RBD proteins generated from the wild type variant.

A positive control and a negative control were included in each microplate. The serum from a cat whose owner recovered from COVID-19 disease and whose serum had high optical density value at 415 nm (OD_{415nm}) and a large percentage of SARS-CoV-2 neutralizing antibodies served as the positive control. The negative control was a cat serum that tested negative for neutralization of the SARS-CoV-2 surrogate virus and showed a low OD_{415nm} value.

Besides the positive and negative control, the microplates also had a secondary antibody control, consisting of a well with horseradish peroxidase (HRP) combined with goat anti-cat IgG Fc (Abcam, Cambridge, United Kingdom, reference ab112801) and no sample serum, and an ABTS control. The ABTS control lacked both the test serum and the secondary antibody.

In order to achieve this, the serum samples were initially inactivated for 1 hour at a temperature of 56°C. Then, 50 µl/well of RBD at a concentration of 2 µg/ml in phosphate buffered saline (PBS) (Gibco™) and 50 µl/well of spike at a concentration of 0.5 µg/ml in PBS were used to coat a 96 Well EIA/RIA Assay Microplate (Corning Inc., New York, United States of America, reference CLS9018-100EA), followed by covering and incubation for an hour at 37°C. The microplate was therefore blocked with 150 µl of PBS containing 2% of bovine serum albumin (BSA) (Sigma-Aldrich, Missouri, United States of America, reference A9576-50ML) and incubated for 1 hour at 37°C, after being washed three times with 200 µl of PBS containing 0.01% Tween® 20 (PanReac AppliChem, Illinois, United States of America, reference 142312) (PBS/Tween® 20 0.01%). Next, the microplate was washed 3 times with PBS/Tween® 20 0.01% and then, added 100 µl of serum, diluted 1:50 in PBS containing 0.05% Tween® 20 and 1% BSA (PBS/Tween20 0.05%/BSA 1%) and incubated for an hour under a room temperature of 25°C. A new wash procedure was performed with a subsequent addition of 100 µl of goat anti-cat IgG Fc HRP (Abcam, Cambridge, United Kingdom, reference ab112801), diluted 1:10000 in PBS/Tween® 20 0.05%/BSA 1%, to the microplate's wells and incubated for another hour at RT. Finally, the microplates were washed three more times, it was added 100 µl of 2,2'-Azinobis (3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) substrate and were incubated for 5 minutes without exposure to the light. The last step consisted of measuring the optical density (OD), with an iMark™ Microplate Absorbance Reader (Bio-Rad Laboratories,

California, United States of America) at 415 nm (OD_{415nm}) at 5 minutes, 15 minutes and 30 minutes. The measures were recorded in Microsoft® Office Excel 365 for Windows® spreadsheet.

All samples were assessed in a well that had neither been coated with RBD or spike proteins, but that had been blocked with PBS containing 2% of BSA and went through the exact process as the other wells in order to establish binding specificity.

It was predefined that the cut-off mark should be the double of the OD_{415nm} mean of the BSA. Accordingly, a value of OD_{415nm} of 0.5 was the cut-off for both RBD and spike. The samples with OD_{415nm} readings higher than this cut-off mark were classified as positive. When the OD_{415nm} of the BSA did not differ significantly from the OD_{415nm} of both RBD and spike, the result was considered uncertain. This means that, even though the OD_{415nm} for RBD and spike was above the cut-off mark, these samples were not considered positive because of the unspecific binding. The same parameters, positive and negative controls, and independent ELISA assays were applied to retest serum samples that tested positive in the first round. The OD_{415nm} cut-off mark for RBD and spike in this independent ELISA was also set at 0.5.

3.3. Neutralization assay to detect SARS-CoV-2 neutralizing antibodies

For the neutralization assay a SARS-CoV-2 Surrogate Virus Neutralization Test (sVNT) Kit (GenScript Biotech Corp, New Jersey, United States of America, reference L00847) was used by the CIISA Microbiology and Immunology Laboratory. Just the most recent serum sample per animal was selected to be tested, out of all the samples that tested positive in the RBD, spike, or both in the ELISA independent assay. The purpose of this action was to identify circulating antibodies that blocked the SARS-CoV-2 RBD from binding to the ACE2 receptor.

The two essential components of the kit are the human angiotensin-converting enzyme 2 (hACE2) receptor protein-coated 96-well ELISA microplate and the HRP conjugated recombinant SARS-CoV-2 RBD wild type fragment (HRP-RBD).

The first step was to dilute the serum samples, the positive and the negative control at a volume ratio of 1:50 with Sample Dilution Buffer, and the HRP-RBD at a volume ratio of 1:1000 with HRP Dilution Buffer. To assess the binding of circulating neutralization antibodies to HRP-RBD, the final diluted samples (serum sample, positive control and negative control) were mixed with the diluted HRP-RBD with a volume ratio of 1:1 and incubated for 30 minutes, at 37 °C. Then, 100 µl of the mixtures were added to the corresponding wells of the kit's ELISA microplate and the plate was sealed and put under incubation at 37°C for 15 minutes. Using the Wash Solution, the microplate was washed 5 times and a volume of 100 µl of Tetramethylbenzidine (TMB) Solution was added to every well of the microplate, followed by incubation for 15 minutes, at a room temperature between 20°C and 25°C. To finish the reaction 50 µl of Stop Solution was added.

In case there were no neutralizing antibodies present, the HRP-RBD could bind to the ACE2 receptor, being captured on the microplate. Whenever neutralizing antibodies were present, they bound to HRP-RBD and prevented it from engaging to the ACE2 receptor. These complexes then stayed in the supernatant and were eliminated during the subsequent washing.

This time, also using iMark™ Microplate Absorbance Reader (Bio-Rad Laboratories, California, United States of America), the optical density was measured at 450 nm (OD_{450nm}). The duplicate positive and negative controls on the plate had OD levels that were required to be greater than 1.0 and lower than 0.3, respectively, to guarantee the reliability of the results. All measurements were recorded in a Microsoft® Office Excel 365 for Windows® spreadsheet. Using Equation 1, the inhibition rate of each sample was estimated by using OD_{450nm} of the negative control. If the inhibition rate was equal to or higher than 30%, it revealed that SARS-CoV-2 neutralizing antibodies were present; if the rate was less than 30%, the inference was that no SARS-CoV-2 neutralizing antibodies were detected. This could be due to true absence of neutralizing antibodies or to a level of SARS-CoV-2 neutralizing antibodies below the test's detection limit.

Equation 1. Calculation of the inhibition rate of the tested samples.

$$Inhibition (\%) = 1 - \left(\frac{OD_{450nm} \text{ of sample}}{OD_{450nm} \text{ of negative control}} \right) \times 100$$

3.4. Data analysis

Several types of data analysis were done in this study. First, it was performed an analysis to portray the study population, based on age, gender of the cats and the frequency of positive, negative and uncertain results on ELISA and neutralization assays. Then, a temporal and spatial analysis, describing the sample collection, results' distribution on time, spatial distribution and a comparison of the SARS-CoV-2 prevalence on cats with the SARS-CoV-2 prevalence on humans. This last analysis allowed us to build maps through geocoding and to identify any existing cluster (spatial, temporal or both). Next, possible risk factors to cats' exposure to SARS-CoV-2 virus were identified. The final step was to build a decision model to ascertain if a sample was positive or not, founded by the OD_{415nm} of RBD and spike measured by the ELISA.

All analysis were executed through R programming using RStudio (Version 1.3.1093). The following R packages were used: “usethis” (Wickham et al. 2021), “devtools” (Wickham, Hester, et al. 2020), “FFTrees” (Phillips et al. 2022), “readxl” (Wickham and Bryan 2019), “dplyr” (Wickham, François, et al. 2020), “tidyverse” (Wickham et al. 2019), “data.table” (Dowle and Srinivasan 2020), “tibble” (Müller and Wickham 2020), “ggplot2” (Wickham 2016), “plotly”

(Sievert 2020), “RColorBrewer” (Neuwirth 2014), “reshape2” (Wickham 2017), “ggmap” (Kahle and Wickham 2019), “sf” (Pebesma 2021), “mapdeck” (Cooley 2020), “leaflet” (Cheng et al. 2019), “htmltools” (Cheng et al. 2020), “epitools” (Aragon 2020), “DescTools” (Signorell 2021), “RCurl” (Lang 2020), “broom” (Robinson et al. 2020), “rgdal” (Bivand et al. 2020) and “stats” (R Core Team 2022).

3.4.1. Statistical analysis

To characterize the study population, the cats’ clinical data was summarized using absolute and relative frequencies, dividing the animals into two or three groups, based on the results obtained from the first ELISA assays, independent ELISA assays and neutralization assays. In order to assess a possible correlation between the different categories of clinical data (age, gender, tutor’s postal code, outdoor access, contact with other cats, vaccination status, FIV/FelV positivity, FIP positivity) and a positive sample, the Pearson’s chi-squared test was used applying the function “chisq.test” of package “stats” (R Core Team 2022) on RStudio (Version 1.3.1093). The significance level for this test was p-value <0.05. To quantify the strength of the association between each one of the categories of clinical data and a positive sample, the odds ratio was calculated with the function “oddsratio” from package “epitools” (Aragon 2020).

3.4.1.1. Decision models

The RStudio package “FFTrees” (Phillips et al. 2022) was applied to build a decision model allowing to ascertain if a sample had a positive or negative test result. Three decision trees were developed. The first using the total of samples as data source and as data test, the other two using one-half of the samples as data source and the other half as data test. The samples were randomly split into to 2 groups (A and B), using the function “sample_frac” included in the R package “dplyr” (Wickham, François, et al. 2020). For the first decision model with the samples divided, group A was used as data source and group B as the data test; for the second decision model, the groups were reversed.

The samples used for the development of these decision trees were only the samples submitted to the neutralization assay. The OD_{415nm} of RBD, OD_{415nm} of spike and the ratio between RBD and spike OD_{415nm}, all from the independent ELISA assay, were established as being the decision parameters.

The decision models enable quick and simple analysis of the ELISA assay results through the visual representation of decision trees. The structure of the decision tree allows for intuitive navigation, starting from the root node and progressing towards the leaf nodes, which represent the final decisions or predictions. The nodes in the decision tree are based on

statistical and algorithmic calculations, considering the relevance and significance of each feature or variable in determining the probable neutralization outcome.

3.4.2. Spatial and temporal analysis

One of the pieces of information collected from the cats' medical records was the postal code of each cat's tutor. This allowed for the mapping of the samples collected through geocoding, using a GitHub repository as source (Félix 2022), and the function "leaflet" from the R package "leaflet" (Cheng et al. 2019). The location of the positive and negative samples was also mapped, using the same process.

To compare the prevalence of SARS-CoV-2 in the sampled cats and in humans by municipality, a bar plot was created using the "ggplot" and "ggplotly" functions from the R packages "ggplot2" (Wickham 2016) and "plotly" (Sievert 2020), respectively. Only the municipalities of Amadora, Cascais, Lisbon, Oeiras and Sintra entered this analysis due to the low significance of the cats' results from the remaining municipalities. The data related to SARS-CoV-2 in people was collected from a GitHub repository (Data Science for Social Good 2022) that gathers all the data released by Instituto Nacional de Saúde Doutor Ricardo Jorge [National Health Institute Doctor Ricardo Jorge] and the General Directorate of Health. The prevalence of SARS-CoV-2 in cats was calculated considering the positive results obtained in the independent ELISA assay.

The identification of spatiotemporal clustering was performed using SaTScan software (Version 10.0.2, 64 bit) and the maps to visualize the clusters were built in RStudio (Version 1.3.1093) using the "readOGR" and "leaflet" functions from "rgdal" (Bivand et al. 2020) and "leaflet" (Cheng et al. 2019) packages, respectively.

4. RESULTS

4.1. Sampled population descriptive analysis

4.1.1. Demographic

As previously mentioned, a total of 751 blood samples were collected from 523 cats, ranging from less than 1 one year old to 20 years old, with an average age of 8 ± 4.86 years. Most sampled cats were adults (2-10 years old), representing 51.82% (271/523), 10.71% (56/523) were young cats (0-1 years old) and 37.09% (194/523) were senior cats (more than 10 years old). Only two sampled cats (0.38%) did not have information about their age on GURUVET (Table 4).

Table 4. Summary of the demographic features of the study population (age).

Age group	N	%
0-1 (young)	56	10.71
2-10 (adult)	271	51.82
>10 (senior)	194	37.09
Unknown	2	0.38
Total	523	100

Out of the 523 cats, 54.30% (284/523) were male and 45.51% were female (238/523). The gender of one cat (0.19%) was not possible to retrieve from GURUVET (Table 5).

Table 5. Summary of the demographic features of the study population (gender).

Gender	N	%
Male	284	54.30
Female	238	45.51
Unknown	1	0.19
Total	523	100

4.1.2. Lifestyle description and medical history

In most cases (53.73%; 281/523,) cats lived strictly indoors with, 65 cats (12.43%; 65/523) had full street access, and 30 cats (5.73%; 30/523) only have access to a garden without access to the street (Table 6). It was not possible to obtain lifestyle information from the medical records of 147 cats (28.11%; 147/523).

Regarding the contact with other cats, a little more than half of the cats interacted with other cats (51.82%; 271/523) and 138 (26.38%; 138/523) did not interact with other cats. The other 114 cats (21.80%; 114/523) did not have this information stored on GURUVET (Table 6).

The data about the cats' vaccination status was grouped into three categories: complete vaccination, incomplete vaccination, and no vaccination. The first category includes the animals that followed the vaccination plan with no pending vaccine. The second one joins the cats that took at least one vaccine but have one or more incomplete vaccines or boosters. The third group refers to animals that were never vaccinated (Table 6).

One hundred and seventy-seven cats (33.84%; 177/523) were fully vaccinated, 28.87% (151/523) showed an incomplete vaccination or boosters plan and 12.62% (66/523) were never vaccinated. The vaccination history was missing in 129 cats (24.67%; 129/523) (Table 6).

Regarding their status for Feline Leukaemia Virus (FeLV) and Feline Immunodeficiency Virus (FIV), 256 cats (48.95%; 256/523) showed negative results for both virus, 16 cats (3.06%; 16/523) were FeLV positive, 14 cats (2.68%; 14/523) were FIV positive, and 5 cats (0.95%; 5/523) were positive for both, FeLV and FIV. The other 232 cats (44.36%; 232/523) were never tested for these diseases or the results were not inserted in their clinical records. Only five of the cats had positive diagnosis for FIP (0.96%; 5/523) (Table 6).

Table 6. Summary of the lifestyle and medical history features of the study population (outdoors access, contact with other cats, vaccination, and FIV, FeLV and FIP positivity).

Outdoors access	N	%	Vaccination	N	%
Indoor cat (no access)	281	53.73	Complete	177	33.84
Only garden access	30	5.73	Incomplete	151	28.87
Full outdoors access	65	12.43	No vaccines	66	12.62
Unknown	147	28.11	Unknown	129	24.67
Total	523	100	Total	523	100

Contact with other cats	N	%	FIV and/or FeLV	N	%
No	138	26.38	Both negative	256	48.95
Yes	271	51.82	FIV positive	14	2.68
Unknown	114	21.80	FeLV positive	16	3.06
Total	523	100	Both positive	5	0.95
			Untested/unknown	232	44.36
			Total	523	100

FIP	N	%
Positive	5	0.96
Negative	518	99.04
Total	523	100

4.1.3. Sampling temporal and spatial distribution

The sampling took place between May and October 2021. From the 523, 81 animals (15.49%; 81/523) were sampled in May, 102 in June (19.50%; 102/523), 87 in July, August and September (16.63%; 87/523), and 79 in October (15.11%; 79/523) (Figure 7).

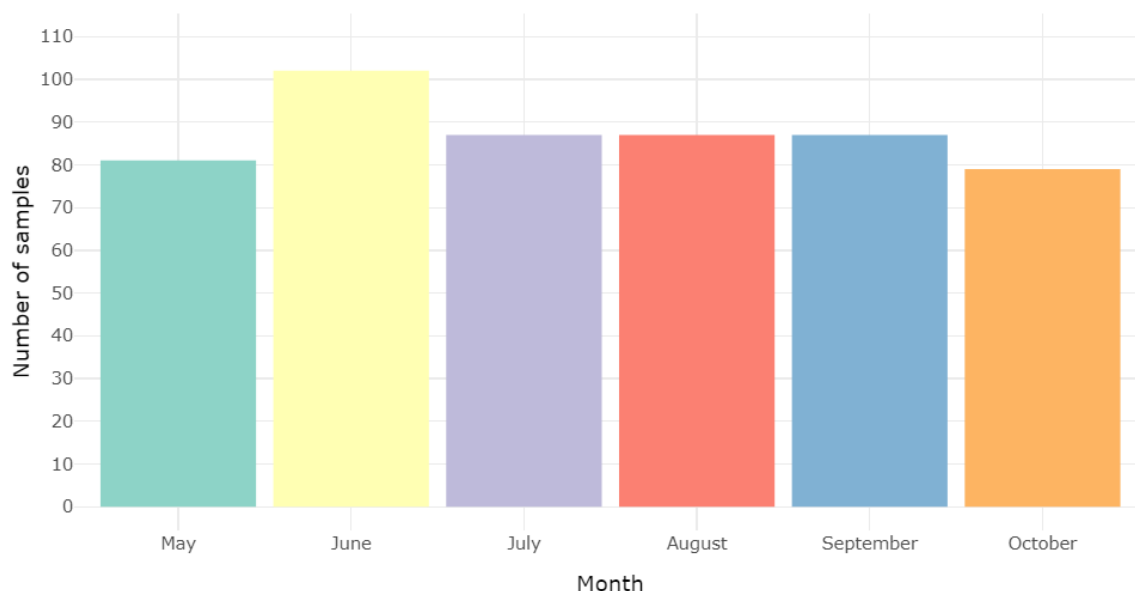


Figure 7. Temporal distribution of the samples' collection between May and October 2022.

15.49% of the samples were collected in May (n = 81), 19.50% in June (n = 102), 16.63% in July, August and September (n = 87), and 15.11% in October (n = 79).

Based on the cats' tutors' postal code, it was possible to obtain the cats' home street locations from 504 of the cats (96.37%; 504/523). The postal code of the remaining 19 cats' tutors (3.63%; 19/523) was not recorded on the animal medical record.

All cats were based in Portugal and the large majority (96.03%; 484/504) lived in the Metropolitan Area of Lisbon (MAL), spread over 18 municipalities: Lisbon (40.08%; 202/504), Oeiras (13.49%; 68/504), Amadora (11.31%; 57/504), Sintra (10.91%; 55/504), Cascais (5.36%; 27/504), Loures (2.98%; 15/504), Almada (1.98%; 10/504), Odivelas (1.98%; 10/504), Seixal (1.79%; 9/504), Mafra (1.19%; 6/504), Moita (0.99%; 5/504), Setúbal (0.99%; 5/504), Palmela (0.79%; 4/504), Vila Franca de Xira (0.79%; 4/504), Barreiro (0.60%; 3/504), Sesimbra (0.40%; 2/504), Alcochete (0.20%; 1/504) and Montijo (0.20%; 1/504) (Table 7).

The remaining cats (3.97%; 20/504) lived in municipalities outside the Metropolitan Area of Lisbon: Loulé (0.40%; 2/504), and Abrantes, Alenquer, Almeirim, Arruda dos Vinhos, Azambuja, Benavente, Coruche, Entroncamento, Figueira da Foz, Lourinhã, Matosinhos, Moura, Óbidos, Odemira, Portimão, Sines, Torres Novas and Vila Nova de Gaia, all with only 1 cat sampled (0.20%; 1/504) (Table 7).

Table 7. Tutors' home location of the cat study population.

Municipality	N	%	Municipality	N	%
Abrantes	1	0.20	Matosinhos	1	0.20
Alcochete (MAL)	1	0.20	Moita (MAL)	5	0.99
Alenquer	1	0.20	Montijo (MAL)	1	0.20
Almada (MAL)	10	1.98	Moura	1	0.20
Almeirim	1	0.20	Óbidos	1	0.20
Amadora (MAL)	57	11.31	Odemira	1	0.20
Arruda dos Vinhos	1	0.20	Odivelas (MAL)	10	1.98
Azambuja	1	0.20	Oeiras (MAL)	68	13.49
Barreiro (MAL)	3	0.60	Palmela (MAL)	4	0.79
Benavente	1	0.20	Portimão	1	0.20
Cascais (MAL)	27	5.36	Seixal (MAL)	9	1.79
Coruche	1	0.20	Sesimbra (MAL)	2	0.40
Entroncamento	1	0.20	Setúbal (MAL)	5	0.99
Figueira da Foz	1	0.20	Sines	1	0.20
Lisbon (MAL)	202	40.08	Sintra (MAL)	55	10.91
Loulé	2	0.40	Torres Novas	1	0.20
Loures (MAL)	15	2.98	Vila Franca de Xira (MAL)	4	0.79
Lourinhã	1	0.20	Vila Nova de Gaia	1	0.20
Mafra (MAL)	6	1.19	Total	504	100

4.2. Detection of SARS-CoV-2 antibodies

The existence of IgG antibodies against SARS-CoV-2 RBD and spike proteins of the wild type variation was examined using an ELISA. Depending on whether the plate had the controls or not, either 28 or 32 serum samples were evaluated per plate. At 5, 15, and 30 minutes, the ELISA reaction outcomes were translated to OD_{415nm} values based on the colour density of each well. As referred previously, the cut-off for RBD and spike protein was established at OD_{415nm} of 0.5. If a serum sample expressed OD_{415nm} values for RBD and/or spike proteins above 0.5, it was considered as positive for SARS-CoV-2 specific antibodies. Similar OD_{415nm} values to RBD and/or spike, as well as to BSA were used to identify samples with nonspecific binding, and their positivity was labelled as inconclusive. While the negative controls had low OD_{415nm} values for both proteins, the positive controls showed OD_{415nm} values above 0.5 for both RBD and spike proteins.

Out of the 523 cats tested, 119 animals (22.75%) had positive results, 334 (63.86%) negative results and the remaining 70 cats (13.39%) inconclusive results (complete data on Annex A). As a confirmatory step, the 119 positive samples were tested in an independent ELISA assay. Ninety samples were positive (17.21%; 90/523) (complete data on Annex B).

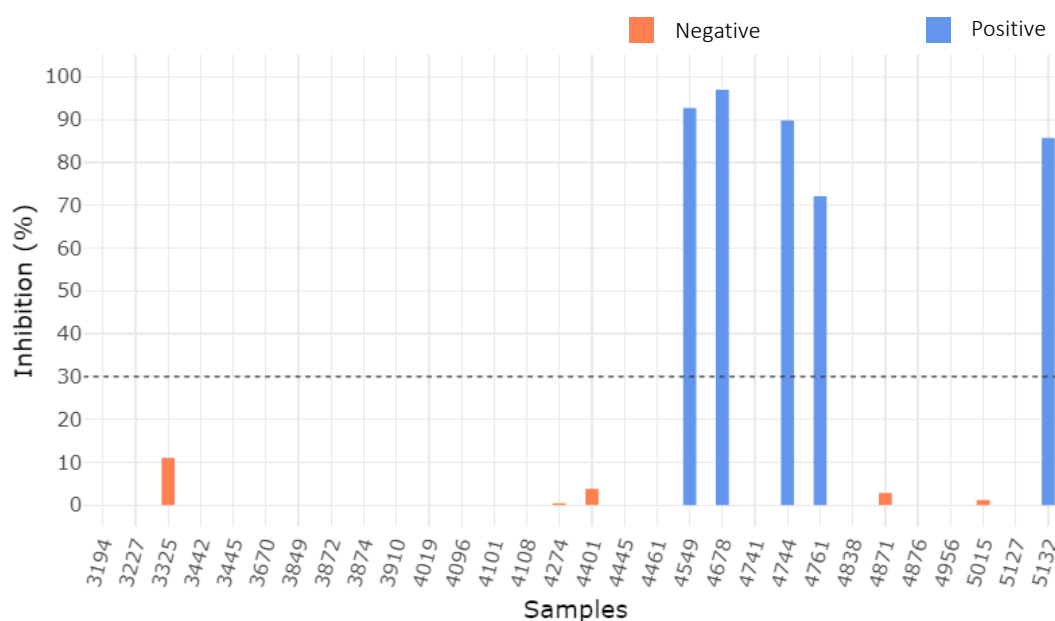
4.3. Detection of neutralizing antibodies against SARS-CoV-2 wild type variant RBD

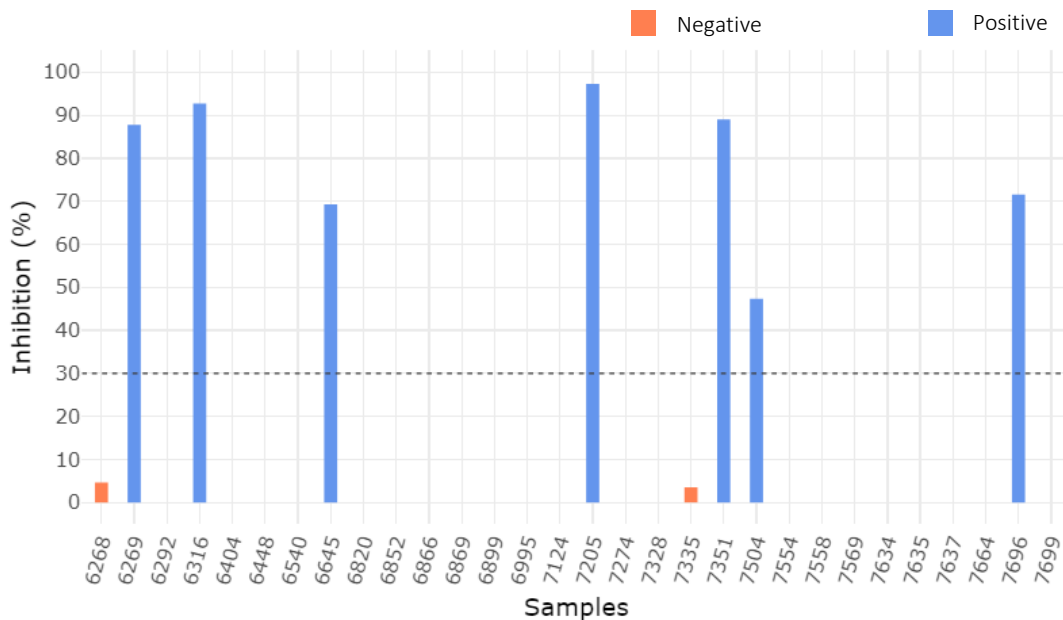
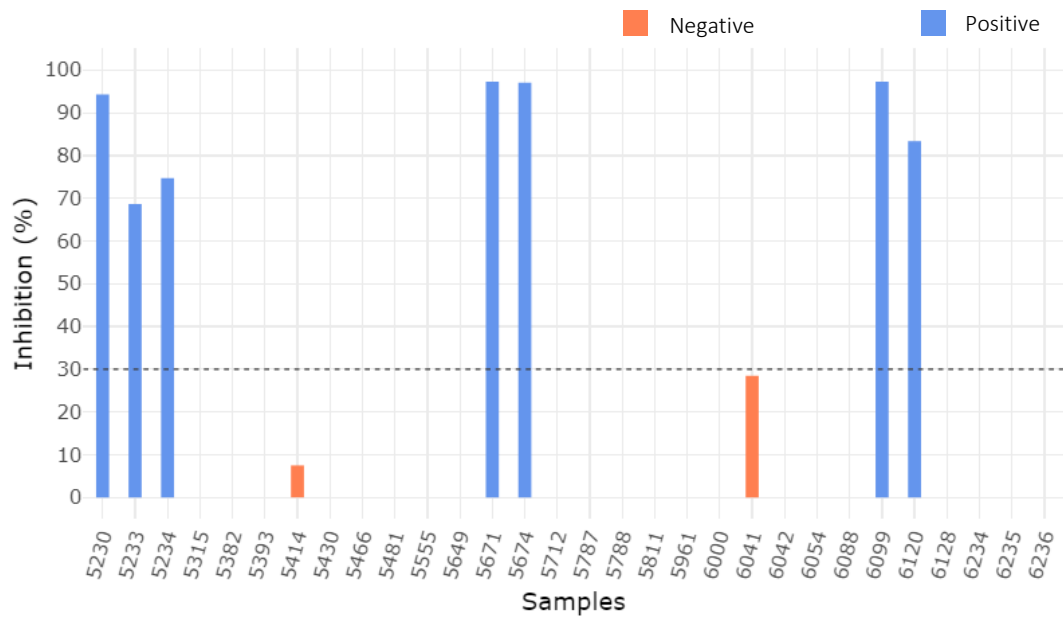
This step was applied to the 90 positive serum in the independent ELISA assay to identify circulating antibodies with the ability to block the SARS-CoV-2 RBD from binding to the ACE2 receptor.

Each plate's reaction outcomes were estimated by the colour density of each well and extrapolated to an OD_{450nm} value. The percentages of inhibition were calculated using these OD_{450nm} measures. In line with the manufacturer's protocol of the kit, samples with a percentage of inhibition equal to or greater than 30% indicated the existence of SARS-CoV-2 neutralizing antibodies.

The sVNT detected antibodies with neutralizing activity against the wild type variant RBD of SARS-CoV-2 in nineteen serum samples (21.11%; 19/90). The inhibition outcomes of the positive samples vary from 47.37% to 97.28%. This means that among the 523 cats, 3.63% had antibodies with the capacity to neutralize the SARS-CoV-2 wild type variant RBD (Figure 8).

Figure 8. Neutralizing antibodies in the SARS-CoV-2 Surrogate Virus Neutralization Test (N=90).





Identical distributions of OD_{415nm} levels were found in the neutralizing samples for RBD, with an OD_{415nm} between 1.881 and 2.552, and spike, with an OD_{415nm} between 1.403 and 2.330, suggesting a considerable uniformity. Thus, in comparison with the samples lacking neutralizing antibodies, the 19 samples with this neutralization capacity displayed greater OD_{415nm} values for RBD and spike in the ELISA assay. With the availability of this attribute, samples with OD_{415nm} values greater than 1 for RBD and spike might point the circulation of antibodies with neutralizing activity.

Figure 9 summarizes the results on the first ELISA, independent ELISA and neutralization assays.

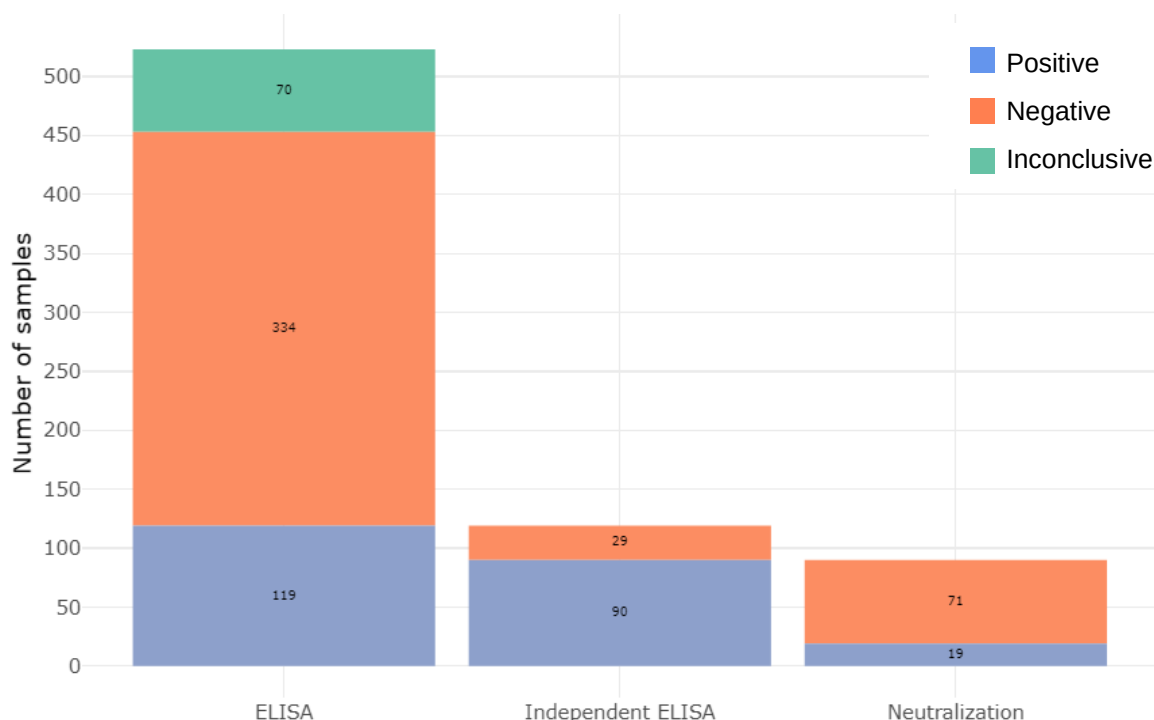


Figure 9. Summary of testing results.

119 (22.75%; 119/523) samples tested positive, 334 (63.86%; 334/523) negative and 70 (13.39%; 70/523) showed uncertain results. The positive samples were reassessed through an independent ELISA and 90 (75.63%; 90/119) were positive, while the remaining 29 (24.37%; 29/119) show negative outcomes. In the final VN testing, 19 (21.11%; 19/90) samples were positive and 71 (78.89%; 71/90) negative.

4.3.1. Temporal distribution of the neutralizing samples

The sampling started on May 9 of 2021 and ended on October 25, of the same year.

Four samples collected in June tested positive (21, 05%; 4/19), another 4 in July (21.05%; 4/19), 3 in August (15.79%; 3/19), 4 in September (21.05%; 4/19), and 4 in October (21.05%; 4/19). No positive results were obtained from samples collected in May (Figure 10).

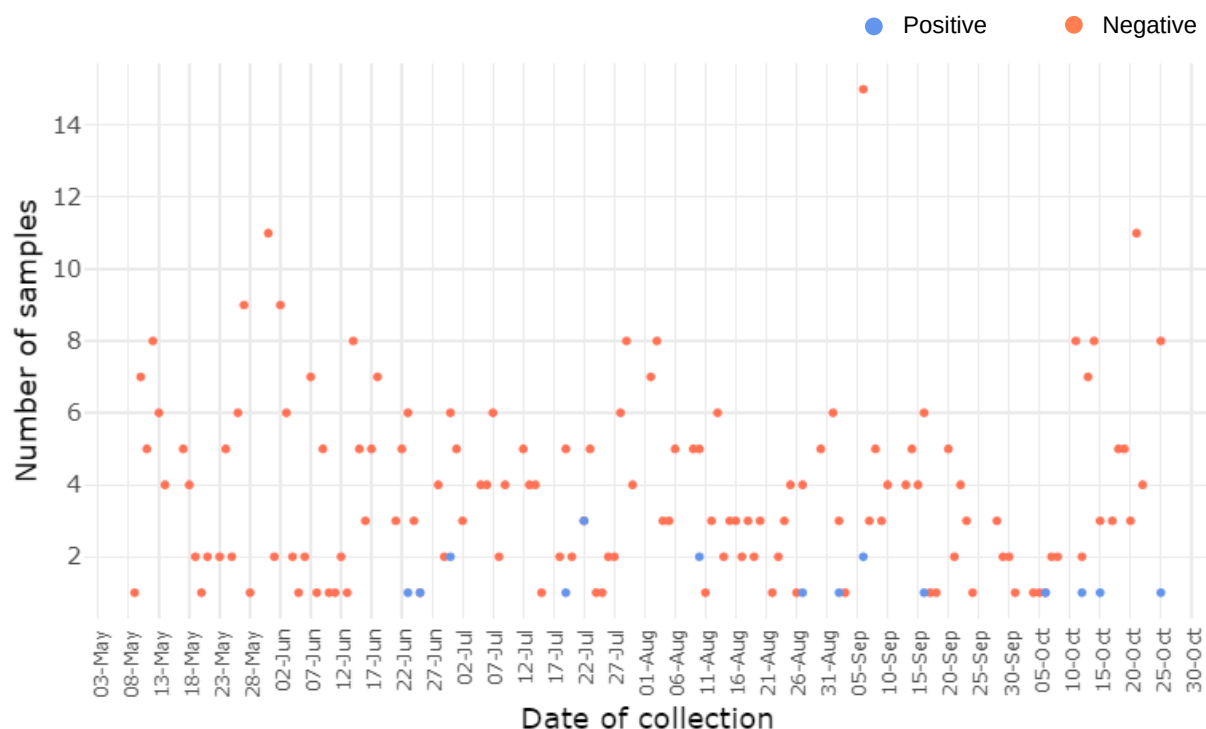


Figure 10. Sampling date and surrogate Virus Neutralization Test results.

The first positive sample was collected on 23rd of June, followed by another on the 25th and a third on the 30th. In July, one positive sample was obtained on the 19th and three on the 22nd. Two positive samples were collected on the 10th of August, another on the 27th of August. One on the 2nd of September, two on the 6th and one on the 16th. The remaining four positive samples were collected on the 6th, 10th, 12th and 25th of October.

4.3.2. Spatial distribution of the neutralizing samples

Using tutors' postal codes stored in GURUVET, cats' locations were plotted in most cases (96.37%; 504/523).

The majority of cats with positive results on the SARS-CoV-2 Surrogate Virus Neutralization Tests lived at the Metropolitan Area of Lisbon (94.74%; 18/19). Five cats lived in Lisbon (26,32%; 5/19), four in Amadora (21,05%; 4/19), two in Cascais (10,53%; 2/19), two in Oeiras (10,53%; 2/19), two in Sintra (10,53%; 2/19), one in Loures (5,25%; 1/19), one in Moura (5,25%; 1/19), one in Seixal (5,25%; 1/19), and one in Setúbal (5,25%; 1/19) (Figure 11).

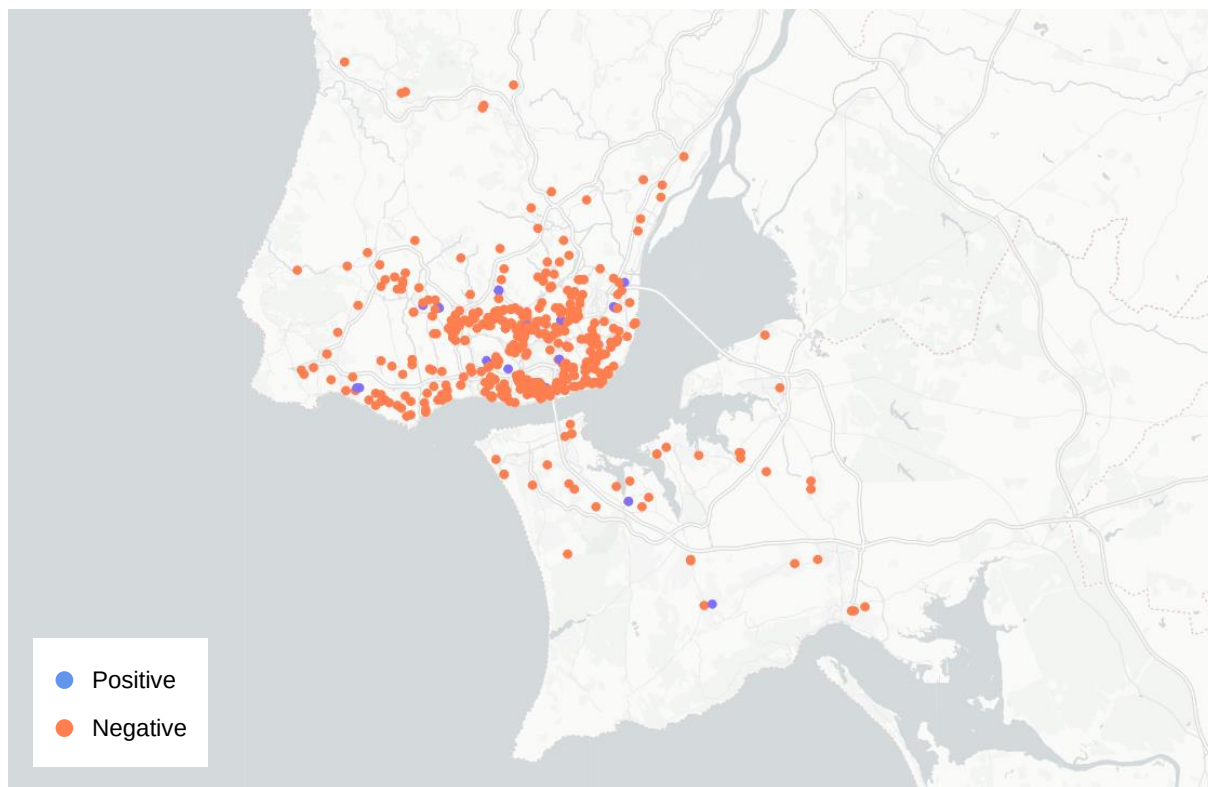


Figure 11. Location of the cats' tutors with residence in the Metropolitan Area of Lisbon (MAL) and respective result on the SARS-CoV-2 Surrogate Virus Neutralization Test.

From the 504 tutors with registered postal code, 484 cats lived with their tutors in the MAL (96.03%; 484/504). Among these, 18 cats (3.72%; 18/484) tested positive for the presence of neutralizing antibodies against the wild type variant RBD of SARS-CoV-2.

4.4. Human COVID-19 prevalence *versus* cats' COVID-19 prevalence on the study population

Based on the COVID-19 pandemic data published by the health authorities in Portugal (Data Science for Social Good 2022), the prevalence of COVID-19 among the human population from the municipalities of Amadora, Cascais, Lisbon, Oeiras, and Sintra, was calculated and compared with the prevalence estimated for the cats study population, based upon the independent ELISA results. For this analysis, the sVNT results were not used due to the low number of samples.

In February of 2022, the prevalence of SARS-CoV-2 among the human population was 17.54% *versus* 2.08% in sampled cats in Amadora, 11.11% *versus* 2.86% in Cascais, 17.16% *versus* 1.23% in Lisbon, 19.12% *versus* 3.57% in Oeiras, and 21.82% *versus* 1.18% in Sintra (Figure 12).

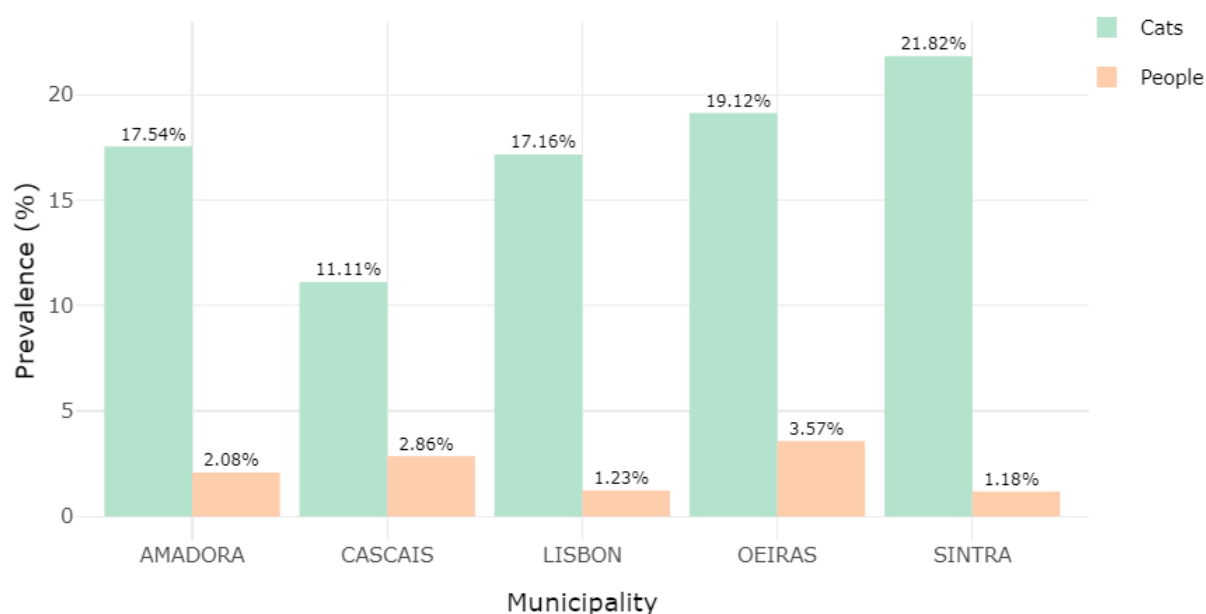


Figure 12. Comparison between SARS-CoV-2 prevalence among the human population and the cats' study population, in February 2022, in Amadora, Cascais, Lisbon, Oeiras and Sintra.

4.5. Risk factors

Based on the clinical data retrieved from cats' medical records and on the results from both independent ELISA and neutralization tests, it was estimated a correlation between the cats' features and the assays' results, employing Pearson's chi-squared test. The main purpose of this statistical test is to determine if both of the categorical variables (cats' features and assays' results) have independent effects on the test statistic, allowing predicting if the cats' characteristics or lifestyle might affect the contact with SARS-CoV-2. The odds ratio (OR) was also estimated to measure the strength of the association between a specific feature or behaviour of the cats and the likelihood of a positive result in the independent ELISA assay and the in the neutralization test.

4.5.1. Risk factors analysis based upon the independent ELISA assay

Generally, based on the results of Pearson's chi-squared test, there is no evidence to suggest a statistical association between any of the evaluated features or behaviours of the cats and the positivity observed in the independent ELISA assay (Table 8).

Table 8. Summary of cats' risk factors analysis based upon ELISA results.

Features		No. of positives	% of positives	No. of negatives	% of negatives	χ^2	p-value
Gender	<i>Female</i>	45	18.91%	193	81.09%	0.62	0.43
	<i>Male</i>	45	15.90%	238	84.10%		
Age	<i>Young</i>	7	12.50%	49	87.50%	2.44	0.30
	<i>Adult</i>	43	15.87%	228	84.13%		
	<i>Senior</i>	39	20.21%	154	79.79%		
Outdoors access	<i>Full access</i>	8	12.31%	57	87.69%	0.24	0.89
	<i>Garden access</i>	4	13.36%	26	86.67%		
	<i>Indoor</i>	40	14.29%	240	85.71%		
Contact with other cats	<i>Yes</i>	43	15.87%	228	84.13%	0.00	1.00
	<i>No</i>	21	15.34%	116	84.66%		
Vaccination	<i>Complete</i>	26	14.33%	150	85.67%	0.76	0.68
	<i>Incomplete</i>	22	14.57%	129	85.43%		
	<i>No vaccines</i>	7	10.61%	59	89.39%		
FIV/FelV	<i>Both positive</i>	0	0.00%	5	100.00%	2.72	0.26
	<i>FIV positive</i>	1	7.14%	13	92.86%		
	<i>FelV positive</i>	1	6.25%	15	93.75%		
	<i>Both negative</i>	48	18.75%	208	81.25%		
FIP	<i>Positive</i>	2	40.00%	3	60.00%	0.58	0.45
	<i>Negative</i>	88	17.02%	429	82.98%		

The first cat feature investigated was gender. The Chi-Squared Test yielded a result of 0.62 and a p-value of 0.43, suggesting that there is no significant association between the gender of the cats and the positivity to the ELISA.

Next, the relationship between age and ELISA positivity was examined. The Chi-Squared Test was 2.44, the p-value 0.30. Again, no statistical association between the age of the cats and the ELISA positivity was found.

The cats' access to outdoors was also investigated. The Chi-Squared Test yielded a result of 0.24, with a p-value of 0.89. These outcomes imply that there is no statistical association between the cats' access to outdoors and the ELISA positivity.

Same results were obtained for the cats' contact with other cats and the ELISA positivity. The Chi-Squared Test was 0.00, with a p-value of 1.00. For the cats' vaccination status. The Chi-Squared Test was 0.76, along with a p-value of 0.69. For the cats' FIV and FeLV diagnosis. The Chi-Squared Test yielded a result of 2.72, with a p-value of 0.26. Equally for the cats' FIP diagnosis. The Chi-Squared Test was 0.58, with a p-value of 0.45.

As we did not find any statistical association between host factors and host lifestyle and positivity to ELISA, odds ratio were not calculated.

This lack of finding cat risk factors for SARS-CoV-2 infection could be due to the small size of the study population and/or to variables not investigated in our analysis that may play a more prominent role in SARS-CoV-2 transmission to cats.

4.5.2. Risk factors analysis based upon the surrogate virus neutralization test

The same statistical methodology used for the independent ELISA assay was applied to the results obtained on the virus neutralization test (sVNT), and again no statistical association between the variables investigated and the positivity to sVTN was found (Table 9).

Table 9. Summary of cats' risk factors analysis based upon Virus Neutralization Test results.

Features		No. of positives	% of positives	No. of negatives	% of negatives	χ^2	P-value
Gender	<i>Female</i>	10	4.20%	228	95.80%	0.15	0.69
	<i>Male</i>	9	3.17%	275	86.83%		
Age	<i>Young</i>	2	3.57%	54	96.43%	0.12	0.94
	<i>Adult</i>	10	3.69%	261	96.31%		
	<i>Senior</i>	6	3.09%	188	96.91%		
Outdoors access	<i>Full access</i>	2	3.08%	63	96.92%	0.03	0.99
	<i>Garden access</i>	1	3.33%	29	96.67%		
	<i>Indoor</i>	8	2.85%	273	97.15%		
Contact with other cats	<i>Yes</i>	10	3.69%	261	96.31%	0.02	0.90
	<i>No</i>	4	2.90%	134	97.10%		
Vaccination	<i>Complete</i>	7	3.95%	170	96.05%	1.64	0.44
	<i>Incomplete</i>	3	1.99%	148	98.01%		
	<i>No vaccines</i>	1	1.52%	6	98.48%		
FIV/FelV	<i>Both positive</i>	0	0.00%	5	100.00%	-	-
	<i>FIV positive</i>	0	0.00%	14	100.00%		
	<i>FelV positive</i>	0	0.00%	16	100.00%		
	<i>Both negative</i>	12	4.69%	244	95.31%		
FIP	<i>Positive</i>	1	20.00%	4	80.00%	0.59	0.45
	<i>Negative</i>	18	3.47%	500	96.53%		

4.6. Identification of spatiotemporal clusters

A spatiotemporal cluster analysis was performed using the SaTScan software (Version 10.0.2, 64 bit). This software employs a statistical method that evaluates the likelihood of detecting the observed number of cases within specific clusters, taking into account the expected number of cases based on the underlying population at risk. By comparing the observed and expected values, the software identifies areas and periods with significantly higher or lower case counts than expected by chance.

This analysis aimed to identify clusters of SARS-CoV-2 cases based on tutors' postal codes, providing the spatial component and the dates of cats' blood sample collection added the temporal dimension. By utilizing both spatial and temporal information, this approach allowed for the detection of clusters that were not only close together geographically but also occurred within a specific period.

Two separate analyses were performed. The first utilized the results obtained from the independent ELISA, while the second used the results from the neutralization test. The purpose of conducting these separate analyses was to investigate potential clustering patterns specific to each test. By conducting two distinct analyses, it was possible to assess if there were any discrepancies or variations in the clustering patterns between the two tests. This information provides insights into the agreement or discordance between the ELISA and neutralization test results regarding the spatial and temporal distribution of SARS-CoV-2 cases.

4.6.1. Results of the spatiotemporal analysis using ELISA results

This analysis uncovered four clusters (Table 10).

Table 10. Cluster analysis using ELISA results.

Cluster	Radius	Relative Risk	P-value	Samples
1	0.00 km	6.11	0.51	3/3
2	0.26 km	6.11	0.80	3/3
3	0.70 km	6.11	0.80	3/3
4	0.89 km	3.11	0.98	6/12

Cluster #1 comprised three positive samples out of three, exhibiting a concentrated pattern with a radius of 0 kilometres (km). The relative risk (RR) associated with this cluster was 6.11, indicating a significantly higher likelihood of positive results within this confined area yet, the p-value of 0.51 indicated that this cluster was not statistically significant.

Cluster #2 also consisted of three positive samples out of three with a slightly larger radius of 0.26 km, suggesting a broader geographic distribution of positive samples compared to cluster number 1. The RR was also 6.11, indicating an elevated probability of positive results within the area. However, the p-value of 0.80 suggested no statistical significance.

Cluster #3, also comprised three positive samples out of three, with a radius of 0.70 km. This cluster displayed a larger spatial extent compared to the previous clusters, signalling a more dispersed distribution of positive cats. The RR remained at 6.11, indicating a heightened likelihood of positive cats within the cluster. Then again, the p-value of 0.80 indicated no statistical significance.

Cluster #4 stood out from the previous clusters by encompassing six positive samples out of twelve, with a higher number of positive cases compared to the previous clusters. This cluster exhibited a radius of 0.89 km, implying a broader geographic distribution. Its RR was 3.11, suggesting a moderate increase in the likelihood of positive cats within the cluster. The p-value of 0.98 did not reach statistical significance.

Figure 13 summarizes the spatiotemporal cluster analysis using ELISA results revealing four clusters with varying spatial extents and numbers of positive cats. While some clusters exhibited higher relative risks, none of them achieved statistical significance based on the calculated p-values. These findings only suggest the presence of geographical clusters.

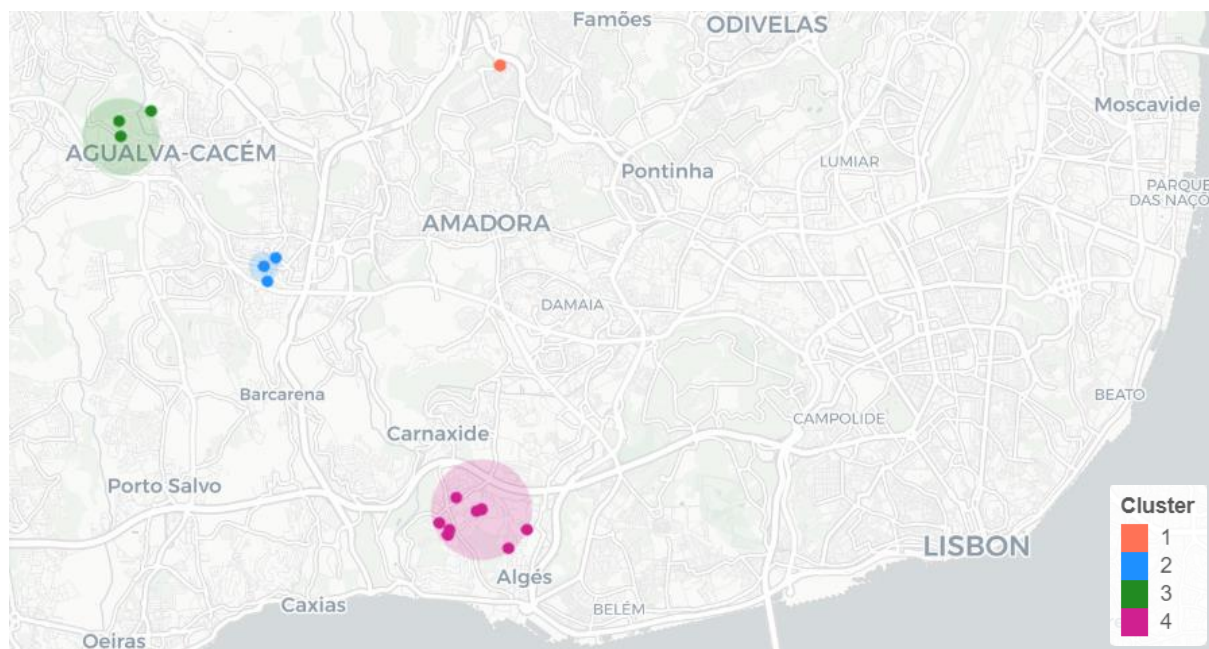


Figure 13. Map with the identified clusters using ELISA results.

The three cats inside Cluster #1 were all located in the postal code of Amadora. Cluster #2 cats were located in a radius of 0.26 km, with two samples belonging to the municipality of Sintra and the third in Oeiras. Cluster #3 had a radius of 0.70 km and the cats' tutors had postal codes from Sintra. Cluster #4, with a radius of 0.89 km, was constituted by 6 cats sited in the municipality of Oeiras. All clusters were set in the Metropolitan Area of Lisbon.

4.6.2. Results of the spatiotemporal analysis using the surrogate virus neutralization test

The outcomes of the neutralization test disclosed the existence of more four distinct clusters (Table 11).

Table 11. Cluster analysis using virus neutralization test results.

Cluster	Radius	Relative Risk	P-value	Samples
1	0.00 km	31.31	0.02	3/3
2	0.23 km	29.53	0.42	2/2
3	1.35 km	11.74	0.97	2/5
4	1.22 km	8.35	1.00	2/7

Cluster #1 grouped three out of the three positive samples with an enclosed radius of 0 km. The RR was 31.31, indicating a markedly increased possibility of positive results in this area. A p-value of 0.02 demonstrated statistical significance.

Cluster #2 displayed two out of two positive samples and a radius of 0.23 km. RR was 29.53 though, not statistically significant due to a p-value of 0.42.

Cluster #3, with a radius of 1.35 kilometres, included two of the five positive samples. RR was 11.74 though, not statistically significant due to a p-value of 0.97.

Cluster #4 gathered two out of seven positive samples with a radius of 1.22 km. RR was 8.35, but the estimated p-value of 1.00 confirmed no statistical significance.

In conclusion, four clusters were found using the spatiotemporal cluster analysis of the results of the neutralization test, each with different spatial extents and numbers of positive samples. While Clusters 2, 3, and 4 displayed varying degrees of heightened relative risks but did not achieve statistical significance based on the estimated p-values, Cluster #1 demonstrated a significant clustering of positive outcomes (Figure 14).



Figure 14. Map with the identified clusters using virus neutralization test results.

The three cats in Cluster #1 were all tracked down to the same postal code, in Amadora. The two samples from Cluster #2 cats had locations in the municipality of Cascais and were collected from cats located within a 0.23 km radius from each other. Cluster #3 occupied a radius of 1.35km, and the two cats were registered with Sintra postal codes. Cluster #4 joined two cats, a radius of 1.22 km and were located in municipalities of Lisbon and Loures. All clusters were in the Lisbon Metropolitan Area.

4.7. Decision models

In order to streamline the interpretation of ELISA results and facilitate quick decision-making regarding the neutralization test outcome, decision models were constructed. They were designed to generate and visualize decision trees, providing a straightforward and efficient way to draw conclusions based on the results obtained from the ELISA.

Decision trees serve as powerful tools for organizing and analysing complex data. They consist of nodes being decision points and branches representing possible outcomes or paths.

By constructing decision trees based on ELISA results, it became possible to visually the factors influencing the probable neutralization outcome and reach conclusions in a simplified manner.

The decision trees incorporated specific decision parameters derived from the ELISA results. These parameters included the OD_{415nm} values of RBD and spike, as well as the ratio between the OD_{415nm} for RBD and the OD_{415nm} of spike. These three parameters were key factors considered in this decision-making process. The ratio between the OD_{415nm} for RBD and the OD_{415nm} of spike complements the decision parameters by assessing the relative proportions or levels of RBD and spike in the ELISA results. This ratio serves as an additional indicator to aid in decision-making, as it offers insights into the balance or discrepancy between the two components.

To develop the decision trees, a specific methodology was followed, involving data splitting and the development of three different decision models, in order to achieve the most optimized and accurate one.

The data used to develop the decision trees consisted on the samples submitted to sVNT (N=90) and their respective results from the independent ELISA and from the neutralization test.

The first decision tree was constructed using all available data as prediction data. Next, the dataset was randomly divided into two distinct groups: Group A and Group B, with 45 samples each. This division created subsets of data for training and prediction purposes.

The second decision tree was built using Group A as the prediction data and Group B as the training data. This arrangement allowed the tree to learn from the patterns and features present in Group B while using Group A as the basis for generating predictions.

Conversely, the third decision tree was developed using Group B as the prediction data and Group A as the training data.

This iterative process facilitated a deeper understanding of the dataset by considering different subsets of data and capturing diverse patterns within the decision trees.

Tree #1 consisted of three nodes or criteria that were evaluated in a sequential manner to classify the samples. The first criterion examined the OD_{415nm} for the spike protein. If the OD for spike was equal to or less than 1.239, the predicted outcome of the neutralization test was negative. If the OD_{415nm} for Spike is greater than 1.239, the second criterion considered was the OD_{415nm} for RBD. If the OD_{415nm} for RBD was equal to or less than 1.80, the predicted outcome of the neutralization test was also negative. When both the OD_{415nm} for spike and RBD were greater than their respective thresholds, the last criterion was assessed. This criterion involved the ratio between the OD_{415nm} of RBD and the OD_{415nm} of spike. If the ratio was less than 1.61, the predicted outcome of the neutralization test was negative. If the ratio was equal to or higher than the referred value, the predicted outcome was positive. Tree #1

was able to correctly classify the majority of the samples, with only two false alarms where negative samples were incorrectly identified as positive (Figure 15).

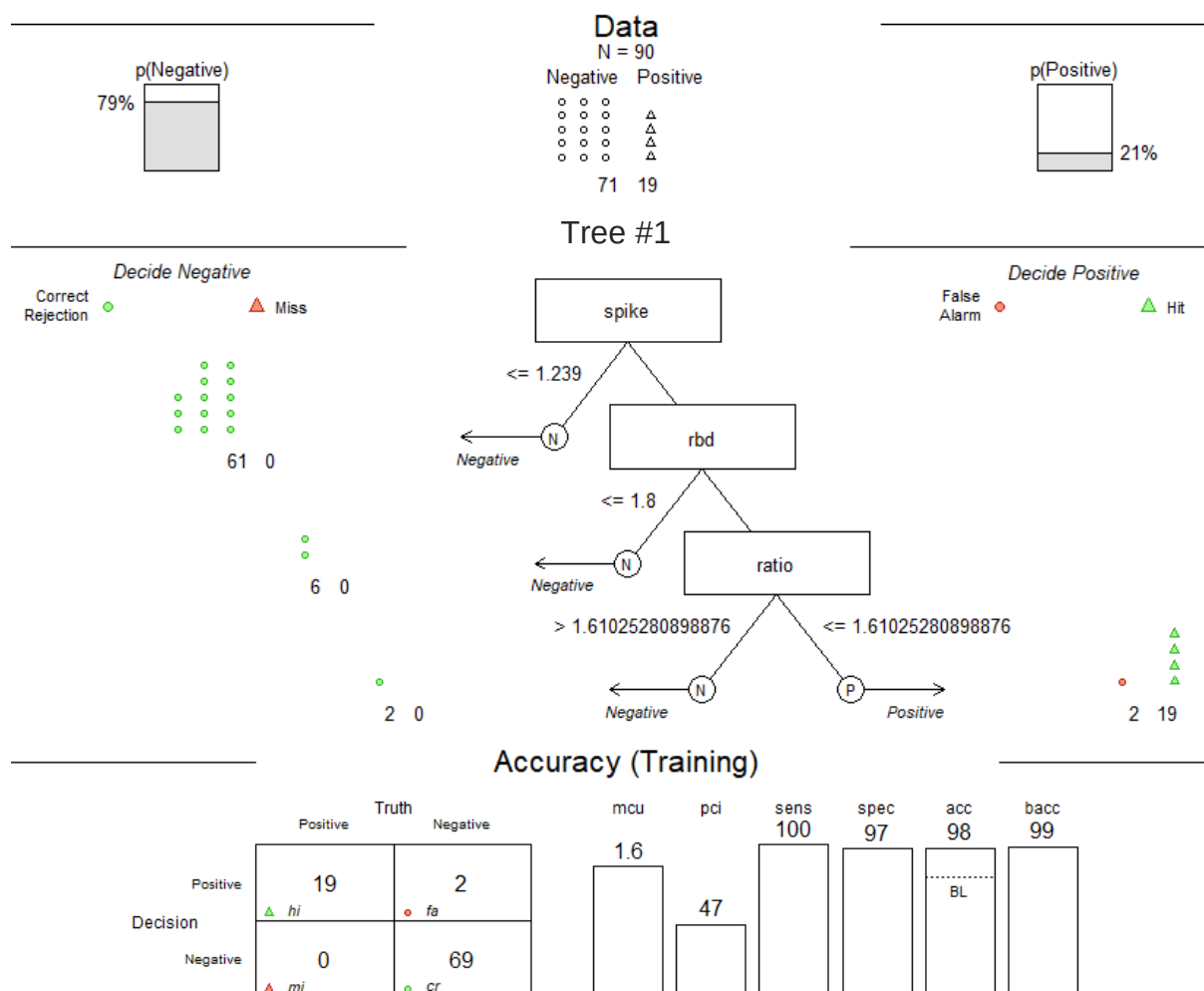


Figure 15. First decision tree (Tree #1) developed using the independent ELISA results to predict the neutralization test outcome.

In this decision tree, 100% of samples assessed for sVNT were used as prediction data, being 79% (N=71) negative in the neutralization test and 21% (N=19) positive. The established nodes were the OD_{415nm} of spike, OD_{415nm} of RBD and the ratio between OD_{415nm} of RBD and the OD_{415nm} of spike, following this order, with respective cut-off values of 1.239, 1.8 and 1.61. The referred values determine the expected outcome in the sVNT. There were no samples incorrectly identified as negative, however 2 positive false alarms were registered. The average number of cue values (mcu) used to define the criteria was 1.6 and the percentage of ignored cues (pci) was 47%. Tree#1 displayed a sensitivity (sens) of 100%, a specificity (spec) of 97%, an accuracy (acc) of 98% and a balanced accuracy (bacc).

There were no instances of missing negative results, indicating that all negative samples were correctly classified. The average number of cue values used to make the criteria was 1.6, indicating that, on average, 1.6 cues or parameters were considered for each decision. The proportion of ignored cues was 47%, suggesting that approximately 47% of the available cues were not utilized in the decision-making process. The performance metrics of the decision tree indicates its effectiveness. It achieved an accuracy of 98%, meaning that it correctly predicted the outcome for 98% of the samples. The balanced accuracy, which

considers the performance across both positive and negative classes, was 99%. The sensitivity (ability to correctly identify positive cases) was 100%, while the specificity (ability to correctly identify negative cases) was 97%.

Tree #2 was developed using the information from Group A, 45 samples (Figure 16).

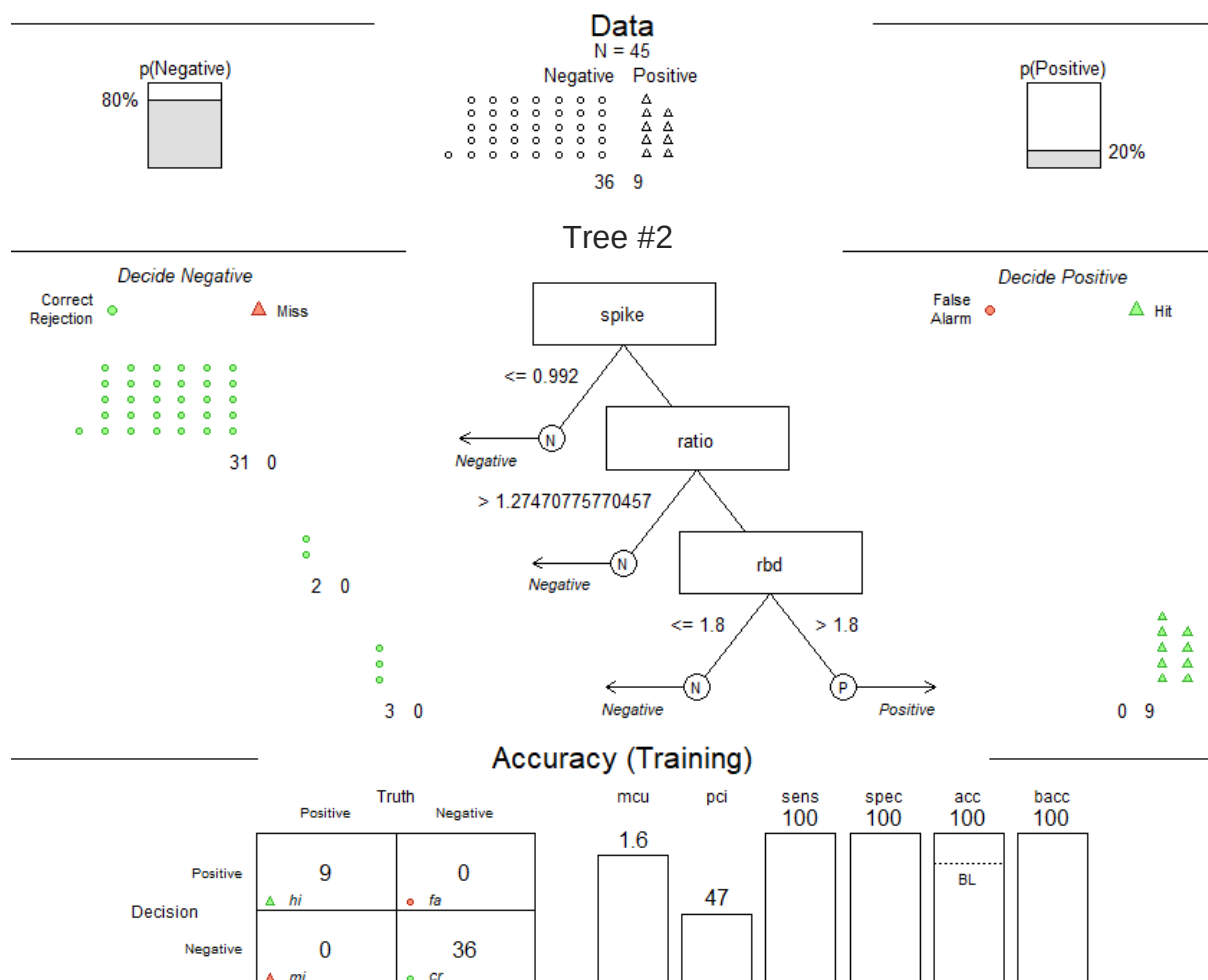


Figure 16. Second decision tree (Tree #2) developed using the independent ELISA results to predict the neutralization test outcome.

Only samples belonging to Group A were used as prediction data, being 80% (N=36) negative in the neutralization test and 20% (N=9) positive. Group B was used as testing data. 3 criteria were defined, including the OD_{415nm} of spike, the ratio between OD_{415nm} of RBD and the OD_{415nm} of spike and the OD_{415nm} of RBD, sequentially and with cut-off values of 0.992, around 1.27 and 1.8, respectively. These marks decided the predicted result in the neutralization test. There were no samples incorrectly unidentified as negative or positive false alarms. The average number of cue values (mcu) used to define the criteria was 1.6, the proportion of ignored cues (pci) was 47% and the sensitivity (sens), specificity (spec), accuracy (acc) and balanced accuracy (bacc) were all at 100%.

The initial criterion relied on the OD_{415nm} for the spike protein, just like Tree#1. If the score was 0.992 or less, a negative result of the neutralization test was expected. The second criterion, considering the ratio between the OD_{415nm} of RBD and the OD_{415nm} of spike, was taken into account if the OD_{415nm} for spike exceeded 0.992. The neutralization test was expected to deliver a negative result when this ratio went above 1.27. The final criterion was employed, though, when the ratio was equal to or lower than 1.27. The OD_{415nm} for RBD was

reviewed in the final criterion. The neutralization test predicted outcome was negative if the value was less than or equal to 1.8. On the other hand, samples were expected to test positive in the neutralization test if their OD_{415nm} for RBD was higher than 1.8. In this decision tree, there were no instances of missing negative results or false alarms, indicating that all negative samples were correctly classified as negative. The average number of cue values applied for generating the conditions was 1.6, just as Tree#1, and 47% of cues were ignored. Tree#2 great accuracy and reliability is evident from its performance measures. All metrics, sensitivity, specificity, accuracy, and balanced accuracy were 100%, revealing that Tree#2 accurately predicted the result for each investigated sample.

Tree#3 was made using data from Group B, consisting of 45 samples (Figure 17).

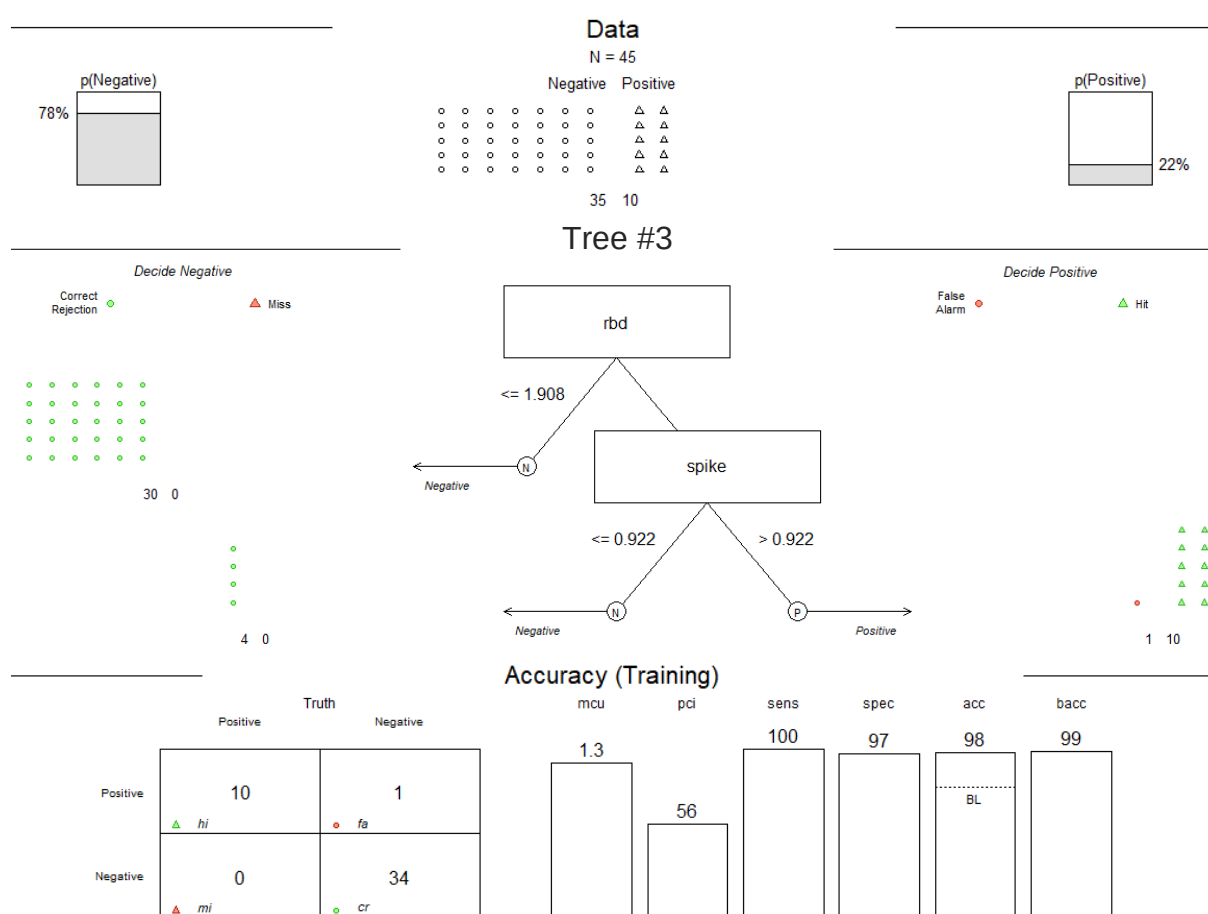


Figure 17. Third decision tree (Tree #3) developed using the independent ELISA results to predict the neutralization test outcome.

Only samples belonging to Group B were used as prediction data, being 78% (N=35) negative in the neutralization test and 22% (N=10) positive. Group A was used as testing data. Only two nodes were set, including the OD_{415nm} of spike followed by the OD_{415nm} of RBD, with cut-off marks at 1.908 and 0.922, respectively. These scores settle the anticipated result in the neutralization test. One sample was misidentified as positive, but no samples were missing from the negative samples. The average number of cue values (mcu) used to define the criteria was 1.3 and the proportion of ignored cues (pci) was 56%, higher than in Tree#1 and Tree#2. The sensitivity (sens), specificity (spec), accuracy (acc) and balanced accuracy (bacc) were 100%, 97%, 98% and 99% respectively.

It used two criteria to predict the outcome of the neutralization test. The first criterion was based on the OD_{415nm} for RBD. If the sample scored, an OD_{415nm} for RBD equal to or less than 1.908, the predicted outcome of the sample's neutralization test was negative. Otherwise, if the OD_{415nm} for RBD was greater than 1.908, the second and final criterion comes, concerning the OD_{415nm} for spike protein. When the OD_{415nm} for spike was equal to or lower than 0.922, the predicted outcome of the neutralization test was negative. In opposition, if the registered value was higher than 0.922, the sample was predicted to be positive in the neutralization test. No missing negative results were identified in Tree#3, indicating that all negative samples were appropriately labelled as such. There was one false alarm misidentifying a sample as positive. The average number of cue values employed to determine this decision tree's qualifiers was 1.3, and the proportion of cues ignored was 56%, reflecting that around 56% of the cues that were available were unapplied in the decision-making process. The performance metrics of Tree#3 point to an accuracy of 98%, showing that it correctly predicted the outcome for 98% of the samples, with 100% sensitivity, 97% specificity, and 99% balanced accuracy.

5. DISCUSSION AND CONCLUSIONS

The first documented case of SARS-CoV-2 infection in a cat occurred in Belgium in mid-March 2020, when a cat displayed digestive and respiratory symptoms following its owner's positive COVID-19 test (Garigliany et al. 2020; WOA 2020b). This initial case raised concerns about the transmission probability of SARS-CoV-2 from humans to cats, particularly considering the close proximity in which many people live with their feline companions. Natural infections in cats living in households with COVID-19-positive people have been reported across multiple regions, particularly in Western countries (Hosie et al. 2021). These findings suggest that human-to-cat transmission was underestimated, emphasizing the need for a comprehensive understanding of cats' role in the epidemiology of COVID-19 (Bessi re et al. 2021). Given the significant number of households with cats and the potential for interaction between humans and feline companions, further research is important to expand insights into the interface of human-to-cat transmission, the susceptibility of cats to SARS-CoV-2, and the implications of such transmission for public health and the overall COVID-19 epidemiology.

The purposes of this project were to assess potential risk factors associated with the transmission of the virus to cats, investigate the presence of spatial or temporal clusters of infection, and develop decision models applicable to neutralization tests using ELISA outcomes of the collected samples.

The primary focus was to identify factors that may contribute to the transmission of the virus to cats, shedding light on the potential routes of infection and understanding the underlying risk factors. Despite the comprehensive analysis of various host variables and cat lifestyle, our data did not identify risk factors associated with the transmission of SARS-CoV-2 to cats. This could be due to the small size of the study population and/or to variables not investigated in our analysis that may play a more prominent role in SARS-CoV-2 transmission to cats, which are probably related to the extent and nature of the human-cat interaction (Bienzle et al. 2022). Some behaviours that reflect the close contact between people and cats and that have been identified as risk factors to the transmission of SARS-CoV-2 to cats, such as the presence of minor in the household, which usually have a closer contact with the animals, the licking of tutors' hands and/or faces, and the frequent feeding and cleaning of droppings (Kuhlmeier et al. 2023), were evaluated in this study. Further research and investigation are necessary to explore cat risk factors to deepen our understanding of the transmission dynamics in feline populations.

Additionally, the project aimed to explore the presence of spatial or temporal clusters of infection among the studied cat population. Although several clusters were identified with high relative risks, only Cluster#1 located in the county of Amadora reached statistical significance. This suggests that while some clusters appeared to have a higher likelihood of a particular outcome, the observed differences could have occurred by chance alone. The small

sample size and the short duration of the study (6 months) may have influenced these results. A study conducted in Illinois, USA (Chen et al. 2023), utilized serum samples collected from cats for 1 year and 7 months, summing a total of 1715 sampled cats, and where 3 significant spatiotemporal clusters were identified. These results emphasize the importance that the sample size and timeframe might have in the clustering results, therefore, further investigation is needed to determine the significance and reliability of these findings and to draw robust conclusions regarding the association between the identified clusters and the specific outcomes under consideration. This information will be valuable in understanding the spread of the virus within a particular area or over time.

Finally, the project aimed to develop decision models specifically tailored for neutralization tests using the ELISA outcomes on the collected blood samples. Throughout the traineeship, three decision models were constructed, each displaying good performance indicators. Notably, Tree #2 exhibited exceptional ratings across all key evaluation metrics, as sensitivity, specificity, accuracy, and balanced accuracy reached 100%. These results indicate that Tree #2 accurately classified events with precision and ensuring minimal false-positives and false-negatives. This highlights its potential as a reliable tool for making accurate predictions for the presence of neutralizing antibodies based on the spike and RBD obtained OD_{415nm} from the ELISA assay, which can be quite beneficial, especially on a large scale, both from a financial point of view as well as from a time and work perspective.

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7. ANNEXES

Annex A: Complete ELISA results (N=523 cats)

Sample	Date of collection	OD _{415nm}								
		5 minutes			15 minutes			30 minutes		
		RBD	Spike	BSA	RBD	Spike	BSA	RBD	Spike	BSA
2575	13/5/2021	0.097	0.098	0.095	0.171	0.162	0.155	0.202	0.192	0.182
3189	9/5/2021	0.243	0.076	0.095	0.340	0.093	0.105	0.404	0.101	0.110
3192	14/5/2021	0.153	0.114	0.069	0.236	0.151	0.081	0.278	0.166	0.086
3194	10/5/2021	0.880	0.129	0.071	1.406	1.193	0.506	1.639	0.224	0.107
3201	10/5/2021	0.075	0.070	0.061	0.098	0.084	0.071	0.106	0.090	0.076
3207	10/5/2021	0.097	0.143	0.064	0.130	0.201	0.074	0.144	0.235	0.079
3213	10/5/2021	0.127	0.131	0.082	0.189	0.192	0.106	0.218	0.220	0.117
3215	10/5/2021	0.208	0.106	0.068	0.326	0.158	0.081	0.390	0.181	0.089
3218	12/5/2021	0.437	0.358	0.298	0.656	0.557	0.420	0.718	0.610	0.479
3226	11/5/2021	0.189	0.194	0.104	0.265	0.274	0.138	0.312	0.324	0.159
3227	11/5/2021	0.506	0.139	0.073	0.895	0.218	0.086	1.094	0.259	0.094
3232	10/5/2021	0.093	0.116	0.075	0.118	0.162	0.084	0.129	0.185	0.092
3238	10/5/2021	0.297	0.319	0.178	0.431	0.473	0.240	0.517	0.548	0.271
3250	11/5/2021	0.102	0.076	0.065	0.149	0.093	0.076	0.172	0.103	0.082
3257	11/5/2021	0.137	0.103	0.067	0.207	0.144	0.082	0.236	0.165	0.089
3261	11/5/2021	0.299	0.238	0.137	0.494	0.385	0.204	0.595	0.467	0.235
3264	14/5/2021	0.336	0.338	0.170	0.566	0.539	0.253	0.694	0.662	0.294
3274	12/5/2021	0.070	0.087	0.056	0.107	0.142	0.070	0.119	0.164	0.075
3304	12/5/2021	0.092	0.073	0.064	0.113	0.110	0.084	0.128	0.123	0.097
3310	12/5/2021	0.103	0.167	0.081	0.140	0.269	0.103	0.157	0.319	0.114
3314	12/5/2021	0.115	0.112	0.071	0.170	0.193	0.086	0.195	0.213	0.093
3319	12/5/2021	0.076	0.089	0.067	0.120	0.145	0.088	0.135	0.166	0.096
3320	13/5/2021	0.137	0.129	0.087	0.267	0.273	0.136	0.314	0.329	0.157
3322	12/5/2021	0.080	0.172	0.062	0.123	0.339	0.086	0.138	0.407	0.097
3325	12/5/2021	0.502	0.511	0.062	1.050	0.794	0.075	1.281	0.963	0.080
3330	17/5/2021	NA	NA	NA	NA	NA	NA	0.127	0.131	0.095
3342	27/5/2021	NA	NA	NA	NA	NA	NA	0.199	0.179	0.100
3343	27/5/2021	0.073	0.077	0.067	0.083	0.091	0.074	0.089	0.101	0.079
3351	31/5/2021	0.146	0.145	0.108	0.203	0.205	0.143	0.272	0.267	0.183
3355	13/5/2021	0.128	0.124	0.079	0.249	0.224	0.122	0.294	0.265	0.141
3361	13/5/2021	0.078	0.075	0.068	0.116	0.107	0.088	0.132	0.120	0.096
3363	13/5/2021	0.057	0.085	0.053	0.073	0.125	0.062	0.079	0.141	0.066
3378	13/5/2021	0.074	0.076	0.066	0.113	0.115	0.087	0.130	0.132	0.095
3390	14/5/2021	NA	NA	NA	NA	NA	NA	0.143	0.221	0.110
3398	14/5/2021	NA	NA	NA	NA	NA	NA	0.076	0.091	0.069
3441	17/5/2021	NA	NA	NA	NA	NA	NA	0.085	0.083	0.086
3442	17/5/2021	NA	NA	NA	NA	NA	NA	0.880	0.188	0.077
3444	17/5/2021	NA	NA	NA	NA	NA	NA	0.098	0.088	0.081
3445	17/5/2021	NA	NA	NA	NA	NA	NA	0.556	0.664	0.160
3458	18/5/2021	NA	NA	NA	NA	NA	NA	0.101	0.133	0.099

3474	18/5/2021	NA	NA	NA	NA	NA	NA	0.179	0.210	0.159
3493	18/5/2021	NA	NA	NA	NA	NA	NA	0.182	0.330	0.150
3498	18/5/2021	NA	NA	NA	NA	NA	NA	0.099	0.099	0.092
3558	19/5/2021	NA	NA	NA	NA	NA	NA	0.177	0.216	0.107
3568	19/5/2021	NA	NA	NA	NA	NA	NA	0.098	0.104	0.079
3576	20/5/2021	NA	NA	NA	NA	NA	NA	0.135	0.302	0.133
3608	21/5/2021	NA	NA	NA	NA	NA	NA	0.567	0.592	0.248
3633	23/5/2021	NA	NA	NA	NA	NA	NA	0.123	0.148	0.093
3637	23/5/2021	NA	NA	NA	NA	NA	NA	0.251	0.309	0.202
3645	24/5/2021	0.087	0.116	0.085	0.105	0.148	0.108	0.117	0.164	0.119
3648	24/5/2021	NA	NA	NA	NA	NA	NA	0.441	0.417	0.121
3652	24/5/2021	NA	NA	NA	NA	NA	NA	0.153	0.151	0.106
3653	21/5/2021	NA	NA	NA	NA	NA	NA	0.230	0.233	0.158
3656	24/5/2021	0.107	0.093	0.096	0.135	0.121	0.129	0.150	0.135	0.146
3668	24/5/2021	NA	NA	NA	NA	NA	NA	0.089	0.084	0.074
3670	31/5/2021	NA	NA	NA	NA	NA	NA	1.044	0.177	0.128
3685	25/5/2021	0.164	0.306	0.112	0.294	0.478	0.162	0.355	0.575	0.187
3688	25/5/2021	NA	NA	NA	NA	NA	NA	0.132	0.204	0.131
3714	26/5/2021	0.150	0.202	0.114	0.252	0.327	0.171	0.293	0.381	0.187
3718	26/5/2021	NA	NA	NA	NA	NA	NA	0.159	0.179	0.135
3727	26/5/2021	NA	NA	NA	NA	NA	NA	0.165	0.150	0.112
3730	26/5/2021	NA	NA	NA	NA	NA	NA	0.154	0.102	0.075
3735	26/5/2021	0.122	0.164	0.098	0.182	0.255	0.102	0.209	0.288	0.111
3761	27/5/2021	0.132	0.099	0.090	0.183	0.127	0.111	0.269	0.187	0.148
3770	27/5/2021	0.234	0.147	0.081	0.372	0.225	0.108	0.440	0.263	0.122
3784	27/5/2021	NA	NA	NA	NA	NA	NA	0.074	0.074	0.075
3793	27/5/2021	0.146	0.080	0.065	0.210	0.110	0.080	0.233	0.122	0.086
3796	27/5/2021	NA	NA	NA	NA	NA	NA	0.117	0.104	0.100
3797	27/5/2021	0.169	0.129	0.103	0.271	0.174	0.133	0.394	0.244	0.179
3799	27/5/2021	0.067	0.128	0.063	0.089	0.188	0.075	0.097	0.211	0.081
3828	28/5/2021	NA	NA	NA	NA	NA	NA	0.092	0.100	0.082
3839	31/5/2021	0.130	0.117	0.098	0.202	0.185	0.145	0.283	0.264	0.200
3849	31/5/2021	0.918	0.276	0.079	1.582	0.479	0.105	2.107	0.717	0.134
3851	31/5/2021	NA	NA	NA	NA	NA	NA	0.135	0.130	0.110
3854	7/6/2021	0.192	0.313	0.171	0.327	0.549	0.282	0.462	0.816	0.404
3856	31/5/2021	0.258	0.153	0.103	0.473	0.262	0.146	0.591	0.371	0.187
3857	31/5/2021	0.134	0.078	0.089	0.205	0.096	0.099	0.239	0.106	0.105
3859	31/5/2021	0.089	0.213	0.111	0.119	0.355	0.163	0.147	0.475	0.216
3863	31/5/2021	0.153	0.137	0.102	0.232	0.210	0.144	0.332	0.297	0.192
3872	31/5/2021	0.610	0.499	0.281	0.766	0.633	0.349	0.957	0.784	0.431
3874	31/5/2021	0.414	0.182	0.121	0.690	0.274	0.175	1.014	0.370	0.226
3878	2/6/2021	0.116	0.134	0.091	0.172	0.200	0.124	0.236	0.270	0.153
3879	2/6/2021	0.099	0.090	0.079	0.136	0.121	0.101	0.183	0.153	0.123
3897	21/6/2021	0.146	0.136	0.091	0.204	0.181	0.118	0.278	0.244	0.149
3900	23/6/2021	0.327	0.280	0.224	NA	NA	NA	1.070	0.826	0.537
3901	24/6/2021	0.152	0.207	0.095	0.187	0.262	0.111	0.240	0.336	0.135
3910	1/6/2021	0.682	0.165	0.103	1.177	0.231	0.142	1.636	0.323	0.175
3979	1/6/2021	0.082	0.123	0.101	0.104	0.164	0.133	0.126	0.206	0.168

3990	2/6/2021	0.287	0.217	0.146	0.422	0.321	0.212	0.579	0.440	0.280
3991	2/6/2021	0.146	0.141	0.099	0.195	0.192	0.129	0.254	0.251	0.161
4013	2/6/2021	0.372	0.174	0.122	0.476	0.219	0.149	0.600	0.284	0.185
4019	2/6/2021	0.439	0.134	0.122	0.597	0.171	0.146	0.793	0.222	0.183
4020	2/6/2021	0.131	0.194	0.122	0.162	0.252	0.141	0.206	0.325	0.178
4023	2/6/2021	0.329	0.298	0.153	0.507	0.428	0.218	0.673	0.580	0.288
4031	2/6/2021	0.145	0.120	0.068	0.172	0.138	0.076	0.208	0.165	0.086
4035	3/6/2021	0.206	0.224	0.138	0.256	0.289	0.167	0.322	0.366	0.208
4036	3/6/2021	0.206	0.190	0.133	0.308	0.254	0.177	0.400	0.329	0.233
4037	3/6/2021	0.109	0.115	0.094	0.154	0.161	0.129	0.190	0.206	0.152
4040	3/6/2021	0.231	0.261	0.093	0.382	0.384	0.121	0.555	0.501	0.154
4041	3/6/2021	0.114	0.102	0.084	0.148	0.132	0.103	0.186	0.159	0.125
4045	3/6/2021	0.137	0.155	0.105	0.167	0.198	0.124	0.212	0.233	0.149
4053	4/6/2021	0.145	0.139	0.114	0.177	0.172	0.134	0.222	0.219	0.166
4065	4/6/2021	0.431	0.440	0.254	0.561	0.603	0.338	0.730	0.761	0.434
4078	5/6/2021	0.190	0.119	0.098	0.227	0.138	0.118	0.278	0.169	0.135
4080	6/6/2021	0.153	0.234	0.134	0.213	0.295	0.175	0.285	0.393	0.226
4081	6/6/2021	0.126	0.288	0.095	0.180	0.418	0.123	0.251	0.524	0.156
4094	7/6/2021	0.210	0.187	0.148	0.266	0.227	0.165	0.356	0.301	0.195
4096	7/6/2021	0.693	0.309	0.177	1.112	0.518	0.268	1.417	0.681	0.355
4098	7/6/2021	0.252	0.141	0.073	0.389	0.226	0.090	0.641	0.289	0.113
4101	7/6/2021	0.513	0.200	0.081	1.009	0.349	0.104	1.345	0.456	0.118
4108	7/6/2021	0.597	0.483	0.095	0.778	0.630	0.120	0.973	0.802	0.137
4120	7/6/2021	0.409	0.401	0.110	0.601	0.565	0.146	0.913	0.852	0.189
4145	8/6/2021	0.243	0.122	0.102	0.295	0.175	0.118	0.347	0.220	0.142
4162	9/6/2021	0.104	0.087	0.060	0.147	0.118	0.073	0.182	0.141	0.081
4166	9/6/2021	0.303	0.289	0.120	0.443	0.427	0.160	0.601	0.542	0.182
4168	9/6/2021	0.119	0.120	0.093	0.160	0.165	0.113	0.246	0.227	0.155
4176	9/6/2021	0.148	0.138	0.082	0.175	0.173	0.097	0.216	0.210	0.110
4189	9/6/2021	0.097	0.102	0.091	0.111	0.132	0.097	0.136	0.165	0.105
4196	10/6/2021	0.145	0.125	0.076	0.247	0.203	0.097	0.357	0.276	0.112
4220	11/6/2021	0.117	0.372	0.148	0.165	0.511	0.215	0.250	0.816	0.319
4232	12/6/2021	0.186	0.191	0.114	0.283	0.283	0.151	0.387	0.360	0.192
4233	12/6/2021	0.238	0.368	0.130	0.366	0.596	0.200	0.486	0.740	0.246
4238	13/6/2021	0.123	0.158	0.070	0.190	0.191	0.084	0.235	0.228	0.095
4244	14/6/2021	0.281	0.220	0.078	0.386	0.339	0.100	0.643	0.528	0.127
4246	14/6/2021	0.148	0.112	0.104	0.190	0.144	0.118	0.248	0.159	0.132
4250	14/6/2021	0.273	0.281	0.101	0.390	0.415	0.142	0.667	0.681	0.201
4256	14/6/2021	0.121	0.146	0.087	0.162	0.191	0.108	0.203	0.240	0.130
4268	14/6/2021	0.199	0.174	0.155	0.281	0.258	0.225	0.371	0.344	0.299
4271	14/6/2021	0.156	0.146	0.146	NA	NA	NA	0.339	0.285	0.216
4272	14/6/2021	0.209	0.216	0.165	NA	NA	NA	0.734	0.685	0.393
4274	14/6/2021	0.310	0.316	0.183	NA	NA	NA	1.091	1.117	0.504
4286	15/6/2021	0.281	0.198	0.205	NA	NA	NA	1.064	0.633	0.636
4288	15/6/2021	0.163	0.182	0.160	NA	NA	NA	0.373	0.499	0.316
4289	15/6/2021	0.213	0.214	0.191	NA	NA	NA	0.675	0.613	0.485
4302	15/6/2021	0.108	0.137	0.090	0.147	0.183	0.114	0.197	0.251	0.142
4364	15/6/2021	0.260	0.172	0.147	NA	NA	NA	0.991	0.487	0.324

4378	16/6/2021	0.191	0.218	0.214	NA	NA	NA	0.427	0.603	0.455
4383	16/6/2021	0.278	0.205	0.168	NA	NA	NA	1.113	0.718	0.507
4391	16/6/2021	0.160	0.152	0.124	NA	NA	NA	0.511	0.414	0.268
4401	17/6/2021	0.715	0.239	0.210	NA	NA	NA	2.684	0.678	0.496
4416	17/6/2021	0.202	0.190	0.219	NA	NA	NA	0.519	0.405	0.358
4417	17/6/2021	0.090	0.075	0.063	0.121	0.092	0.077	0.154	0.110	0.087
4419	17/6/2021	0.257	0.176	0.176	NA	NA	NA	0.740	0.522	0.402
4433	17/6/2021	0.175	0.153	0.105	0.256	0.209	0.140	0.352	0.289	0.180
4435	18/6/2021	0.064	0.062	0.056	0.070	0.066	0.058	0.078	0.071	0.062
4443	18/6/2021	0.165	0.144	0.104	NA	NA	NA	0.593	0.447	0.222
4444	18/6/2021	0.089	0.121	0.079	0.132	0.179	0.103	0.185	0.253	0.128
4445	18/6/2021	0.669	0.353	0.140	0.946	0.469	0.165	1.203	0.605	0.204
4446	18/6/2021	0.213	0.202	0.168	NA	NA	NA	0.801	0.741	0.540
4461	18/6/2021	0.646	0.118	0.086	1.208	0.198	0.125	1.523	0.241	0.145
4466	18/6/2021	0.167	0.177	0.109	0.234	0.240	0.147	0.319	0.336	0.191
4473	21/6/2021	0.456	0.367	0.083	0.675	0.536	0.104	0.932	0.727	0.129
4483	21/6/2021	0.095	0.113	0.080	0.123	0.135	0.099	0.164	0.168	0.122
4509	22/6/2021	0.289	0.264	0.126	0.357	0.310	0.140	0.459	0.398	0.166
4510	22/6/2021	0.153	0.322	0.142	0.196	0.425	0.173	0.260	0.567	0.224
4516	22/6/2021	0.335	0.165	0.101	0.568	0.248	0.146	0.837	0.372	0.201
4529	22/6/2021	0.210	0.297	0.129	0.396	0.513	0.197	0.623	0.791	0.282
4531	23/6/2021	0.223	0.216	0.150	NA	NA	NA	0.801	0.730	0.375
4535	22/6/2021	0.527	0.223	0.160	0.717	0.282	0.194	1.012	0.367	0.243
4541	23/6/2021	0.415	0.228	0.155	0.558	0.292	0.176	0.770	0.380	0.212
4549	23/6/2021	0.779	0.717	0.114	1.420	1.344	0.159	1.984	1.896	0.219
4588	23/6/2021	0.131	0.133	0.079	0.170	0.198	0.093	0.231	0.282	0.112
4591	23/6/2021	0.164	0.188	0.071	0.219	0.254	0.084	0.287	0.331	0.095
4592	23/6/2021	0.169	0.288	0.137	0.236	0.425	0.192	0.330	0.612	0.265
4609	24/6/2021	0.130	0.290	0.125	0.204	0.412	0.173	0.298	0.591	0.238
4624	24/6/2021	0.147	0.165	0.102	0.230	0.238	0.143	0.339	0.339	0.193
4638	25/6/2021	0.268	0.101	0.110	0.338	0.120	0.122	0.437	0.146	0.139
4650	28/6/2021	0.135	0.143	0.090	0.197	0.217	0.117	0.278	0.307	0.151
4661	7/7/2021	0.166	0.085	0.062	0.276	0.113	0.072	0.354	0.137	0.083
4664	12/7/2021	0.117	0.106	0.081	0.176	0.151	0.108	0.233	0.203	0.133
4678	25/6/2021	0.857	0.850	0.081	1.333	1.225	0.100	1.846	1.694	0.124
4680	26/5/2021	0.153	0.169	0.078	0.197	0.235	0.098	0.255	0.314	0.122
4699	29/6/2021	0.101	0.234	0.135	0.119	0.309	0.150	0.142	0.427	0.174
4706	28/6/2021	0.151	0.161	0.081	0.262	0.247	0.110	0.362	0.345	0.143
4709	1/7/2021	0.242	0.291	0.225	NA	NA	NA	0.905	1.011	0.399
4715	28/6/2021	0.158	0.145	0.114	NA	NA	NA	0.605	0.447	0.289
4721	28/6/2021	0.213	0.211	0.193	NA	NA	NA	0.581	0.507	0.367
4732	29/6/2021	0.249	0.240	0.181	NA	NA	NA	0.818	0.883	0.547
4735	30/6/2021	0.436	0.443	0.202	0.559	0.561	0.254	0.743	0.745	0.327
4739	30/6/2021	0.315	0.208	0.175	0.405	0.263	0.214	0.543	0.343	0.272
4741	30/6/2021	1.029	0.439	0.183	1.438	0.635	0.243	1.919	0.878	0.328
4744	30/6/2021	1.167	1.099	0.084	1.498	1.407	0.094	1.961	1.854	0.109
4749	30/6/2021	0.271	0.173	0.126	0.332	0.222	0.148	0.424	0.288	0.178
4755	30/6/2021	0.433	0.389	0.163	0.516	0.465	0.185	0.683	0.607	0.234

4761	30/6/2021	0.882	0.874	0.162	1.295	1.256	0.229	1.732	1.687	0.310
4765	30/6/2021	0.193	0.164	0.134	NA	NA	NA	0.464	0.416	0.333
4784	8/7/2021	0.104	0.140	0.076	0.144	0.173	0.090	0.184	0.206	0.102
4788	1/7/2021	0.182	0.095	0.084	0.256	0.126	0.103	0.395	0.179	0.131
4790	1/7/2021	0.138	0.162	0.079	0.199	0.277	0.105	0.260	0.401	0.132
4792	1/7/2021	0.100	0.112	0.081	0.137	0.153	0.095	0.171	0.184	0.106
4794	1/7/2021	0.109	0.110	0.107	0.166	0.172	0.150	0.221	0.230	0.198
4801	2/7/2021	0.128	0.097	0.079	0.191	0.139	0.097	0.240	0.171	0.115
4804	2/7/2021	0.218	0.265	0.244	0.370	0.453	0.402	0.522	0.633	0.560
4813	2/7/2021	0.223	0.099	0.101	0.387	0.141	0.131	0.551	0.186	0.160
4825	5/7/2021	0.147	0.090	0.069	0.235	0.124	0.085	0.303	0.155	0.103
4838	5/7/2021	0.692	0.107	0.124	1.182	0.150	0.159	1.598	0.194	0.193
4846	5/7/2021	0.331	0.188	0.071	0.574	0.303	0.082	0.796	0.403	0.099
4856	5/7/2021	0.213	0.108	0.081	0.359	0.154	0.100	0.464	0.191	0.114
4860	6/7/2021	0.257	0.100	0.072	0.447	0.140	0.087	0.605	0.176	0.097
4871	6/7/2021	0.340	0.219	0.113	0.546	0.345	0.146	0.757	0.471	0.186
4876	6/7/2021	0.383	0.137	0.070	0.627	0.211	0.085	0.930	0.280	0.101
4881	6/7/2021	0.349	0.106	0.067	0.749	0.152	0.081	1.029	0.204	0.098
4897	7/7/2021	0.122	0.113	0.103	0.183	0.157	0.122	0.237	0.205	0.154
4904	7/7/2021	0.310	0.066	0.071	0.488	0.079	0.082	0.654	0.091	0.092
4907	7/7/2021	0.173	0.175	0.109	0.279	0.288	0.154	0.382	0.389	0.201
4911	7/7/2021	0.099	0.082	0.069	0.134	0.101	0.075	0.160	0.117	0.084
4915	7/7/2021	0.255	0.225	0.117	NA	NA	NA	0.983	0.809	0.228
4935	8/7/2021	0.290	0.277	0.077	0.435	0.460	0.100	0.585	0.620	0.128
4941	9/7/2021	0.112	0.080	0.071	0.172	0.105	0.082	0.214	0.128	0.091
4956	9/7/2021	1.358	0.317	0.058	2.034	0.472	0.067	2.345	0.562	0.072
4965	9/7/2021	0.115	0.109	0.070	0.150	0.147	0.085	0.213	0.194	0.106
4967	9/7/2021	0.158	0.126	0.079	0.238	0.186	0.101	0.312	0.246	0.122
4981	12/7/2021	0.234	0.214	0.081	0.366	0.345	0.110	0.529	0.487	0.135
4991	12/7/2021	0.092	0.089	0.076	0.116	0.111	0.089	0.143	0.135	0.106
5001	12/7/2021	0.113	0.097	0.094	0.155	0.135	0.118	0.203	0.178	0.145
5002	12/7/2021	0.270	0.085	0.080	0.476	0.111	0.102	0.651	0.140	0.126
5013	13/7/2021	0.126	0.095	0.094	0.178	0.131	0.115	0.227	0.166	0.137
5015	13/7/2021	0.750	0.130	0.073	1.129	0.189	0.091	1.666	0.250	0.108
5028	13/7/2021	0.185	0.115	0.069	0.270	0.159	0.079	0.367	0.207	0.089
5033	13/7/2021	0.397	0.338	0.079	0.643	0.586	0.104	0.801	0.707	0.117
5052	14/7/2021	0.609	0.191	0.084	1.006	0.266	0.104	1.269	0.310	0.117
5054	14/7/2021	0.389	0.172	0.066	0.644	0.241	0.074	0.797	0.296	0.079
5056	14/7/2021	0.085	0.091	0.074	0.115	0.126	0.088	0.142	0.160	0.103
5063	14/7/2021	0.197	0.210	0.148	NA	NA	NA	0.690	0.712	0.375
5070	15/7/2021	0.204	0.189	0.096	0.295	0.306	0.138	0.367	0.390	0.160
5109	22/7/2021	0.211	0.136	0.080	0.318	0.210	0.106	0.381	0.243	0.121
5117	18/7/2021	0.143	0.241	0.119	0.279	0.437	0.193	0.340	0.513	0.226
5120	18/7/2021	0.148	0.155	0.085	0.213	0.218	0.115	0.279	0.276	0.132
5121	19/7/2021	0.188	0.097	0.075	0.327	0.135	0.103	0.396	0.159	0.115
5124	19/7/2021	0.179	0.112	0.139	0.271	0.182	0.186	0.337	0.211	0.217
5127	19/7/2021	0.778	0.104	0.109	1.434	0.178	0.155	1.803	0.213	0.180
5128	19/7/2021	0.107	0.082	0.071	0.154	0.114	0.095	0.182	0.130	0.106

5129	19/7/2021	0.153	0.173	0.085	0.281	0.311	0.128	0.356	0.398	0.151
5132	19/7/2021	1.134	1.045	0.097	1.498	1.348	0.128	1.862	1.727	0.146
5136	22/7/2021	0.439	0.507	0.238	0.698	0.745	0.382	0.894	0.924	0.451
5168	20/7/2021	0.179	0.198	0.101	0.298	0.337	0.149	0.298	0.337	0.149
5170	20/7/2021	0.151	0.264	0.135	0.296	0.442	0.246	0.364	0.564	0.294
5210	29/7/2021	0.203	0.251	0.115	0.337	0.427	0.165	0.459	0.572	0.209
5227	9/8/2021	0.226	0.167	0.101	0.364	0.261	0.136	0.535	0.346	0.175
5230	22/7/2021	1.014	0.572	0.116	1.597	0.966	0.169	2.028	1.313	0.216
5233	22/7/2021	0.975	0.632	0.200	1.552	1.017	0.315	1.898	1.210	0.371
5234	22/7/2021	0.965	0.574	0.165	1.395	0.829	0.256	1.760	1.097	0.312
5244	22/7/2021	0.152	0.115	0.095	0.223	0.180	0.135	0.286	0.235	0.168
5256	23/7/2021	0.239	0.121	0.090	0.372	0.165	0.114	0.509	0.215	0.140
5257	23/7/2021	0.113	0.097	0.081	0.161	0.133	0.099	0.194	0.155	0.111
5261	23/7/2021	0.140	0.130	0.082	0.267	0.235	0.119	0.331	0.299	0.154
5269	26/7/2021	0.162	0.119	0.081	0.227	0.169	0.105	0.280	0.206	0.122
5283	23/7/2021	0.179	0.151	0.109	0.255	0.229	0.146	0.305	0.286	0.167
5284	23/7/2021	0.146	0.201	0.159	0.217	0.336	0.250	0.263	0.424	0.299
5293	24/7/2021	0.089	0.084	0.065	0.127	0.110	0.078	0.157	0.139	0.092
5295	25/7/2021	0.291	0.177	0.105	0.450	0.277	0.159	0.579	0.371	0.193
5315	26/7/2021	1.092	0.326	0.154	1.841	0.546	0.246	2.279	0.739	0.321
5331	27/7/2021	0.110	0.161	0.093	0.151	0.235	0.125	0.180	0.288	0.144
5341	27/7/2021	0.071	0.099	0.062	0.108	0.144	0.077	0.132	0.183	0.090
5348	28/7/2021	0.164	0.103	0.077	0.304	0.162	0.106	0.396	0.215	0.134
5350	28/7/2021	0.104	0.086	0.063	0.164	0.127	0.080	0.214	0.162	0.094
5357	28/7/2021	0.151	0.154	0.098	0.234	0.233	0.138	0.310	0.299	0.171
5360	28/7/2021	0.093	0.099	0.074	0.131	0.130	0.097	0.160	0.152	0.113
5364	28/7/2021	0.287	0.267	0.137	0.533	0.434	0.201	0.797	0.601	0.268
5365	28/7/2021	0.136	0.108	0.083	0.216	0.164	0.121	0.273	0.204	0.144
5381	29/7/2021	0.163	0.089	0.072	0.273	0.133	0.094	0.391	0.166	0.112
5382	29/7/2021	0.623	0.217	0.107	0.995	0.336	0.147	1.342	0.473	0.184
5384	29/7/2021	0.203	0.231	0.094	0.295	0.362	0.145	0.385	0.482	0.178
5388	29/7/2021	0.146	0.242	0.099	0.283	0.480	0.150	0.367	0.648	0.196
5393	29/7/2021	0.661	0.642	0.176	1.096	1.046	0.310	1.308	1.258	0.370
5397	29/7/2021	0.161	0.104	0.082	0.249	0.152	0.117	0.346	0.202	0.150
5398	29/7/2021	0.113	0.096	0.073	0.205	0.138	0.095	0.261	0.179	0.115
5407	30/7/2021	0.226	0.107	0.075	0.449	0.170	0.088	0.581	0.219	0.107
5414	30/7/2021	1.190	0.258	0.078	1.841	0.521	0.099	2.310	0.651	0.120
5430	30/7/2021	0.358	0.139	0.102	0.646	0.176	0.131	0.903	0.209	0.153
5450	2/8/2021	0.090	0.099	0.069	0.124	0.140	0.085	0.151	0.171	0.099
5454	2/8/2021	0.136	0.067	0.061	0.200	0.077	0.064	0.260	0.087	0.070
5466	2/8/2021	0.458	0.062	0.063	0.780	0.070	0.067	1.026	0.078	0.074
5467	2/8/2021	0.113	0.096	0.083	0.158	0.119	0.093	0.181	0.133	0.102
5477	2/8/2021	0.087	0.111	0.080	0.111	0.145	0.087	0.127	0.167	0.093
5481	2/8/2021	0.700	0.113	0.078	1.341	0.177	0.106	1.764	0.214	0.124
5485	2/8/2021	0.125	0.087	0.091	0.176	0.105	0.103	0.226	0.121	0.113
5495	3/8/2021	0.171	0.200	0.108	0.280	0.333	0.141	0.360	0.438	0.169
5497	3/8/2021	0.070	0.078	0.077	0.089	0.093	0.085	0.102	0.103	0.093
5501	3/8/2021	0.110	0.173	0.093	0.158	0.242	0.116	0.194	0.301	0.140

5504	3/8/2021	0.218	0.295	0.167	0.383	0.457	0.290	0.482	0.569	0.358
5505	3/8/2021	0.394	0.175	0.087	0.670	0.291	0.104	0.913	0.395	0.121
5508	3/8/2021	0.107	0.090	0.076	0.163	0.127	0.079	0.209	0.152	0.087
5509	3/8/2021	0.598	0.135	0.091	0.974	0.200	0.122	1.299	0.258	0.151
5511	3/8/2021	0.354	0.170	0.107	0.660	0.273	0.143	0.921	0.347	0.184
5523	4/8/2021	0.164	0.075	0.062	0.285	0.103	0.071	0.344	0.118	0.075
5525	5/8/2021	0.401	0.232	0.112	0.596	0.372	0.153	0.756	0.446	0.177
5533	4/8/2021	0.279	0.409	0.194	0.448	0.678	0.287	0.540	0.815	0.333
5548	4/8/2021	0.147	0.147	0.135	0.234	0.235	0.178	0.270	0.270	0.207
5555	5/8/2021	1.272	0.297	0.131	1.804	0.550	0.190	2.077	0.650	0.237
5584	5/8/2021	0.071	0.093	0.063	0.089	0.103	0.079	0.095	0.107	0.084
5590	6/8/2021	0.079	0.081	0.062	0.113	0.111	0.076	0.129	0.126	0.081
5594	6/8/2021	0.103	0.114	0.078	0.144	0.163	0.093	0.171	0.187	0.104
5595	6/8/2021	0.199	0.441	0.168	0.315	0.778	0.257	0.373	0.970	0.312
5597	6/8/2021	0.100	0.147	0.080	0.142	0.233	0.093	0.171	0.274	0.101
5598	6/8/2021	0.055	0.055	0.053	0.063	0.061	0.058	0.068	0.065	0.061
5647	9/8/2021	0.066	0.081	0.056	0.082	0.098	0.062	0.090	0.107	0.065
5648	9/8/2021	0.092	0.090	0.096	0.124	0.129	0.127	0.144	0.146	0.143
5649	9/8/2021	0.458	0.083	0.103	0.750	0.110	0.194	0.876	0.118	0.204
5654	9/8/2021	0.147	0.119	0.106	0.178	0.142	0.121	0.219	0.172	0.139
5662	10/8/2021	0.114	0.085	0.081	0.167	0.113	0.109	0.201	0.126	0.123
5671	10/8/2021	1.203	1.237	0.544	1.732	1.700	0.784	2.414	2.351	1.193
5674	10/8/2021	0.396	0.287	0.054	0.807	0.538	0.065	0.897	0.622	0.069
5678	10/8/2021	0.232	0.103	0.097	0.388	0.138	0.127	0.461	0.160	0.148
5679	10/8/2021	0.085	0.077	0.059	0.116	0.102	0.067	0.137	0.117	0.071
5692	6/9/2021	0.092	0.085	0.085	0.147	0.137	0.131	0.180	0.174	0.151
5693	8/9/2021	0.063	0.066	0.061	0.076	0.085	0.071	0.089	0.099	0.080
5695	14/9/2021	0.260	0.273	0.179	0.313	0.349	0.220	0.393	0.425	0.254
5696	13/9/2021	0.079	0.083	0.075	0.096	0.104	0.089	0.130	0.138	0.115
5697	13/9/2021	0.093	0.096	0.083	0.144	0.155	0.118	0.171	0.185	0.136
5701	17/9/2021	0.219	0.251	0.138	0.367	0.421	0.200	0.455	0.533	0.241
5706	10/8/2021	0.442	0.462	0.216	0.688	0.788	0.361	1.169	1.276	0.576
5708	13/8/2021	0.427	0.531	0.215	0.696	0.869	0.324	1.087	1.273	0.505
5712	10/8/2021	0.892	0.477	0.304	1.416	0.730	0.427	1.965	1.078	0.637
5742	11/8/2021	0.108	0.162	0.094	0.132	0.193	0.106	0.166	0.243	0.119
5745	12/8/2021	0.293	0.271	0.138	0.444	0.401	0.203	0.774	0.689	0.327
5748	12/8/2021	0.354	0.191	0.111	0.453	0.242	0.129	0.531	0.293	0.150
5762	12/8/2021	0.440	0.483	0.242	0.704	0.719	0.354	1.070	1.093	0.504
5764	13/8/2021	0.128	0.198	0.092	NA	NA	NA	0.294	0.502	0.177
5774	13/8/2021	0.176	0.164	0.098	0.223	0.213	0.125	0.274	0.268	0.143
5780	13/8/2021	0.274	0.210	0.150	0.460	0.320	0.198	0.570	0.392	0.236
5782	13/8/2021	0.318	0.185	0.117	NA	NA	NA	0.756	0.388	0.236
5787	13/8/2021	0.284	0.554	0.220	0.494	0.948	0.384	0.637	1.151	0.482
5788	14/8/2021	0.545	0.671	0.286	0.831	1.082	0.442	1.376	1.618	0.647
5789	14/8/2021	0.190	0.316	0.166	0.276	0.508	0.233	0.325	0.631	0.277
5795	15/8/2021	0.226	0.158	0.185	0.349	0.227	0.202	0.430	0.275	0.225
5797	15/8/2021	0.196	0.122	0.106	NA	NA	NA	0.473	0.263	0.230
5800	15/8/2021	0.243	0.079	0.074	NA	NA	NA	0.669	0.140	0.120

5811	16/8/2021	0.409	0.207	0.135	0.698	0.317	0.199	1.011	0.423	0.245
5812	16/8/2021	0.458	0.461	0.176	0.752	0.771	0.282	1.180	1.224	0.436
5814	16/8/2021	0.116	0.115	0.105	0.127	0.121	0.114	0.153	0.145	0.124
5865	17/8/2021	0.193	0.152	0.110	NA	NA	NA	0.466	0.340	0.223
5880	17/8/2021	0.113	0.080	0.078	0.167	0.106	0.103	0.206	0.127	0.123
5882	18/8/2021	0.309	0.161	0.158	NA	NA	NA	0.775	0.368	0.358
5885	18/8/2021	0.370	0.176	0.103	NA	NA	NA	0.918	0.517	0.261
5900	18/8/2021	0.134	0.110	0.087	NA	NA	NA	0.317	0.228	0.161
5911	24/8/2021	0.236	0.168	0.147	0.384	0.234	0.193	0.478	0.276	0.229
5928	19/8/2021	0.131	0.085	0.078	NA	NA	NA	0.291	0.157	0.120
5945	19/8/2021	0.135	0.126	0.083	NA	NA	NA	0.312	0.279	0.164
5953	20/8/2021	0.132	0.131	0.134	0.180	0.187	0.182	0.207	0.222	0.210
5958	20/8/2021	NA	NA	NA	NA	NA	NA	0.183	0.370	0.282
5960	20/8/2021	0.074	0.072	0.072	NA	NA	NA	0.138	0.132	0.112
5961	24/8/2021	0.500	0.141	0.122	0.795	0.204	0.167	0.996	0.239	0.196
5977	22/8/2021	0.131	0.126	0.088	0.212	0.181	0.118	0.273	0.229	0.143
6000	23/8/2021	0.657	0.959	0.363	1.037	1.357	0.536	1.591	1.895	0.807
6003	30/7/2021	0.202	0.149	0.086	0.321	0.195	0.111	0.428	0.277	0.137
6006	23/8/2021	0.226	0.290	0.104	0.355	0.471	0.133	0.429	0.592	0.149
6015	24/8/2021	0.351	0.337	0.189	0.501	0.507	0.282	0.710	0.674	0.362
6041	25/8/2021	0.515	0.381	0.100	1.149	0.958	0.137	2.111	1.829	0.185
6042	25/8/2021	0.567	0.390	0.288	0.891	0.601	0.433	1.398	0.881	0.621
6044	25/8/2021	1.572	1.443	0.599	2.609	2.372	0.932	(+)	(+)	1.389
6054	25/8/2021	0.466	0.222	0.136	0.793	0.347	0.203	0.947	0.423	0.232
6064	6/9/2021	0.081	0.086	0.072	0.107	0.115	0.090	0.122	0.131	0.100
6081	26/8/2021	0.208	0.291	0.216	0.340	0.465	0.338	0.489	0.706	0.496
6088	6/9/2021	1.000	0.539	0.287	1.567	0.740	0.307	2.289	1.088	0.410
6095	6/9/2021	0.163	0.154	0.175	0.261	0.244	0.205	0.329	0.312	0.237
6097	27/8/2021	0.121	0.119	0.086	0.181	0.163	0.111	0.228	0.202	0.132
6098	27/8/2021	0.142	0.100	0.084	NA	NA	NA	0.343	0.212	0.171
6099	27/8/2021	0.711	0.535	0.123	NA	NA	NA	1.807	1.425	0.286
6101	27/8/2021	0.307	0.299	0.201	NA	NA	NA	0.840	0.862	0.557
6111	27/8/2021	0.125	0.121	0.093	0.166	0.156	0.115	0.195	0.182	0.134
6120	6/9/2021	1.220	0.916	0.252	2.091	1.637	0.355	3.215	2.508	0.498
6128	6/9/2021	0.648	0.218	0.186	1.051	0.301	0.229	1.624	0.406	0.281
6131	6/9/2021	0.366	0.200	0.122	0.589	0.268	0.148	0.814	0.358	0.178
6148	30/8/2021	0.245	0.305	0.254	0.367	0.474	0.361	0.444	0.582	0.431
6149	30/8/2021	0.190	0.242	0.158	0.259	0.361	0.216	0.337	0.471	0.264
6156	30/8/2021	0.199	0.182	0.158	0.286	0.256	0.210	0.375	0.332	0.266
6158	30/8/2021	0.179	0.159	0.112	0.282	0.224	0.149	0.378	0.277	0.173
6165	30/8/2021	0.141	0.152	0.096	0.201	0.221	0.127	0.237	0.265	0.148
6220	1/9/2021	0.281	0.341	0.156	0.359	0.457	0.195	0.441	0.567	0.235
6230	1/9/2021	0.109	0.203	0.107	0.148	0.313	0.143	0.202	0.447	0.188
6234	1/9/2021	0.664	0.321	0.204	1.241	0.567	0.333	1.691	0.701	0.439
6235	1/9/2021	0.600	0.234	0.215	1.383	0.470	0.362	2.548	0.784	0.577
6236	1/9/2021	0.929	0.295	0.191	1.303	0.394	0.250	2.073	0.676	0.415
6241	1/9/2021	0.154	0.150	0.143	0.217	0.200	0.175	0.279	0.256	0.216
6268	2/9/2021	0.417	0.249	0.141	0.824	0.486	0.211	1.362	0.788	0.290

6269	2/9/2021	0.680	0.603	0.111	1.358	1.093	0.146	2.414	1.873	0.184
6274	2/9/2021	0.110	0.128	0.095	0.153	0.183	0.124	0.190	0.223	0.146
6275	6/9/2021	0.123	0.120	0.089	0.152	0.159	0.112	0.222	0.234	0.155
6277	2/9/2021	0.173	0.149	0.127	0.262	0.242	0.190	0.332	0.315	0.220
6292	6/9/2021	0.687	0.646	0.334	1.079	0.855	0.465	1.607	1.129	0.633
6297	3/9/2021	0.289	0.263	0.196	0.411	0.371	0.245	0.583	0.500	0.313
6303	6/9/2021	0.070	0.084	0.064	0.090	0.119	0.078	0.107	0.139	0.088
6305	6/9/2021	0.101	0.111	0.086	0.142	0.171	0.121	0.182	0.216	0.148
6311	6/9/2021	0.086	0.084	0.068	0.099	0.098	0.074	0.114	0.111	0.079
6316	6/9/2021	0.851	0.746	0.115	1.544	1.484	0.157	2.440	2.365	0.197
6317	14/9/2021	0.290	0.662	0.182	0.407	0.924	0.240	0.504	1.153	0.290
6322	6/9/2021	0.224	0.172	0.147	0.351	0.234	0.192	0.431	0.277	0.225
6329	6/9/2021	0.108	0.102	0.071	0.181	0.170	0.097	0.223	0.208	0.113
6337	6/9/2021	0.210	0.214	0.154	0.307	0.309	0.218	0.484	0.483	0.311
6358	6/9/2021	0.138	0.109	0.087	0.166	0.124	0.096	0.194	0.142	0.107
6369	7/9/2021	0.117	0.167	0.089	0.139	0.206	0.109	0.157	0.247	0.125
6375	7/9/2021	0.132	0.253	0.123	0.168	0.337	0.137	0.200	0.406	0.159
6393	7/9/2021	0.253	0.282	0.117	0.292	0.324	0.143	0.350	0.381	0.163
6404	8/9/2021	0.568	0.859	0.268	0.757	1.162	0.384	0.959	1.498	0.481
6413	8/9/2021	0.132	0.142	0.105	0.167	0.177	0.119	0.194	0.206	0.136
6417	8/9/2021	0.157	0.303	0.159	0.207	0.398	0.182	0.250	0.483	0.229
6420	8/9/2021	0.079	0.097	0.071	0.124	0.163	0.099	0.150	0.202	0.113
6448	9/9/2021	0.842	0.472	0.213	1.111	0.671	0.290	1.428	0.873	0.363
6453	9/9/2021	0.126	0.131	0.102	0.143	0.162	0.116	0.164	0.188	0.131
6455	9/9/2021	0.179	0.316	0.107	0.327	0.628	0.191	0.452	0.863	0.234
6461	10/9/2021	0.116	0.158	0.099	0.181	0.283	0.167	0.236	0.315	0.235
6467	10/9/2021	0.191	0.334	0.099	0.249	0.474	0.113	0.297	0.554	0.127
6468	10/9/2021	0.147	0.205	0.010	0.267	0.340	0.143	0.362	0.458	0.181
6469	10/9/2021	0.157	0.151	0.148	0.181	0.181	0.181	0.208	0.203	0.209
6498	13/9/2021	0.073	0.073	0.073	0.010	0.102	0.097	0.125	0.126	0.118
6507	13/9/2021	0.097	0.108	0.087	0.146	0.165	0.119	0.169	0.183	0.136
6540	14/9/2021	0.530	0.269	0.130	1.378	0.431	0.192	2.872	0.744	0.266
6541	14/9/2021	0.237	0.187	0.145	0.329	0.263	0.206	0.540	0.434	0.277
6546	14/9/2021	0.246	0.309	0.136	0.369	0.452	0.179	0.599	0.699	0.262
6582	21/9/2021	0.126	0.137	0.106	0.178	0.190	0.136	0.266	0.274	0.179
6588	15/9/2021	0.189	0.233	0.110	0.329	0.433	0.179	0.437	0.572	0.217
6602	15/9/2021	0.084	0.103	0.076	0.122	0.176	0.101	0.150	0.226	0.122
6603	15/9/2021	0.102	0.144	0.087	0.169	0.262	0.135	0.203	0.327	0.155
6605	15/9/2021	0.098	0.111	0.107	0.155	0.163	0.138	0.227	0.243	0.194
6628	16/9/2021	0.131	0.131	0.093	0.164	0.171	0.118	0.249	0.250	0.164
6631	16/9/2021	0.141	0.137	0.111	0.180	0.178	0.139	0.280	0.279	0.203
6632	16/9/2021	0.083	0.143	0.095	0.127	0.208	0.135	0.160	0.281	0.175
6633	16/9/2021	0.150	0.157	0.105	0.252	0.272	0.176	0.331	0.350	0.222
6637	16/9/2021	0.088	0.091	0.074	0.136	0.138	0.106	0.165	0.168	0.121
6643	16/9/2021	0.170	0.213	0.116	0.271	0.366	0.172	0.319	0.468	0.204
6645	16/9/2021	0.405	0.487	0.090	0.758	0.966	0.140	0.922	1.193	0.164
6714	18/9/2021	0.184	0.214	0.129	0.258	0.289	0.171	0.396	0.429	0.234
6734	20/9/2021	0.089	0.160	0.080	0.138	0.296	0.120	0.163	0.361	0.139

6736	20/9/2021	0.062	0.069	0.065	0.076	0.088	0.079	0.090	0.107	0.093
6738	20/9/2021	0.081	0.063	0.059	0.117	0.076	0.071	0.126	0.087	0.080
6747	20/9/2021	0.069	0.093	0.070	0.098	0.130	0.093	0.120	0.167	0.115
6750	21/9/2021	0.073	0.088	0.079	0.103	0.133	0.112	0.127	0.171	0.138
6773	13/10/2021	0.102	0.107	0.089	0.154	0.156	0.122	0.173	0.173	0.136
6777	14/10/2021	0.378	0.432	0.118	0.727	0.802	0.181	0.870	0.997	0.216
6781	20/9/2021	0.612	0.305	0.295	0.826	0.411	0.364	1.212	0.607	0.530
6820	22/9/2021	1.272	0.406	0.415	1.746	0.519	0.528	2.238	0.738	0.712
6821	22/9/2021	0.390	0.718	0.245	0.567	0.964	0.343	0.703	1.193	0.426
6851	22/9/2021	0.841	0.939	0.667	1.112	1.172	0.863	1.412	1.458	1.106
6852	22/9/2021	0.944	0.432	0.383	1.213	0.574	0.497	1.535	0.721	0.614
6866	23/9/2021	1.097	0.393	0.264	1.715	0.576	0.337	2.262	0.858	0.468
6869	23/9/2021	0.321	0.930	0.306	0.426	1.249	0.401	0.540	1.583	0.496
6883	23/9/2021	0.270	0.576	0.201	0.354	0.750	0.265	0.428	0.926	0.324
6899	24/9/2021	0.610	0.153	0.114	0.962	0.202	0.140	1.426	0.283	0.187
6937	6/10/2021	0.245	0.539	0.233	0.319	0.783	0.303	0.442	1.122	0.415
6970	28/9/2021	0.091	0.082	0.077	0.118	0.106	0.095	0.149	0.132	0.116
6984	28/9/2021	0.133	0.103	0.089	0.185	0.141	0.115	0.242	0.180	0.143
6995	28/9/2021	0.544	0.307	0.296	0.940	0.426	0.474	1.590	0.684	0.721
7006	29/9/2021	0.101	0.093	0.093	0.134	0.126	0.121	0.181	0.164	0.152
7069	29/9/2021	0.275	0.686	0.273	0.362	0.892	0.338	0.447	1.129	0.407
7070	30/9/2021	0.272	0.603	0.281	0.347	0.817	0.349	0.467	1.070	0.468
7074	30/9/2021	0.226	0.652	0.196	0.295	0.890	0.245	0.353	1.115	0.301
7124	1/10/2021	0.534	0.254	0.162	0.939	0.408	0.245	1.568	0.713	0.365
7179	4/10/2021	0.515	0.379	0.318	0.777	0.461	0.427	1.144	0.641	0.601
7184	5/10/2021	0.107	0.097	0.097	0.148	0.135	0.124	0.182	0.168	0.150
7205	6/10/2021	1.516	1.460	0.305	2.009	1.852	0.392	2.412	2.188	0.522
7219	8/10/2021	0.188	0.121	0.102	0.268	0.183	0.144	0.361	0.240	0.187
7266	7/10/2021	0.225	0.206	0.212	0.354	0.318	0.315	0.451	0.410	0.379
7274	7/10/2021	0.343	0.264	0.151	NA	NA	NA	1.098	0.816	0.459
7287	8/10/2021	0.406	0.252	0.217	NA	NA	NA	0.934	0.590	0.441
7298	11/10/2021	0.135	0.226	0.114	NA	NA	NA	0.374	0.703	0.327
7302	11/10/2021	0.078	0.079	0.069	0.097	0.096	0.080	0.117	0.112	0.092
7318	11/10/2021	0.145	0.170	0.121	0.207	0.235	0.176	0.261	0.284	0.218
7320	11/10/2021	0.134	0.130	0.129	0.193	0.191	0.186	0.247	0.241	0.236
7321	11/10/2021	0.091	0.089	0.089	0.116	0.117	0.115	0.143	0.140	0.137
7328	11/10/2021	0.886	0.875	0.200	1.451	1.499	0.306	1.926	1.947	0.369
7332	11/10/2021	0.188	0.150	0.126	0.272	0.233	0.181	0.352	0.298	0.226
7335	11/10/2021	0.414	0.200	0.113	NA	NA	NA	1.174	0.657	0.267
7351	12/10/2021	0.912	0.762	0.137	1.546	1.300	0.192	1.885	1.653	0.228
7356	12/10/2021	0.115	0.113	0.091	0.171	0.171	0.123	0.197	0.193	0.135
7361	12/10/2021	0.094	0.092	0.089	0.149	0.122	0.113	0.202	0.160	0.148
7402	13/10/2021	0.107	0.120	0.090	0.146	0.153	0.116	0.181	0.182	0.134
7406	13/10/2021	0.163	0.166	0.113	0.280	0.277	0.161	0.327	0.322	0.182
7409	13/10/2021	0.129	0.104	0.103	0.228	0.138	0.138	0.371	0.186	0.187
7410	13/10/2021	0.111	0.116	0.096	0.165	0.167	0.130	0.183	0.182	0.141
7421	13/10/2021	0.331	0.331	0.191	0.574	0.605	0.363	0.726	0.723	0.428
7424	13/10/2021	0.177	0.194	0.133	0.288	0.297	0.198	0.347	0.353	0.227

7429	14/10/2021	0.090	0.085	0.079	0.125	0.119	0.106	0.140	0.130	0.117
7431	14/10/2021	0.073	0.074	0.065	0.096	0.094	0.078	0.105	0.102	0.085
7432	14/10/2021	0.104	0.098	0.078	0.153	0.146	0.109	0.177	0.165	0.119
7443	14/10/2021	0.238	0.239	0.210	0.422	0.411	0.323	0.507	0.504	0.383
7452	14/10/2021	0.188	0.165	0.111	0.350	0.277	0.180	0.412	0.337	0.206
7456	14/10/2021	0.271	0.181	0.134	0.441	0.271	0.183	0.621	0.389	0.252
7459	14/10/2021	0.120	0.126	0.126	0.205	0.204	0.206	0.239	0.246	0.239
7470	15/10/2021	0.136	0.118	0.096	0.189	0.169	0.127	0.252	0.226	0.162
7472	15/10/2021	0.087	0.082	0.092	0.110	0.107	0.117	0.141	0.137	0.147
7479	15/10/2021	0.134	0.100	0.083	0.206	0.140	0.106	0.276	0.202	0.134
7489	22/10/2021	0.274	0.298	0.137	NA	NA	NA	NA	NA	NA
7490	25/10/2021	0.183	0.212	0.120	0.358	0.413	0.201	0.678	0.724	0.336
7504	15/10/2021	0.518	0.153	0.078	0.829	0.218	0.097	1.155	0.288	0.122
7520	17/10/2021	0.107	0.113	0.101	0.151	0.161	0.140	0.181	0.180	0.153
7521	17/10/2021	0.093	0.095	0.101	0.142	0.138	0.147	0.162	0.155	0.164
7532	17/10/2021	0.325	0.188	0.146	0.559	0.317	0.257	0.703	0.365	0.292
7535	18/10/2021	0.227	0.238	0.157	0.397	0.411	0.259	0.477	0.481	0.292
7544	18/10/2021	0.246	0.239	0.175	0.421	0.401	0.291	0.486	0.468	0.330
7549	18/10/2021	0.225	0.215	0.250	0.379	0.355	0.402	0.435	0.415	0.458
7554	18/10/2021	0.733	0.199	0.161	1.312	0.317	0.256	1.485	0.360	0.295
7558	18/10/2021	0.799	0.367	0.143	1.387	0.652	0.233	2.213	1.150	0.407
7569	19/10/2021	0.610	0.342	0.127	1.038	0.590	0.188	1.670	0.953	0.287
7573	19/10/2021	0.388	0.367	0.114	0.723	0.601	0.166	1.229	0.954	0.257
7579	19/10/2021	0.248	0.222	0.099	0.409	0.412	0.142	0.699	0.735	0.209
7589	19/10/2021	0.453	0.357	0.183	0.815	0.613	0.285	1.441	1.071	0.493
7594	19/10/2021	0.433	0.546	0.206	0.720	0.903	0.329	1.304	1.494	0.542
7607	20/10/2021	0.358	0.285	0.133	0.538	0.456	0.197	0.977	0.804	0.326
7615	20/10/2021	0.550	0.520	0.315	0.921	0.916	0.501	1.457	1.429	0.776
7629	20/10/2021	0.368	0.371	0.177	NA	NA	NA	NA	NA	NA
7632	21/10/2021	0.227	0.153	0.089	NA	NA	NA	NA	NA	NA
7633	21/10/2021	0.493	0.524	0.247	NA	NA	NA	NA	NA	NA
7634	21/10/2021	0.568	0.297	0.189	1.099	0.588	0.360	1.870	1.043	0.630
7635	21/10/2021	0.620	1.005	0.321	NA	NA	NA	NA	NA	NA
7637	21/10/2021	0.894	1.106	0.536	NA	NA	NA	NA	NA	NA
7638	21/10/2021	0.523	0.615	0.326	NA	NA	NA	NA	NA	NA
7644	21/10/2021	0.652	0.806	0.430	NA	NA	NA	NA	NA	NA
7646	21/10/2021	0.548	0.503	0.247	NA	NA	NA	NA	NA	NA
7651	21/10/2021	0.306	0.380	0.158	NA	NA	NA	NA	NA	NA
7660	21/10/2021	0.234	0.335	0.119	NA	NA	NA	NA	NA	NA
7663	21/10/2021	0.324	0.253	0.153	NA	NA	NA	NA	NA	NA
7664	22/10/2021	1.140	0.480	0.205	NA	NA	NA	NA	NA	NA
7672	22/10/2021	0.536	0.550	0.152	NA	NA	NA	NA	NA	NA
7675	22/10/2021	0.336	0.447	0.146	NA	NA	NA	NA	NA	NA
7695	25/10/2021	0.437	0.514	0.184	NA	NA	NA	NA	NA	NA
7696	25/10/2021	1.565	1.051	0.147	NA	NA	NA	NA	NA	NA
7698	25/10/2021	0.610	0.788	0.347	NA	NA	NA	NA	NA	NA
7699	25/10/2021	0.914	1.052	0.439	NA	NA	NA	NA	NA	NA
7708	25/10/2021	1.075	1.058	0.731	NA	NA	NA	NA	NA	NA

7709	25/10/2021	0.331	0.356	0.141	NA	NA	NA	NA	NA	NA
7711	25/10/2021	0.302	0.268	0.106	0.471	0.475	0.159	0.784	0.740	0.249
7726	25/10/2021	0.332	0.178	0.133	0.652	0.327	0.221	1.164	0.549	0.352

Annex B: OD415nm results obtained from the independent ELISA assay (N=119 samples)

